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CIRCULAR DICHROISM OF CATTLE RHODOPSIN AND BATHORHODOPSIN AT LIQUID NITROGEN TEMPERATURES

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Summary

The photoevent in vision has been considered to be the conversion of rhodopsin to bathorhodopsin, which is caused by photoisomerization of the chromophoric retinal. Recently some objections were raised to this hypothesis. The reliability of the hypothesis was verified by measurement of circular dichroism of bathorhodopsin.

The measurement of circular dichroism of rhodopsin extract (containing 66% or 75% of glycerol) at liquid nitrogen temperatures (—195°C) by a conventional spectropolarimeter induced an extraordinary large signal, owing to linear dichroism originated from conversion of rhodopsin to bathorhodopsin by the measuring light. The similar linear dichroism can be induced by irradiation of rhodopsin extract at —195°C with polarized light or natural light. At photosteady state the linear dichroism disappeared.

Circular dichroism spectrum of cattle rhodopsin displayed two positive peaks ($[\theta]_{max}$ = 80 800 degrees at 335 nm, and $[\theta]_{max}$ = 42 600 degrees at 500 nm) at —195°C, whereas, bathorhodopsin displayed a positive peak ($[\theta]_{max}$ = 43 100 degrees at 334 nm) and a negative peak ($[\theta]_{max}$ = 163 000 degrees at 540 nm).

The change of the positive sign to negative one at α-band of circular dichroism spectrum supports the hypothesis that the conversion of rhodopsin is due to rotation of the chromophoric retinal about C-11—12 double bond ('photoisomerization model').

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Introduction

When rhodopsin absorbs a photon, it bleaches over a series of intermediates [1]. The photoconversion of rhodopsin to bathorhodopsin has been attributed to the photoisomerization of chromophoric 11-*cis* retinal to a twisted 11-*trans* form [2]. Recently, some strong objections were raised to the 'photoisomerization model' of the chromphore and a so-called 'proton translocation model' [3—5] was proposed, in which bathorhodopsin had 11-*cis* form [3,4] or a structure very close to 11-*cis* form [5]. In order to determine which model is correct, we tried to measure circular dichroic change in photoconversion of rhodopsin to bathorhodopsin at liquid nitrogen temperatures, since the circular dichroism (CD) has been regarded as a signal which reflects the asymmetric structure of the chromophoric retinal.

It has been reported that a free retinal solution does not show CD, while a retinal bound with specific protein such as retinol-binding protein [6] or aporetinochrome [7] displays CD. Rhodopsin and its intermediates in the bleaching also show CD at α- and β-bands [8—11]. Horwitz and Heller [12] reported that rhodopsin displayed no CD at liquid nitrogen temperatures and irradiation of rhodopsin at liquid nitrogen temperatures brought extraordinarily large signals, which were an artefact due to linear dichroism induced by polarized light as they mentioned later [13]. We shall call the artefact apparent circular dichroism (apparent CD). Now, we have examined the apparent CD in detail and measured the intrinsic CD of rhodopsin and bathorhodopsin at liquid nitrogen temperatures. The experimental results obtained support the isomerization model, i.e. the conversion of rhodopsin to bathorhodopsin is the photoisomerization of the chromophoric retinal from 11-*cis* form to a twisted 11-*trans* one.

Materials and Methods

Preparation of rhodopsin. All the experiments were carried out under dim red light. About 50 cattle retinas were shaken in M/15 phosphate buffer (pH 6.5) in order to detach rod outer segments from the inner segments. Rod outer segments were precipitated by centrifugation at $10\,000 \times g$ for 15 min and rinsed with the same buffer. The rod outer segments were purified by a sucrose floatation method (37% sucrose). After rinsing it with phosphate buffer several times and then with deionized water once, it was treated with 4% alum. After lyophylizating the tanned rod outer segments, the lipid in the rod outer segments was extracted with petroleum ether. Then, rhodopsin was extracted from the rod outer segments with 2% digitonin in M/15 phosphate buffer. The extract was concentrated by a colodion bag or by the ultracentrifugation ($105\,000 \times g$, 14 h), and then neutral NH_2OH (final concentration 0.1 M), and 2 or 3 vols. of glycerol were added. The sample was stored in a deepfreeze (−15°C) until use.

Optical Dewar. The two kinds of specially designed Dewars were used for measurements of absorption and circular dichroic spectra. One is made of quartz (Fig. 1a). The sample holder is directly immersed into the liquid nitrogen in the Dewar. The Dewar has a pair of windows made of optically

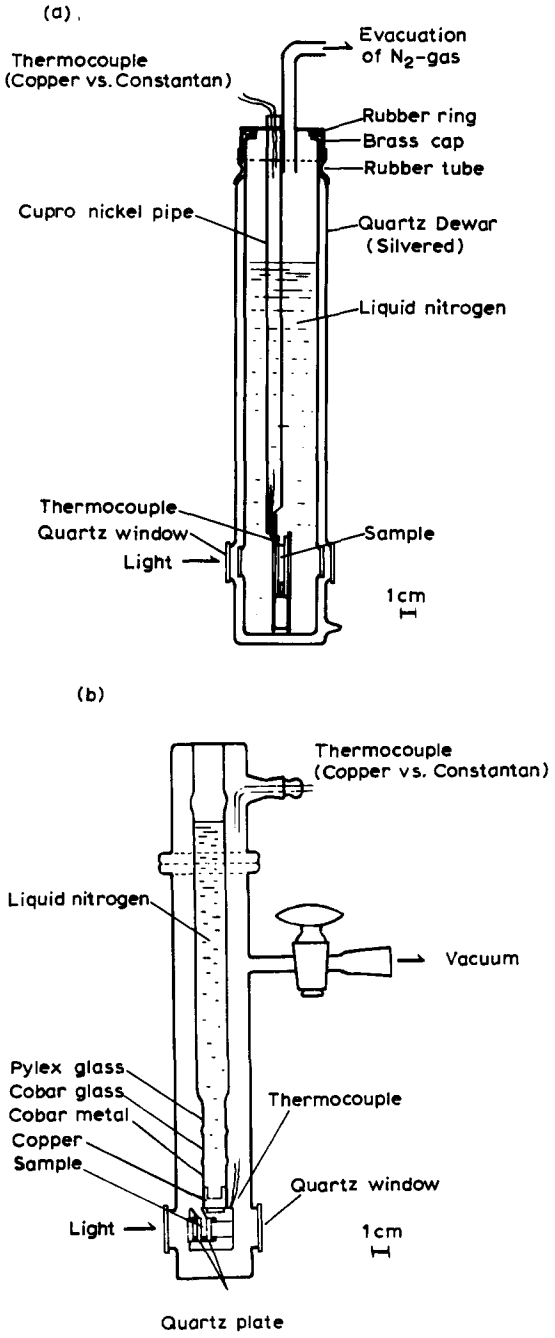

Fig. 1. Diagrams of Dewars for measurements of absorption and CD spectra at liquid nitrogen temperatures. (a) An immersion type: this Dewar is made of quartz. The sample in the optical cell (light path: 2.0 mm) is directly immersed into the coolant, liquid nitrogen. The brass cap and the quartz Dewar are sealed with the rubber tube during the experiment. (b) A heat conduction type: this Dewar is made of Pyrex glass except for optical windows (quartz) and the lower part of the cold finger with the sample holder made of pure copper. The cold finger is joined by the flange with the glass jacket. After injecting the sample into the optical cell (light path: 2.0 mm) which has been cooled by liquid nitrogen, the space between the cold finger and the jacket is evacuated by a rotary pump. In both the Dewars, all the glass parts are silvered except for the windows, and the temperature of the preparation is measured continuousely by a copper vs. constantan thermocouple inserted into the optical cell holder.

polished quartz plate. Since the sample is kept in the liquid nitrogen, the spectral measurement is interfered with bubbles of the nitrogen gas. For preventing the bubbling, the liquid nitrogen was cooled down below the boiling point by reducing the pressure in the liquid nitrogen container with a rotary pump. Absorption cell (light path 2 mm) was fixed at the end of cupro-nickel pipe soldered to a brass cap. The absorption cell is consisting of two pieces of quartz plate and a silicone rubber.

Another Dewar was made of Pylex glass. The optical cell in the Dewar described by Yoshizawa [1] was modified for measuring CD so that one may put the sample quickly into the optical cell cooled by liquid nitrogen (Fig. 1b).

Filling up the sample. One of the technical difficulties in the measurement of the CD spectra at low temperatures is to keep the preparation clear without any cracks, by which the measurement of CD was disturbed. Usually, for low-temperature spectroscopy, an organic solvent, for example ether/isopentane/C_2H_5OH (5 : 5 : 2, v/v), is used as the medium which formed no cracks at liquid nitrogen temperatures. For measuring the absorption spectra of rhodopsin, one cannot use these organic solvents because of denaturation of rhodopsin. Thus the rhodopsin extract was mixed with 2 or 3 vols. of glycerol and used as the sample for measurements of absorption and CD spectra of rhodopsin at liquid nitrogen temperatures. On cooling the sample to liquid nitrogen temperatures, some cracks are usually formed owing to volume contraction of the sample. In the measurement of absorption spectra, dispersion of the measuring light by the cracks has been conquered by use of an opal glass [2]. For measuring the circular dichroic spectra, however, it is necessary to freeze the sample clear without any cracks. When the mixture was injected into the precooled absorption cell (light path approx. 2 mm) by a syringe and then the cell was rapidly immersed into liquid nitrogen, the sample often froze clearly without any cracks (rapid cooling method).

Irradiation. A high-pressure mercury lamp (Ushio Co., HMB-500/B, 500 W) or a xenon lamp (Ushio Co., DSB-2000A, 2 kW) was used as a light source for irradiating the sample. The wavelengths were selected by a glass filter (Toshiba) with or without an interference filter (Nihonshinku Co. or Toshiba) inserted between the light source and the sample. The polarized light for irradiating the sample was obtained by use of a Polaroid filter (an attachment of Hitachi fluorescence spectrophotometer).

Instruments for spectroscopy. Absorption and CD spectra were measured with an automatic recording spectrometer (Hitachi, EPS-3T or 323) and with an automatic recording spectropolarimeter (JASCO, J-20), respectively. To minimize the photoconversion of sample by the measuring light, it was cut by a shutter attached on the instrument immediately after the measurements of the spectra, unless otherwise specified.

Results

Apparent circular dichroism

Since rhodopsin is a photosensitive pigment, it is necessary to check an effect of the measuring light on the sample. In the case of the measurements of absorption spectra and CD spectra, if the second scanning gives the same

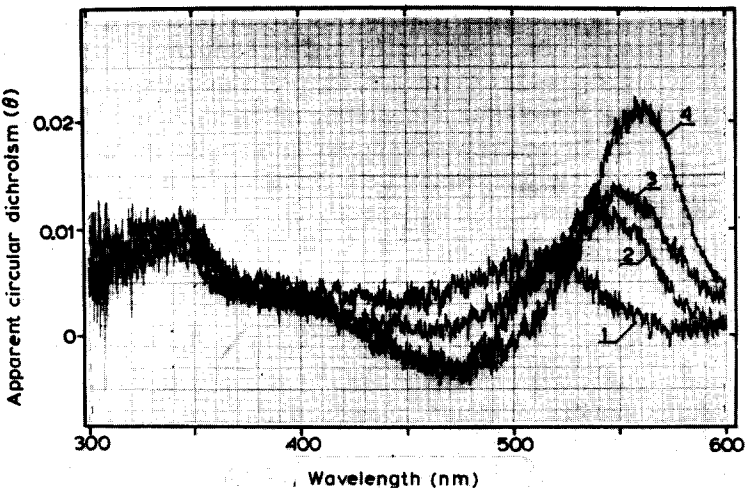

Fig. 2. Spectral change of apparent CD of rhodopsin preparation by scanning repeatedly. Curve 1, rhodopsin in glycerol/H_2O mixture (2 : 1, v/v) at −195°C was scanned from 600 nm to 300 nm. Curve 2, rescanned. Curve 3, the third scanning. After the third scanning (curve 3) the wavelength was reversed from 300 nm to 600 nm without cutting the measuring light by a shutter. Then the spectrum was measured (curve 4).

spectrum as the first one, one can regard it as no effect of the measuring light upon the sample.

Fig. 2 shows the spectral change of rhodopsin at −195°C by repetition of spectral scanning with the automatic recording spectropolarimeter under the same condition that the CD spectra of rhodopsin are usually measured at room temperature, in other words, that one cannot observe any bleaching of rhodopsin by the measuring light. The sample in Fig. 2 had some cracks. Measurements were performed from 600 nm to 300 nm at 4 s of time constant and the scanning speed was 20 nm/min. A peak lay at approx. 510 nm in the first scanned spectrum (curve 1 in Fig. 2). The repetition of the measurement under the same conditions shifted the peak to longer wavelengths with rise of the signal (curves 2 and 3 in Fig. 2). The curve-intersection point located near 520 nm. After the measurement of curve 3, the wavelength of the measuring light was returned to the original wavelength for the next measurement without cutting off the measuring light by the shutter. Then curve 4 was measured, which displayed further enhancement of the signal. Thus it is sure that the measuring light of the spectropolarimeter affected the sample and induced an extraordinarily large signal but it scarcely affected the absorption spectra. On the other hand, the measuring light of the spectrophotometer did not affect the absorption and CD spectra.

Irradiation with polarized or natural light

The spectral change in Fig. 2 is too large to be regarded as CD spectral change due to the formation of bathorhodopsin or isorhodopsin. Disch and Sverdlik [14] described that if one measures oriented pigments with a conventional spectropolarimeter, some linear dichroism is contained in the signal,

which we call apparent CD. Since irradiation of rhodopsin in a rigid solvent with polarized light may form a oriented system, linear dichroism should be induced. This possibility was examined by irradiating the sample with a polarized light at −195°C. In Fig. 3 the conversion of rhodopsin to bathorhodopsin was measured by both spectrophotometer and spectropolarimeter. Fig. 3a shows change of absorption spectrum of rhodopsin to that of a photosteady-state mixture composing rhodopsin, isorhodopsin and bathorhodopsin. As irradiation time went on, the absorbance in the longer wavelength region than 513 nm simply increased and that in the shorter wavelength region than 513 nm decreased (Fig. 3a and d).

On the other hand, the apparent CD spectrum increased at the beginning of the irradiation, reached the maximum and then decreased (Fig. 3b—d). At the maximum, the absorbance change was just at half-way (Fig. 3). This fact supports the view that the apparent CD change is mainly originated from photoinduced anisotropy, that is, linear dichroism in the sample [14]. If the apparent CD originates from only the linear dichroism, it should be equal in

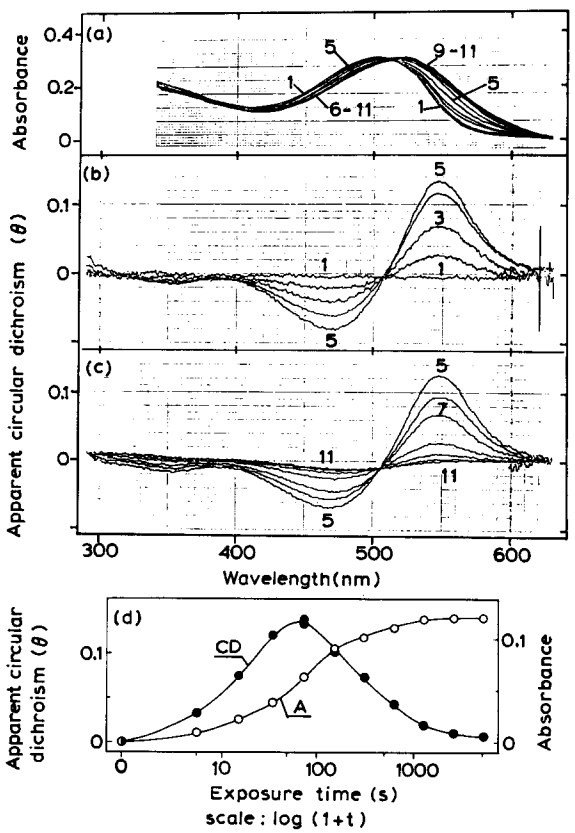

Fig. 3. Courses of spectral changes of absorbance (a) and apparent CD (b and c) in the conversion of rhodopsin to bathorhodopsin by irradiation with polarized light. Curves 1 (a and b), rhodopsin in glycerol/H_2O mixture (2 : 1, v/v) at −195°C. Curves 2—11 (a—c), products of irradiation with polarized light at 437 nm for a total of 5, 15, 35, 75, 155, 315, 635, 1275, 2555 and 5115 s (85 min 15 s). In d, increments of apparent CD (•) and absorbance (*A*) (○) at 550 nm were plotted against exposure time.

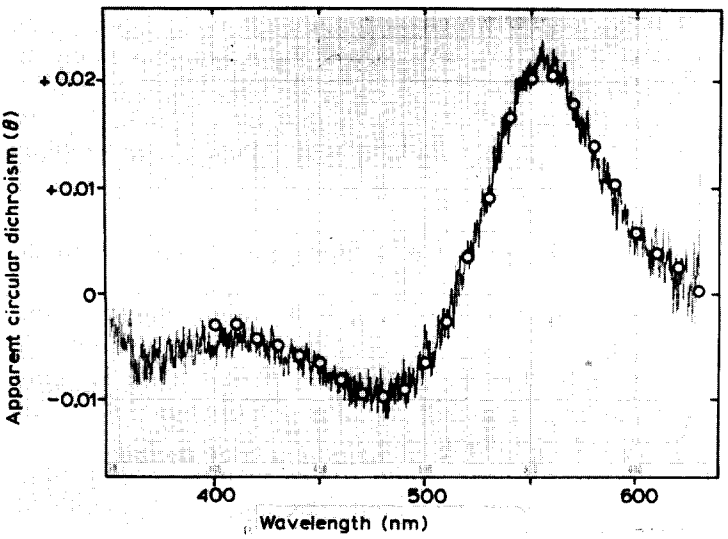

Fig. 4. Comparison of the apparent CD spectrum with the difference spectrum between rhodopsin and bathorhodopsin. The jagged spectrum represents an apparent CD spectrum of rhodopsin/glycerol mixture (1 : 2, v/v) which was obtained by scanning simply repeated 12 times. The spectrum was scanned at 2 nm/min and with a 4-s time constant. ○, the difference spectrum between rhodopsin and bathorhodopsin. The maximum absorbance of the difference spectrum was adjusted to that of the jagged spectrum.

shape to the difference spectrum between rhodopsin and its photoproduct, bathorhodopsin. Fig. 4 shows that this is indeed the case.

The changes of apparent CD signals against the exposure time, were shown in Fig. 5. The irradiation of rhodopsin at −195°C with polarized (curve 1) or even with natural light (curve 2) induced apparent CD, because the natural light

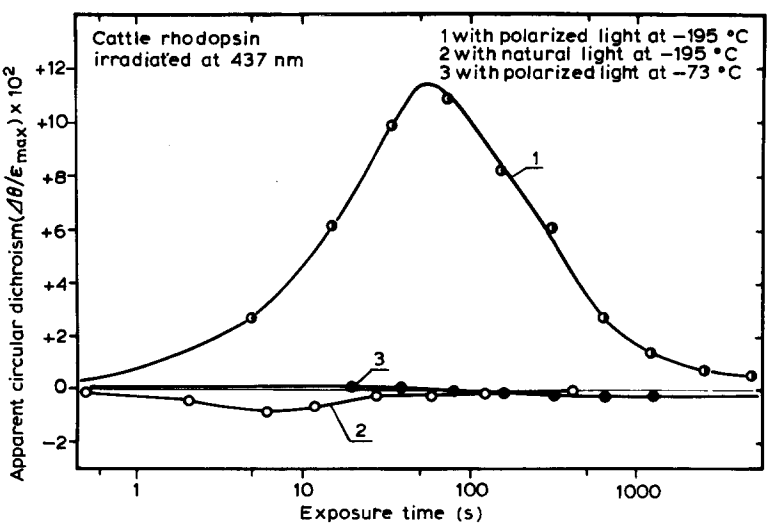

Fig. 5. Time course of formation of apparent CD. Cattle rhodopsin/glycerol mixture (1 : 2, v/v) was irradiated at 437 nm with polarized light at −195°C (curve 1), with natural light at −195°C (curve 2) or with polarized light at −73°C (curve 3). All the samples are equivalent in concentration of rhodopsin.

usually contains some polarized light which was induced by reflection on mirrors and others. Prolonged irradiation diminished the apparent CD signal. At a photosteady state the sample showed only intrinsic CD. While at −73°C the irradiation with polarized light did not induce apparent CD (curve 3), probably owing to movement of rhodopsin molecules and their photoproducts in 66% glycerol.

Circular dichroic spectra of rhodopsin and bathorhodopsin at liquid nitrogen temperatures

After measurements of absorption and CD spectra at room temperatures, the sample was cooled without any cracks by the rapid cooling method. We usually measured first an absorption spectrum and then CD spectrum. We confirmed that the absorption spectra scarcely changed before and after CD measurement at liquid nitrogen temperatures.

Curves 2 in Fig. 6a and b show absorption and CD spectra of rhodopsin at liquid nitrogen temperatures (−180°C), respectively. The absorption maximum of rhodopsin located at 505 nm which coincided with that reported previously [2]. The absorbance increased to 1.19 times by the cooling. The peaks of CD signals located at 335 nm and 500 nm at liquid nitrogen temperatures.

Then, the sample was irradiated at 437 nm. The time course of the photoconversion of rhodopsin to bathorhodopsin was monitored by measuring the absorption spectra. When the sample was irradiated for a total of 160 s, the bathochromic shift stopped. The formation of the photosteady state was confirmed by irradiating the sample for another 160 s. Then absorption and CD spectra of the photosteady-state mixture consisting of rhodopsin, bathorhodopsin and a little isorhodopsin were measured (curve 3 in Fig. 6a and b). In conformation of the view that the CD spectrum contained no linear dichroism, the scanning was repeated (curve 3′ in Fig. 6a). Curves 3 and 3′ in Fig. 6b perfectly coincided with each other. This is an assurance that the CD spectra displayed only intrinsic CD spectra without linear dichroism. Curves 3 and 3′ displayed a large negative CD near 540 nm, while the absorption spectrum showed a λ_{max} at 520 nm (curve 3 in Fig. 6a). Then the sample was warmed to room temperature (18°C) in order to convert bathorhodopsin into retinal-oxime and opsin, and then spectrum was measured. Absorbance at 500 nm (the isosbestic point between rhodopsin and isorhodopsin at room temperatures) decreased to 42% of the initial spectrum at room temperature. The sample was recooled to liquid nitrogen temperature (−180°C) by the rapid cooling method (curve 4). The sample also showed 42% of absorbance at 505 nm (the isosbestic point between rhodopsin and isorhodopsin at liquid nitrogen temperature) of the initial rhodopsin at liquid nitrogen temperature (−180°C). Thus 58% of rhodopsin converted into bathorhodopsin on irradiation at liquid nitrogen temperature.

Since retinal-oxime and opsin display no CD in the visible region [9], the subtraction of curve 4 from curve 3 gives us the intrinsic CD spectrum of bathorhodopsin. Since retinal-oxime has no absorbance in the wavelength region above 420 nm, the absorption spectrum of bathorhodopsin in this region was calculated according to the same method as that of CD.

The calculated spectra are shown in Fig. 7 in which the absorbance of the

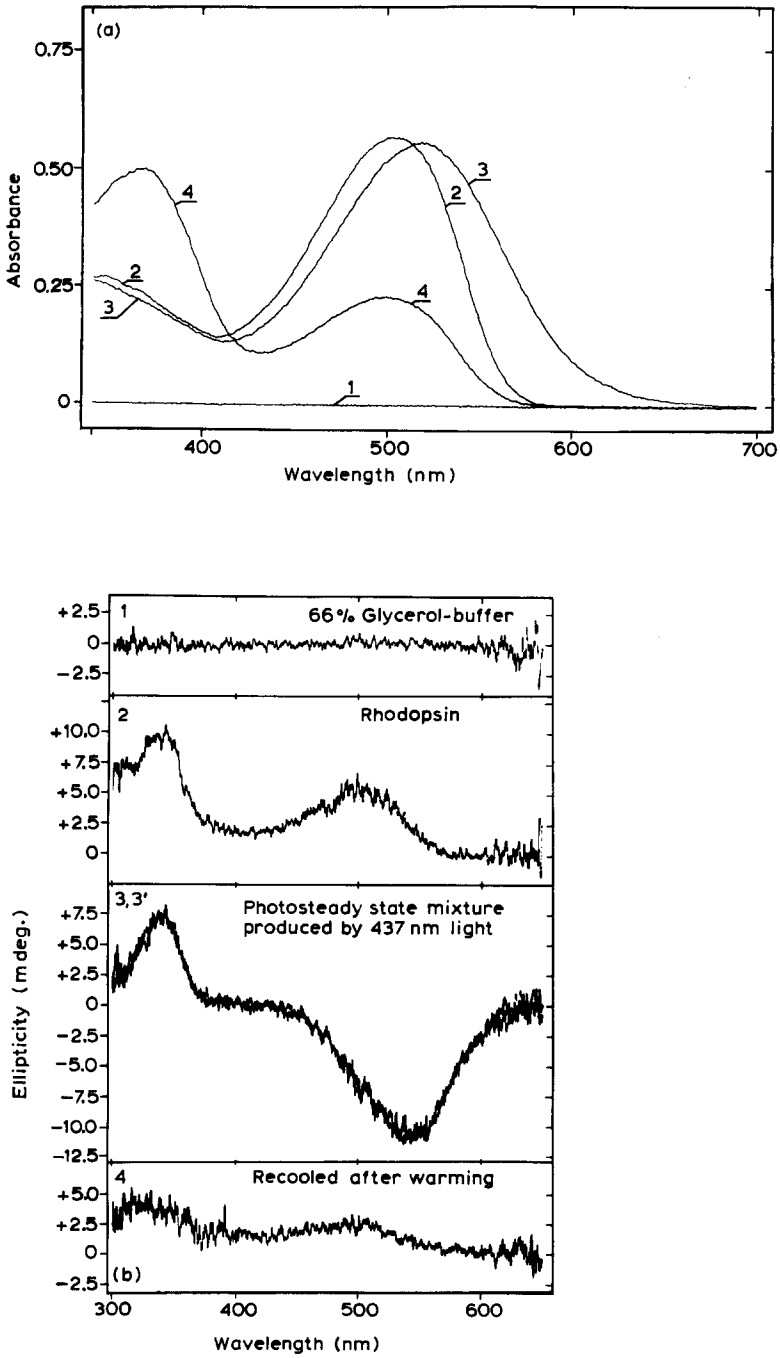

Fig. 6. Absorption (a) and CD (b) spectra. All spectra were measured at −190°C. Curve 1, base line, M/15 phosphate buffer/glycerol mixture (1 : 3, v/v). Curve 2, rhodopsin in the mixture. Curve 3, a photosteady-state mixture (mainly bathorhodopsin with rhodopsin and a small amount of isorhodopsin) produced by irradiating the rhodopsin with natural blue light (437 nm) for 5 min 20 s. Curve 3′ (in b only) rescanned. Curves 3 and 3′ perfectly coincide with each other. Curve 4, recooled to liquid nitrogen temperature after warming to room temperature.

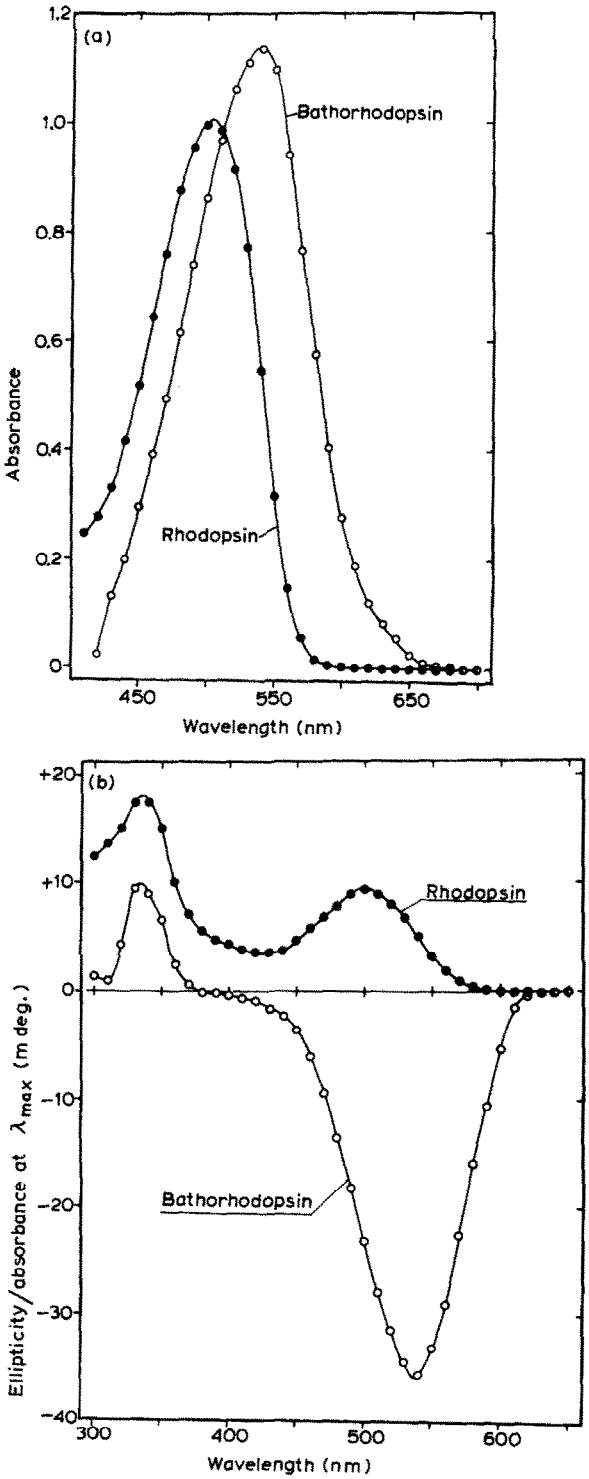

Fig. 7. Absorption spectra (a) and CD spectra (b) of equivalent concentrations of cattle rhodopsin and bathorhodopsin in glycerol mixture (1 : 3, v/v) at liquid nitrogen temperatures.

rhodopsin were normalized at 505 nm. The absorption spectrum of bathorhodopsin showed λ_{max} at 543 nm, and its relative extinction coefficient to rhodopsin was 1.13 (Fig. 7a), that is, the molar extinction coefficients of rhodopsin and bathorhodopsin were perfectly coincident with those reported by Yoshizawa and Wald [2].

The CD spectrum of bathorhodopsin displayed a large negative α-band and a small positive β-band (near 340 nm). The change of CD signal from positive to negative in the photoconversion of rhodopsin to bathorhodopsin, suggests a conformational change of the chromophoric retinal, that is, the photoisomerization of 11-*cis* retinal to a twisted 11-*trans* form.

The CD intensities of rhodopsin were 9.3 mdegrees at 500 nm and 17.6 mdegrees at 335 nm, while those of bathorhodopsin were −35.5 mdegrees at 540 nm and +9.4 mdegrees at 334 nm (Fig. 7b).

Since the molar extinction coefficient of rhodopsin is 40 600 at room temperature [15], at liquid nitrogen temperatures the molar ellipticity ($[\theta]_{max}$) of rhodopsin was estimated to be 42 600 degrees at 500 nm and 80 800 degrees at 335 nm and those of bathorhodopsin are 163 000 degrees at 540 nm and 43 100 degrees at 334 nm.

If the shape of absorption or CD spectrum is gaussian, the dipole strength (D) and the rotational strength (R) are calculated as follows:

$$D = 6.62 \cdot 10^{-34} \cdot \epsilon_{max} \cdot \Delta_{1/2}/\lambda_{max}$$

$$R = 5.01 \cdot 10^{-36} \cdot [\theta]_{max} \cdot \Delta_{1/2}/\lambda_{max}$$

where $\Delta_{1/2}$ is a band width of absorption or CD spectrum which is, in this case, the difference from the peak wavelength to the wavelength where the signal is $1/e$ of the peak absorbance or CD signal in the longer wavelength region than λ_{max} (e: base of natural logarithm). From Fig. 7, $\Delta_{1/2}$, extinction coefficients (ϵ_{max}) and molar ellipticities ($[\theta]_{max}$) were obtained. The dipole and rotational strengths of α-bands of rhodopsin were $82.0 \cdot 10^{-36}$ cgs unit and 0.58 Debye magneton, respectively, while those of bathorhodopsin were $87.0 \cdot 10^{-36}$ cgs unit and 2.08 Debye magneton, respectively.

Discussion

Our experimental results clearly demonstrated that the extraordinarily large CD signal at liquid nitrogen temperatures observed in Fig. 2 is attributed to the linear dichroism due to anisotropy in the sample induced by polarized light. Some years ago, Horwitz and Heller [12] reported that illumination of rhodopsin at 500 nm at −195°C produced a CD spectrum with a positive peak at approx. 560 nm and a negative peak at approx. 480 nm, and further illumination with 560 nm light produced another CD spectrum with a negative peak at approx. 540 nm (this wavelength should be 550 nm, judging from Figs. 4, 6 and 8 in their paper) and a positive peak at approx. 460 nm. According to our estimation, the former spectrum corresponds to the difference absorption spectrum between rhodopsin and bathorhodopsin, and the latter to that between isorhodopsin and bathorhodopsin. Thus, their experimental results can

be fully explained by simple interconversion among rhodopsin, bathorhodopsin and isorhodopsin by light.

They also described that a CD band of rhodopsin at 490 nm disappeared by cooling to liquid nitrogen temperatures. However, our measurement, which had been done by use of the clear sample without any crack, clearly showed CD spectrum of rhodopsin at liquid nitrogen temperatures. Because the apparent CD signal is much bigger than the intrinsic CD signal, they may have failed to observe the CD peak of rhodopsin.

Three possible explanations as to the origin of the induced CD of rhodopsin have been proposed [11,16] : (1) Only one of enantiomers of 11-*cis* retinal can bind with opsin. Since the enantiomer has an asymmetric structure, it must be optical active [17]. (2) Opsin has an asymmetric structure, to which a retinal can bind by changing its conformation. As the results the optical activity of the retinal molecules can be induced. These two explanations can be regarded as 'intrinsic model', because the optical activity is derived from the twisted structure of retinal in the cleft of opsin [8]. According to this model, the big change of CD spectrum, that is, change of the CD signal from positive to negative and increase of the CD intensity, associated with the conversion of rhodopsin to bathorhodopsin, strongly suggests that the chromophore of bathorhodopsin is very different in conformational structure from that in rhodopsin, probably the chromophore in bathorhodopsin twisting with a different direction from that in rhodopsin. Thus the photoconversion of rhodopsin to bathorhodopsin should be photoisomerization of the chromophore from 11-*cis* retinal to a twisted 11-*trans* form.

The third explanation is that the induced CD is derived from coupling of two oscillators. According to Kropf et al. [17], one oscillator is the chromophoric retinal and another is a tryptophan residue (j) in opsin. If the plane of oscillator of tryptophan is assumed to be parallel to that of retinal, the rotational strength can be expressed as follows:

$$R_{ij} = 1.7 \cdot 10^{54} \cdot \frac{\nu_i \nu_j D_i D_j}{r^2(\nu_i^2 - \nu_j^2)} \sin 2\theta$$

where suffix i and j represent retinal and tryptophan, respectively, ν is the wavenumber of the oscillator, D is the dipole strength, r is the distance between two oscillators and θ is an azimuthal angle between two oscillators. The dipole strength of tryptophan (D_j) and its wavenumber (ν_j) were estimated to be $9.5 \cdot 10^{-36}$ cgs unit and 35100 cm^{-1}, respectively [17]. Moreover, we calculated the values of ν_i, and D_i from the experimental results.

If θ is assumed at $\pm 45^\circ$ where the two oscillators most strongly interact on each other, r values are calculated at 2.1 Å in bathorhodopsin and 4 Å in rhodopsin. The calculated distance may be too short to regard the oscillators as only two dipoles. But the CD signal may be constructed from the sum of five interactions since rhodopsin has five residues of tryptophan [18].

The above calculation might suggest that the average distances between retinal and five tryptophans are shortened by the conversion of rhodopsin to bathorhodopsin. In addition, the change of sign from positive to negative strongly suggests the change of the azimuthal angle caused by structural change of the chromophoric retinal, that is, *cis-trans* isomerization about C-11—12

double bond, because rearrangements of tryptophans may not occur at liquid nitrogen temperatures.

The conversion of rhodopsin to bathorhodopsin has been suggested to be photoisomerization of the chromophore from 11-*cis* retinal to a twisted 11-*trans* form since Yoshizawa and Wald reported [2]. Recently some objections to the 'photoisomerization model' were raised and a photo-induced proton or charge translocation model ('proton translocation model') [3,4] without any rotation of C-11—12 double bond in the chromophoric retinal was proposed. If it were correct, the circular dichroism of bathorhodopsin originated from the retinal chromophore should not change remarkably. The present results strongly suggest that the chromophore of bathorhodopsin is a twisted all-*trans* form. Using our preliminary results, Kakitani et al. [19] have calculated the structure of chromophoric retinal in bathorhodopsin by extended Hückel approximation on the assumption that double bonds as well as single bonds in retinal can be twisted, and indicated that the chromophoric retinal in bathorhodopsin may be a twisted all-*trans* form.

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