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Bacteriorhodopsin analogues regenerated with enantiomers of 5,6-epoxyretinal

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Two enantiomers of 5,6-epoxyretinal, (5S, 6R) and (5R, 6S), were optically resolved using a chiral column. Each enantiomer showing the positive circular dichroism (CD) peak at 245 nm ((+)-epoxyretinal) and the negative at 245 nm ((+)-epoxyretinal) was respectively added to apomembranes to regenerate the pigments. The absorption maximum of bacteriorhodopsin analogue complexed with (+)-epoxyretinal was 485 nm and the molecular arrangement in the membrane was the hexagonal lattice as observed in the native purple membrane. Corresponding to the crystalline arrangement of the pigments, the coupling CD peaks, the positive at 460 nm and the negative at 520 nm, were observed. On the other hand, the absorption maximum of bacteriorhodopsin analogue regenerated by addition of (−)-epoxyretinal was at 445 nm and the arrangement of pigments was paracrystalline as observed in the apomembrane. Then, the positive CD peak at 435 nm was observed. The half-time of pigment regeneration was 400 s for (+)-epoxyretinal and 48 s for (−)-epoxyretinal addition at 10 °C. Both pigments were incorporated into lipid vesicles and the light-induced H^+-pumping activity was compared. These bacteriorhodopsin analogues showed a proton-pumping activity with a rather low yield. When a racemic mixture of epoxyretinal was added to apomembranes, the chiral discrimination of chromophores was observed through the pigment formation. The optical purity of extracted chromophores for (+)-/(−)-epoxyretinal was approx. 7/3.

Introduction

Bacteriorhodopsin is the only protein in the purple membrane of *Halobacterium halobium*. A bacteriorhodopsin molecule functions as a light-driven proton pump [1–5]. Its chromophore, all-*trans*- and/or 13-*cis*-retinal, is bound via a protonated Schiff base to the ε-amino group of Lys-216 in the protein [6–10].

Many binding experiments with retinal analogues have been performed to elucidate the structure of the chromophoric site and the effect of the chromophore on the H^+ pumping [1,11]. Oesterhelt and Christoffel [12] showed that 5,6-epoxyretinal bound to bacterioopsin to form a 'yellow pigment', but the pigment did not mediate the photophosphorylation. 5,6-Epoxyretinal contains the oxygen atom bound to carbon-5 and carbon-6 in the cyclohexane ring and two enantiomers, (5S, 6R) and (5R, 6S) as shown in Fig. 1, are present. The increased steric requirement may give differences between both diastereomers, pigments regenerated by addition of each enantiomer, and may provide clues about the structure of the chromophoric site. Also, if the steric or electrostatic

Abbreviation: CD, circular dichroism.

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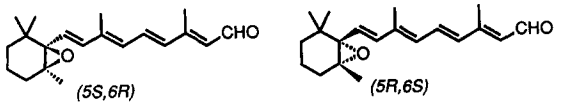

Fig. 1. Two enantiomers of 5,6-epoxyretinal.

interaction in the vicinity of the ring is strong, some chiral discrimination of the enantiomer would be observed during the formation of the pigment.

In this study, two enantiomers of all-*trans*-5,6-epoxyretinal were optically resolved using a chiral column and the pigments were regenerated by respective addition of each enantiomer to apomembranes. Characteristics of both pigments, absorption and CD spectrum, the arrangement of pigments in the membrane, light-dark adaptation and the activity of light-induced H^+ pumping were compared. Also, the chromophores extraced from the pigments which were reconstituted by addition of a racemic mixture of all-*trans*-epoxyretinal were analyzed with a chiral column to investigate the optical purity.

Materials and Methods

5,6-Epoxyretinal. Crystals of all-*trans*-5,6-epoxyretinal, a racemic mixture, were a generous gift of Hoffman-LaRoche Co Ltd. Also, all-*trans*-epoxyretinal synthesized in this laboratory was used. Synthesis and identification were performed according to the literature [13]. The purity of both specimens was more than 99% as judged by HPLC elution.

Protonated Schiff base. The Schiff base was prepared by addition of 5 μl monoethanolamine to 2 ml of 5,6-epoxyretinal in ethanol. Then, 10 μl 10 M HCl was added to prepare the protonated form. The absorption spectrum of protonated Schiff base was observed at pH 1.43.

Optical resolution of 5,6-epoxyretinal. A racemic mixture of epoxyretinal was optically resolved using Chiralcel OK column (4.6 × 250 mm, Daicel Chem. Ind. Ltd., Japan). The movile phase ratio for CH_3CN/H_2O was 45 : 55 (v/v) and the flow rate was 1.5 ml/min. Each of resolved enantiomer was mixed with a small amount of CH_2Cl_2. After shaking in a separatory funnel, dichloromethane layer, the top layer, was decanted. Recovered enantiomers were respectively applied to Zorbax SIL column (4.6 × 250 mm, Dupont Co. Ltd.) in order to separate the stereoisomers. The movile phase ratio for diethylether/ethanol/hexane was 12 : 0.2 : 87.8 (v/v) and the flow rate was 1.5 ml/min. Purified each enantiomer of all-*trans*-epoxyretinal was dissolved in ethanol and stored in the light-tight container at −80 °C.

Preparation of membrane specimens. Purple membranes were isolated from *Halobacterium halobium* R_1 according to the method of Oesterhelt and Stoeckenius [14]. Brown membranes were purified from cells cultured in the presence of 1.5 mM nicotine [15]. Apomembrane was prepared by bleaching the purple membrane in the presence of hydroxylamine as described in the literature [16]. The retinaloxime in membranes was removed by washing with bovine serum albumin solution [17]. Briefly, purple membranes containing 10 mg of bacteriorhodopsin were suspended in 0.5 M NH_2OH/0.1 M Tris-HCl/3 mM NaN_3. The final pH was adjusted to 7.5 with NaOH. The specimen was illuminated using a slide projector equipped with a 500 W tungsten lamp at 25 °C under constant stirring. The light was passed through a Y-52 Toshiba filter ($\lambda > 520$ nm). After bleaching, the specimen was washed twice with 20 mM Tris-HCl/3 mM NaN_3 solution (pH 7.2) by centrifugation to remove free hydroxylamine. The second precipitate was resuspended in 5% bovine serum albumin/3 mM NaN_3/20 mM Tris-HCl solution (pH 7.2). Following the stirring for 5–12 h in ice, the specimen was centrifuged at 100 000 × *g* for 30 min. This procedure was repeated until A_{360} of retinaloxime was almost diminished in the supernatant. Following washing with 20 mM Tris-HCl/3 mM NaN_3 (pH 7.2), the suspension was briefly sonicated. The specimen was chromatographed on a Bio-gel A-0.5 m column (2 × 40 cm) in 20 mM Tris-HCl/3 mM NaN_3 solution (pH 7.2) to remove bovine serum albumin. Fractions containing apomembranes were pooled and used for pigment regeneration.

When the phenomenon of chiral discrimination was investigated, the molar excess of a racemate of 5,6-epoxyretinal was added to apomembranes. Prior to the extraction of chromophores from re-

generated pigments, nonbonding, excess, epoxyretinal was removed by washing with cholic acid. Briefly, reconstituted membranes were centrifuged at 100000 × *g* for 30 min and the precipitate was dispersed in 20 mM Tris-HCl/3 mM NaN_3 solution (pH 7.5) containing 5% (w/v) cholic acid. After 5–12 h incubation in ice in the dark, the specimen was centrifuged. This procedure was repeated until the absorbance at 360 nm in the supernatant almost disappeared. The final specimen was washed twice with 20 mM Tris-HCl/3 mM NaN_3 (pH 7.2) and the chromophore was extracted.

Reconstitution of pigments. All-*trans*-retinal in ethanol was added to aliquots of the prepared apomembrane so as to the retinal being slightly excess. From A_{560}, the concentration of bacteriorhodopsin in the membrane was estimated with the molar extinction coefficient 54000 $M^{-1} \cdot cm^{-1}$ for dark-adapted bacteriorhodopsin [18]. This value was also confirmed in the present study. Then, a racemic mixture or each enantiomer of epoxyretinal dissolved in ethanol was respectively added so that the pigment formation ratio became to the desired value. The concentration of all-*trans*-epoxyretinal was estimated using the molar extinction coefficient 45300 $M^{-1} \cdot cm^{-1}$ [19]. Just before addition, the isomer composition was checked with HPLC. The volume of added ethanol was less than 1% of each specimen.

Chromophore extraction. Chromophores in regenerated pigments were extracted according to the methods of Gärtner et al. [20] and/or Suzuki et al. [21]. For both methods, it was confirmed that over 70% of chromophore in purple membranes could be extracted after three repetitions of the procedure.

Circular dichroism and absorption spectrum. Hitachi 200-20 or Union SM401 spectrophotometer was used for absorption measurements. CD spectrum was recorded with JASCO J20C spectropolarimeter.

X-ray diffraction. Fine focus rotating anode X-ray generator, Rigaku RU-100 unit, was used, which was operated at 40 kV and 20 mA. The Ni-filtered CuKα radiation, $\lambda = 0.1542$ nm, was incident in an Elliott toroid camera [22]. X-ray diffraction patterns were recorded on Sakura cosmic ray film. Unoriented and oriented membranes for X-ray diffractions were prepared according to the literature [23].

Measurement of light-induced H^+-pumping. To compare the H^+-pumping activity of bacteriorhodopsin analogues, pigment-lipid vesicles having the same orientation of pigments were prepared as follows. Retinaloxime-removed apomembranes were incorporated into crude soybean lipid vesicles by the method of Racker et al. [24]. Crude soybean lecitin purchased from Nakarai Chemicals (Japan) was used without further purification. The weight ratio for bacterioopsin/lipid was 1 : 60. Bacterioopsin-lipid vesicles were divided into four portions and all-*trans* isomers of retinal and optically resolved two enantiomers of epoxyretinal were successively added to the aliquots. Following the addition of slight molar excess of chromophores, pH of the vesicle suspension was adjusted to 5.8–5.9 with HCl, and the light-induced pH change was measured. The light source was a 500 W tungsten lamp and irradiation was through the Toshiba Y-48 cut-off filter ($\lambda > 480$ nm). The bacterioopsin-lipid vesicles were used as a control specimen. The light-induced pH change of the suspension was measured using a Horiba 6327-10C combination electrode with a Horiba F-8AT pH meter. When the pH of the solution became to the constant value after turning off the illumination, 5 μl of 0.1 M HCl was added to the solution in order to calibrate the amount of the transfered protons.

Results

Optical resolution of 5,6-epoxyretinal

When a racemic mixture of 5,6-epoxyretinal was applied to Chiralcel OK column, two enantiomers were optically resolved as shown in Fig. 2a. The fraction of each peak was collected and rechromatographed using the same column in order to confirm the purity. As shown in Fig. 2b and c, each enantiomer was almost pure in recovered fractions. Then, all-*trans* and 13-*cis* isomer of each enantiomer were fractionated with Zorbax SIL column.

Fig. 3a and b show the absorption and CD spectrum of two enantiomers of all-*trans* isomer. Absorption maximum was at 365 nm and 249 nm in ethanol. Solid line in Fig. 3b is the CD spec-

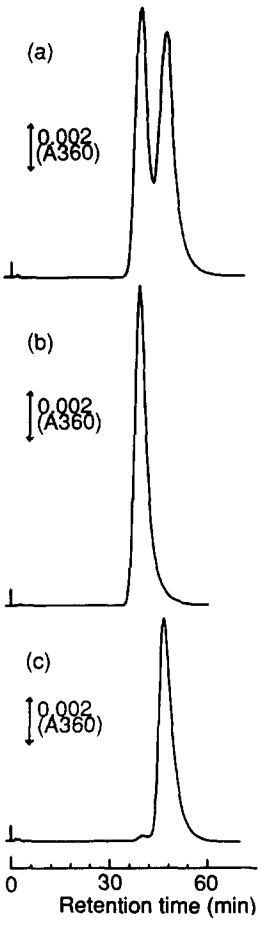

Fig. 2. Optical resolution of 5,6-epoxyretinal. (a) A racemic mixture of epoxyretinal was applied to the Chiralcel OK column and eluted with the movile phase of CH_3CN/H_2O (45:55). Elution was detected by the absorbance at 360 nm. Recovered each enantiomer was reapplied to the chiral column under the same conditions to confirm the purity. The elution profile of the fraction collected from the first peak (b) and the second (c) shows that these fractions are almost pure.

trum of enantiomer recovered from the first peak of the chiral column elution. The positive CD peak was observed around 245 nm and the weak negative peak was around 355 nm. The enantiomer recovered from the second peak showed the symmetrical spectrum as shown in the dashed line. Molar circular dichroism was 5.7 at 245 nm.

Fig. 3c and d show the absorption and CD spectrum of enantiomers of 13-*cis* isomer. Absorption maximum was at 358 nm and 253 nm in ethanol. Solid line and dashed line are the CD spectrum of the enantiomer derived from the first peak and from the second peak of chiral column elution, respectively. Symmetrical positive and negative CD peaks were observed around 250 and 350 nm. Molar circular dichroism at 250 nm was 10.3.

Two enantiomers of all-*trans* isomer were used to regenerate the pigment. In this paper, the enantiomer showing the positive CD peak at 245 nm and the negative peak at the same wavelength are termed as (+)-epoxyretinal and (−)-epoxyretinal, respectively.

Regeneration of pigments

Slight molar excess of all-*trans* retinal, at first, was added to aliquots of prepared apomembranes and the concentration of bacterioopsin in membranes was estimated as described in Materials and Methods. Then, all-*trans* isomer of (+)- or (−)-epoxyretinal was added to the remained membranes so that the pigment regeneration became to the desired ratio. Fig. 4 shows the absorption spectra associated with regeneration of pigments. Curves 1 and 2 represent 19% and 83% regeneration of (+)-epoxyretinal addition, respectively. For (−)-epoxyretinal addition, absorption spectra of 20% and 81% regeneration are shown in curves 3 and 4, respectively. In this paper, the regenerated bacteriorhodopsin analogues, bacteriorhodopsin-(+)-epoxyretinal complex and bacteriorhodopsin-(−)-epoxyretinal complex are termed as [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin, respectively. Despite the difference of regeneration ratio, the absorption maxima, 485 nm for [(+)-epoxy]bacteriorhodopsin and 445 nm for [(−)-epoxy]bacteriorhodopsin, were almost agreed for both pigments. The absorption maximum of the protonated Schiff base of 5,6-epoxyretinal with ethanolamine was 421 nm. The opsin shift, the difference in λ_{max} of pigment and protonated Schiff base in cm^{-1} [25], was 3100 cm^{-1} for [(+)-epoxy]bacteriorhodopsin and 1300 cm^{-1} for [(−)-epoxy]bacteriorhodopsin. The rate of regeneration, the half-time at 10 °C, were 400 s for [(+)-epoxy]bacteriorhodopsin and 48 s for [(−)-epoxy]bacteriorhodopsin.

The absorption spectrum of [(−)-epoxy] bacteriorhodopsin showed the shoulder around 490 nm. Since the absorption maximum of [(+)-epoxy]bacteriorhodopsin was around 485 nm, it was checked

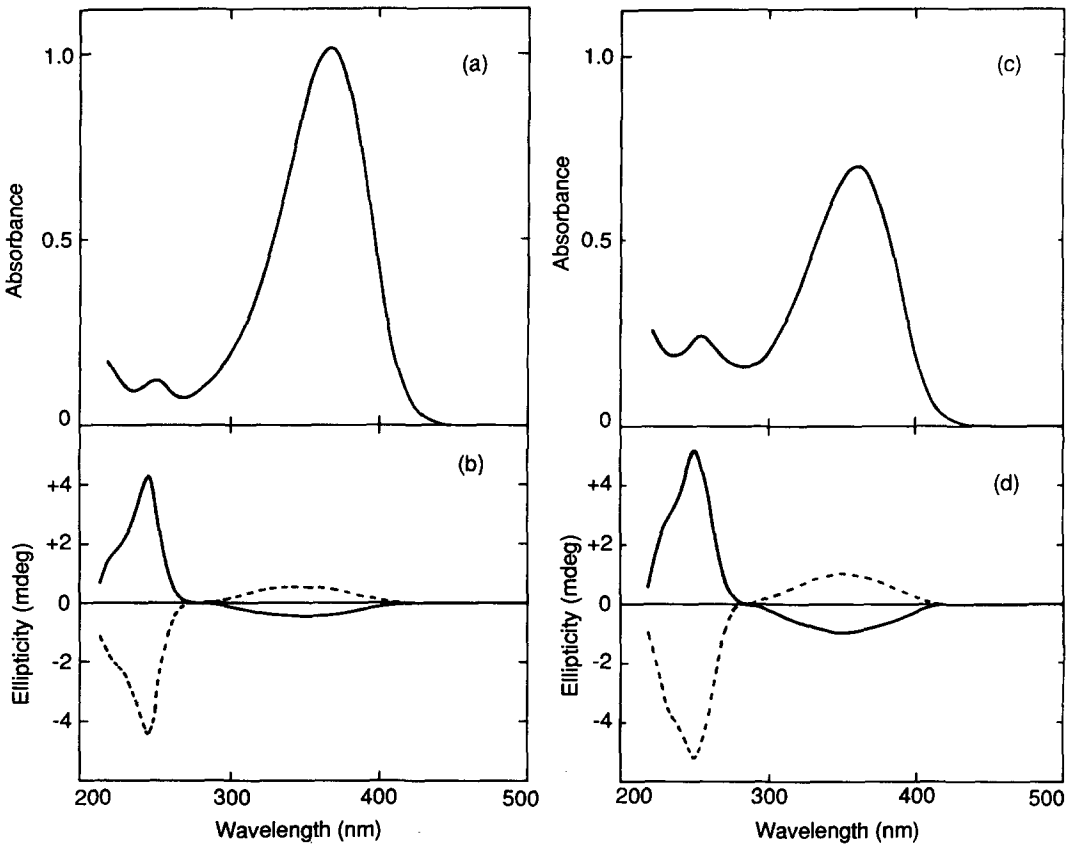

Fig. 3. Absorption and CD spectrum of 5,6-epoxyretinal enantiomers in ethanol. Absorption spectrum of (a) all-*trans* and (c) 13-*cis* isomer and the CD spectrum of two enantiomers of (b) all-*trans* and (d) 13-*cis* forms are shown. The solid and the dashed lines in (b) and (d) correspond to the first peak fraction and the second peak of a chiral column elution, respectively.

whether the racemization could occur during the pigment regeneration. Most of the chromophore extracted from [(−)-epoxy]bacteriorhodopsin was (−)-epoxyretinal and (+)-epoxyretinal was trace amount, which was the similar extent of the prepared enantiomer for pigment regeneration (Fig. 2c). The occurrence of the racemization in the process of pigment regeneration was not observed.

In the regeneration of bacteriorhodopsin analogue from all-*trans* naphthylretinal and bacterioopsin, it has been shown that two kinds of pigments having their absorption maxima around 500 and 440 nm are formed. Interconversion of these pigments takes place depending on the pH of the suspending medium, temperature and the mixing ratio of bacterioopsin and added chromophore [26]. The temperature dependence of absorption spectrum of [(−)-epoxy]bacteriorhodopsin was investigated. No remarkable increment of the 490 nm shoulder was observed in the range 5–45 °C.

Chromophore displacement

Fig. 5 shows the time-course of absorption spectra after addition of all-*trans* retinal to fully regenerated [(+)-epoxy]bacteriorhodopsin and to [(−)-epoxy]bacteriorhodopsin in the dark. The absorbance at 485 nm or 445 nm was slowly displaced by the 560-nm absorbance characteristic to the dark-adapted form of bacteriorhodopsin, indicating that retinal and two enantiomers of 5,6-epoxyretinal compete for a common binding site and that the binding of retinal to the bacterioopsin is stronger than epoxyretinal enantiomers. The half-time of chromophore replacement was about 8000 min for [(+)-epoxy]bacteriorhodopsin and about 260 min for [(−)-epoxy]bacteriorhodopsin

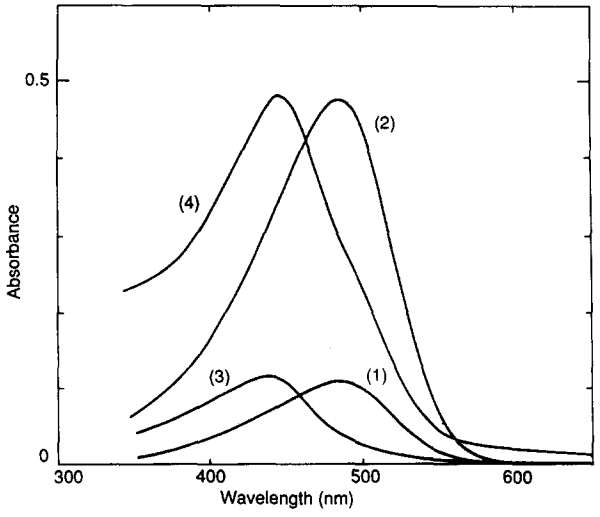

Fig. 4. The absorption spectrum of regenerated pigments. All-*trans* isomer of (+)- and (−)-epoxyretinal were respectively added to apomembranes. The regeneration ratio of (+)-epoxyretinal addition was 19% (1) and 83% (2) of total bacterioopsin. For (−)-epoxyretinal addition, the regeneration ratio was 20% (3) and 81% (4). The absorption spectrum was recorded against apomembranes in the references.

at 25°C. Interaction between (+)-epoxyretinal and bacterioopsin is stronger than that of (−)-epoxyretinal and bacterioopsin.

Molar extinction coefficient of pigments

The molar extinction coefficients (ϵ) of bacteriorhodopsin-epoxyretinal pigments were determined in three ways. Derived values are summarized in Table I. First, method 1, a slight molar excess of all-*trans* retinal, (+)-epoxyretinal and (−)-epoxyretinal were respectively added to apomembranes. From the ratio of absorbance maxima, [(+)-epoxy]bacteriorhodopsin/bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin/bacteriorhodopsin, ϵ of each bacteriorhodopsin analogue was calculated. Second, method 2, a definite amounts of each enantiomer, which were calculated so as to regenerate about 20% and 80% of total bacterioopsin in membranes, were added to apomembranes. From the difference of final absorbance maxima, ϵ was calculated. Two pairs of data are already shown in Fig. 4. The values for each sample represent the means ± SD from the analysis of six experiments. Third, method 3, the chromophore of regenerated [(−)-epoxy]bacterio-

TABLE I

MOLAR EXTINCTION COEFFICIENTS OF BACTERIORHODOPSIN-EPOXYRETINAL PIGMENTS

The three different ways for determining the ϵ of pigments are described in the text as Method 1, 2 and 3. BR, bacteriorhodopsin.

	$\epsilon(M^{-1}\cdot cm^{-1})$		
	method 1	method 2	method 3
[(+)-epoxy]BR	40000	41000 ± 3000	–
[(−)-epoxy]BR	41000	42000 ± 3000	40000

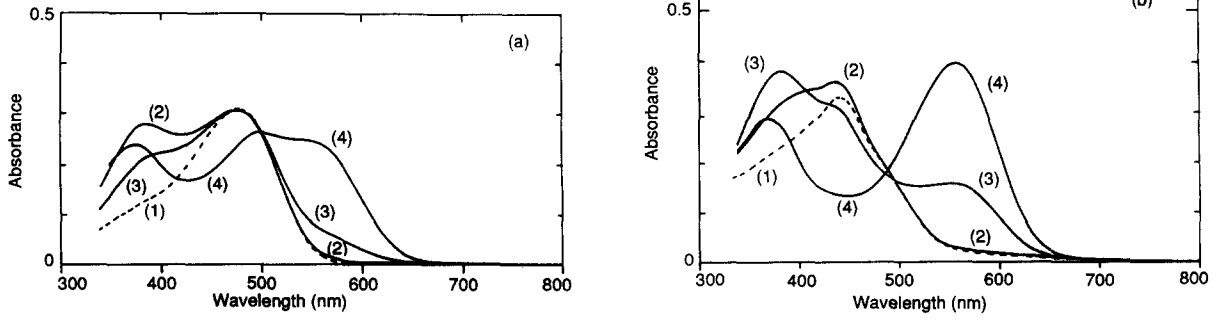

Fig. 5. Chromophore displacement of [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin following the addition of all-*trans* retinal. The time-course of absorption spectra is shown after addition of all-*trans* retinal to fully regenerated [(+)-epoxy] bacteriorhodopsin (a) and [(−)-epoxy]bacteriorhodopsin (b) in the dark at 25°C. The time after addition of retinal was 1 min (2), 150 min (3) and 9 days (4). The absorption spectra before retinal addition of both specimens are shown in curve 1 (dashed line).

rhodopsin was displaced by all-*trans* retinal as described above. Substantial decrease in absorbance at 445 nm and a concomitant absorbance increase at 560 nm were used to calculate ϵ. These values derived by three different ways were in fair agreement. The mean molar extinction coefficient was 41000 $M^{-1} \cdot cm^{-1}$ for ⁴h bacteriorhodopsin analogues.

Arrangement of regenerated bacteriorhodopsin analogues in the membrane

Fig. 6 indicates the CD spectrum of [(+)-epoxy] bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin in the membrane. The absorption spectra of the same specimens are already shown in Fig. 4. When the regeneration ratio of [(+)-epoxy]bacteriorhodopsin increased to 83%, bilobed CD peaks were observed around 520 and 460 nm as shown in Fig. 6a curve 2. In the case of 19% regeneration, the positive CD peak around 480 nm, near the absorption maximum, was observed but the negative 520-nm peak disappeared (curve 1). When retinal is added to apomembranes, it has been known that the shape of CD spectrum is dependent on the percentage of the retinal binding sites occupied, i.e., the percent regeneration of pigment. More than 50% regeneration, the bilobed exciton coupling bands due to the chromophore–chromophore interaction is observed in the visible CD spectrum [27]. Therefore, the positive and negative CD peaks at 460 and 520 nm observed in 83% regeneration of [(+)-epoxy]bacteriorhodopsin could arise from the same origin observed in purple membranes: a positive CD band associated with each (+)-epoxyretinal bound to protein plus a positive-negative pair of bands attributable to the interaction of the chromophores of separate pigments.

Fig. 6b shows the CD spectrum of [(−)-epoxy] bacteriorhodopsin. The regeneration ratios were 20% (curve 1) and 81% (curve 2). The positive CD peak was observed around 440 nm for 20% regenerated specimen. For the specimen of 81% regeneration, the positive peak around 435 nm and the shoulder around 490 nm were observed.

It is not evident if the regenerated [(−)-epoxy] bacteriorhodopsin in the membrane showed the coupling bilobed bands because the negative CD peak would be hindered by the positive CD centered around 435 nm. Also, it is necessary to confirm that the positive-negative CD bands observed in [(+)-epoxy]bacteriorhodopsin is attributable to the exciton interaction between chromophores of crystalline-packed pigments in membranes. Therefore, X-ray diffractions of both regenerated membranes were investigated to clarify the molecular arrangement.

Fig. 7 shows X-ray diffraction photographs and those densitometer tracings. As well known, X-ray diffraction of native purple membrane, shown in Fig. 7(a), indicates a series of crystalline reflections indexed by a two-dimensional hexagonal

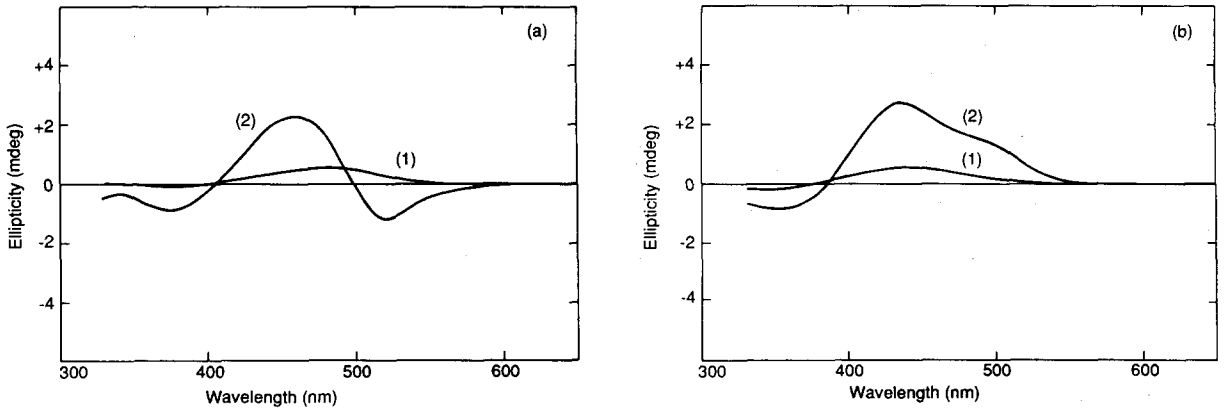

Fig. 6. CD spectra of (a) [(+)-epoxy]bacteriorhodopsin and (b) [(−)-epoxy]bacteriorhodopsin in the membrane. Specimens are the same as stated in the legend of Fig. 4.

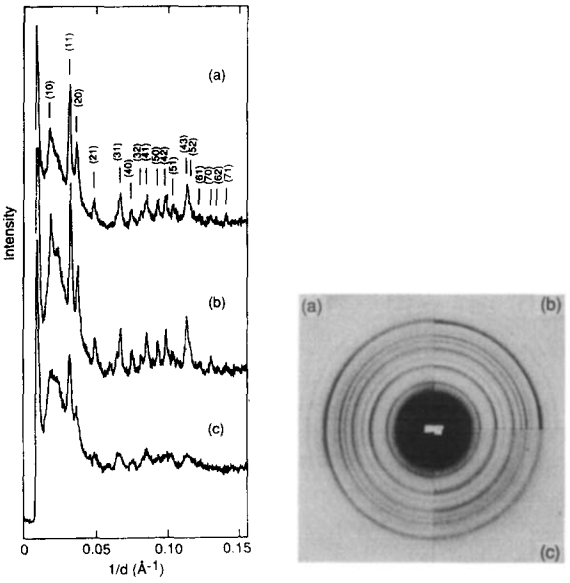

Fig. 7. X-ray diffraction photographs and those densitometer tracings. Diffractions of (b) [(+)-epoxy]bacteriorhodopsin and (c) [(−)-epoxy]bacteriorhodopsin, regenerated by addition of (+)- and (−)-epoxyretinal to apomembrane, are shown. Native purple membrane diffraction patterns (a) are demonstrated for comparison. Arrows and figures in (a) show the positions and the indexes of reflections calculated from a two-dimensional hexagonal lattice with a unit cell length of 6.3 nm. The abscissa is $1/d$, where d is the spacing given by $\lambda/2 \sin\theta$, λ being the wavelength and 2θ being the scattering angle.

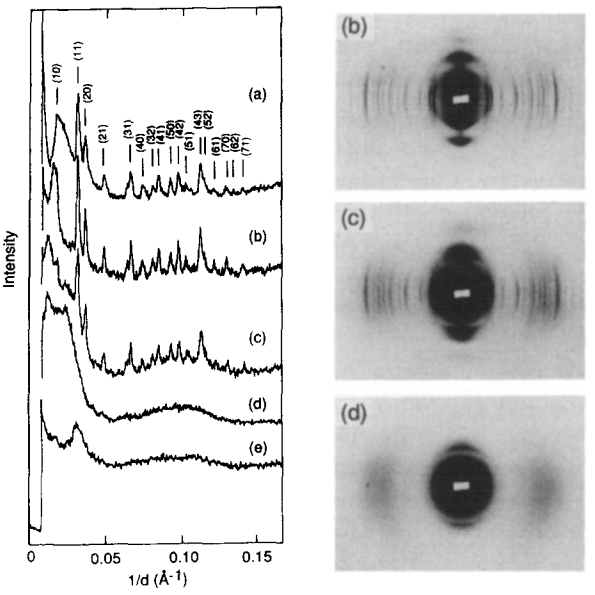

Fig. 8. X-ray diffraction patterns of brown membranes before and after the regeneration of pigments. Diffractions were shown for the specimens regenerated with retinal (b), (+)-epoxyretinal (c) and (−)-epoxyretinal (d) and the brown membrane before regeneration (e). Native purple membrane (a) was shown for comparison. Arrows and figures in (a) are the same as noted in Fig. 7.

lattice with a 6.3 nm cell-edge length [28]. Fig. 7b shows the diffraction of the membrane reconstituted by addition of (+)-epoxyretinal. Regenerated [(+)-epoxy]bacteriorhodopsin in the membrane gives the similar X-ray diffraction pattern as observed in native purple membrane: crystalline reflections were indexed by a hexagonal lattice with a 6.3 nm cell-edge length and the intensity distribution was practically indistinguishable. This diffraction supports that the bilobed CD peaks, shown in Fig. 6a, curve 2, is a consequence of a coupled oscillator type of interaction among the three retinal moieties present in [(+)-epoxy]bacteriorhodopsin trimer of the crystalline lattice.

Fig. 7c indicates the diffraction pattern of [(−)-epoxy]bacteriorhodopsin in the membrane. The width of reflections became broader with increasing the diffraction angle. This pattern is similar to that observed in apomembranes. The molecular arrangement of bacterioopsin is paracrystalline in apomembranes [29,30]. Disordered arrangement of [(−)-epoxy]bacteriorhodopsin would disturb the chromophore–chromophore interaction and cause the observation of positive CD band near the absorption maximum.

Fig. 8b–e show the diffraction patterns of brown membranes before and after the regeneration of pigments. The diffraction of native purple membrane, Fig. 8a, is given for comparison. The diffraction pattern of brown membranes before regeneration, Fig. 8e, showed noncrystalline reflections around $1/3$ nm^{-1} and 1 nm^{-1}, indicating that the bacterioopsin molecules are in the state of monomer and/or a small aggregates such as trimer. When all-*trans* retinal was added, Fig. 8b, the spontaneous crystallization of regenerated bacteriorhodopsin was observed as reported before [30]. Therefore, the brown membrane is favourable for the investigation whether the regenerated pigment forms a crystalline packing or not.

Fig. 8c is the diffraction of [(+)-epoxy]bacte-

riorhodopsin in brown membrane, indicating that the crystalline arrangement of pigments is formed after regeneration. On the other hand, as shown in Fig. 8d, the diffraction pattern of [(−)-epoxy]bacteriorhodopsin in brown membranes did not change appreciably from the original arrangement of bacterioopsin in brown membranes.

From these experiments, it was concluded that [(+)-epoxy]bacteriorhodopsin in the membrane spontaneously aggregated to form a hexagonal lattice as observed in purple membrane, but the regenerated [(−)-epoxy]bacteriorhodopsin remained in noncrystalline arrangement.

Light-dark adaptation

Light-dark adaptation of [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin was investigated bearing on the absorption spectrum and the chromophore composition. The isomer compositions extracted from regenerated pigments are summarized in Table II. The all-*trans* isomer was more than 95% for both dark-adapted pigments. Chromophores were extracted with two different methods [20,21]. Results were in fair agreement. The isomer compositions of used epoxyretinal enantiomers were respectively checked just before addition to apomembranes. They were almost pure all-*trans* isomer of each enantiomer and the 13-*cis* isomer was trace amounts. Therefore, the dark isomerization from the all-*trans* to the 13-*cis* form of chromophore is forbidden in both pigments. The molar extinction coefficients of dark-adapted pigments given in Table I correspond to those of specimens that all-*trans* isomer dominant.

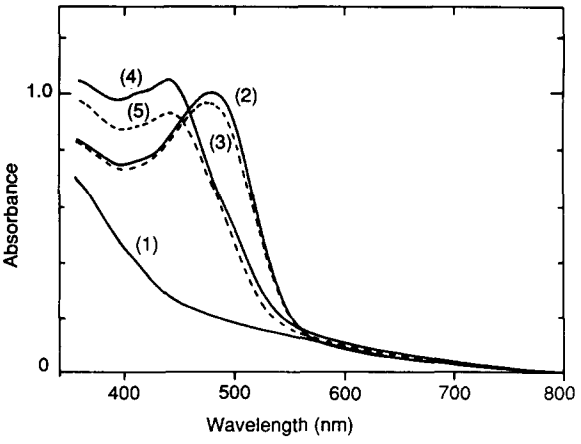

Fig. 9. Absorption spectrum of [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin before and after illumination. [(+)-epoxy]bacteriorhodopsin: (2) in the dark and (3) after 5 min irradiation. [(−)-epoxy]bacteriorhodopsin: (4) in the dark and (5) after 10 min illumination. Light source was a 500-watt tungsten lamp and illuminated through Toshiba Y-48 cut-off filter ($\lambda > 480$ nm). The absorption spectrum was recorded within 3 min after turning off the light. The reference was distilled water. Curve 1 is the absorption spectrum of membranes before regeneration, retinaloxime-removed apomembranes.

Fig. 9 shows the absorption spectra of [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin before and after irradiation. Since these

TABLE II

CHROMOPHORE COMPOSITIONS OF BACTERIORHODOPSIN-EPOXYRETINAL PIGMENTS

Chromophores in regenerated pigments were extracted by the procedures of Gärtner et al. [20] and of Suzuki et al. [21]. These methods were termed as 2-propanol method and HCHO method, respectively. Figures in parentheses are the numbers of experimental runs. BR, bacteriorhodopsin.

	13-*cis*/all-*trans* isomer				
	before illumination		just after illumination	standing in the dark after illumination	
	HCHO method	2-propanol method	HCHO method	24 h HCHO method	168 h HCHO method
[(+)-epoxy]BR	4:96 (2)	5:95 (2)	50:50 (2)	12:88 (1)	–
[(−)-epoxy]BR	8:92 (2)	4:96 (2)	52:48 (2)	–	39:61 (1)

spectra were recorded against distilled water in the reference cell, λ_{max} of each specimen was blue shifted comparing with those shown in Fig. 4. The light-adapted [(+)-epoxy]bacteriorhodopsin showed a 2-nm blue-shift, from 480 to 478 nm, in absorption maximum and the absorbance at λ_{max} (A_{max}) reduced by 5%. In the case of [(−)-epoxy] bacteriorhodopsin the change of λ_{max} did not rise appreciably before and after irradiation, but A_{max} reduced by 16% after yellow light illumination for 10 min. The chromophore composition just after irradiation indicated that 13-*cis* isomer increased to about 50% in both pigments as shown in Table II. Standing in the dark for 24 h at 25°C after irradiation, the isomer ratio for 13-*cis*/all-*trans* of [(+)-epoxy]bacteriorhodopsin restored to 12 : 88. However, for [(−)-epoxy]bacteriorhodopsin, the chromophore composition after 7 days incubation in the dark for 13-*cis*/all-*trans* had a ratio of 39 : 61. Dark-adaptation of [(+)-epoxy]bacteriorhodopsin was almost completed within 24 h, but [(−)-epoxy]bacteriorhodopsin was not even after 7 days under the same conditions.

Proton pumping

Light-induced proton-pumping activity of [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy] bacteriorhodopsin was investigated by incorporating into soybean lipid vesicles. The orientation of pigments in proteoliposomes, the ratio of molecules in inside-out and right-side-out directions, is known to be different from preparation to preparation [31]. Proteoliposomes having the same orientation of pigments were prepared as described in Materials and Methods. At first, bacterioopsins in apomembranes were incorporated into lipid vesicles and divided into four portions. In order to regenerate pigments, a slightly molar excess of all-*trans* retinal, (+)-epoxyretinal and (−)-epoxyretinal were added to each aliquot. Fig. 10 shows the absorption spectra of regenerated bacteriorhodopsin-lipid vesicles (1), [(+)-epoxy]bacteriorhodopsin-lipid vesicles (2) and [(−)-epoxy]bacteriorhodopsin-lipid vesicles (3). The absorption maxima which were observed against bacterioopsin-lipid vesicles in reference were 549 nm for bacteriorhodopsin, 444 nm for [(+)-epoxy]bacteriorhodopsin and 433 nm for [(−)-epoxy]bacteriorhodopsin in lipid vesicles. Visible CD

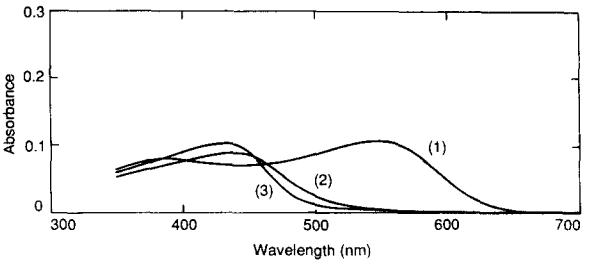

Fig. 10. Absorption spectra of bacteriorhodopsin analogues incorporated into soybean lipid vesicles. Absorption spectrum of bacteriorhodopsin (1), [(+)-epoxy]bacteriorhodopsin (2) and [(−)-epoxy]bacteriorhodopsin (3) in lipid vesicles were recorded against bacterioopsin-lipid vesicles in the reference cell. The weight ratio for protein/lipid was 1 : 60.

spectra of three regenerated specimens showed the positive CD peak around 510, 450 and 440 nm, respectively. The lipid-to-protein ratio was 60 : 1 in weight. Each pigment should dispersed in the monomeric state in proteoliposomes.

Fig. 11 indicates the light-induced pH change of each regenerated specimen under irradiation of yellow light ($\lambda > 480$ nm). The pH value of the vesicle suspension was adjusted to 5.8–5.9 with HCl before measurement. In all specimens, light-induced alkalization of suspension was observed. An extent of maximum proton uptake of unmodified bacteriorhodopsin-lipid vesicles, Fig. 11(1), was about 0.22 pH, i.e., 450 nmol H^+/mg bacteriorhodopsin. Preferentially oriented pigments are well incorporated into vesicles.

Light-induced pH-change of bacterioopsin-lipid vesicles, the control experiment, is shown in Fig. 11(4). A small pH change, an extent of maximum proton uptake of 0.015 pH, was observed. This would be due to a small amount of bacteriorhodopsin remained in apomembranes without bleaching.

Fig. 11(2) and (3) show the light-induced proton-uptake of [(+)-epoxy]BR and of [(−)-epoxy] bacteriorhodopsin, respectively. A maximum proton-uptake was 0.029 pH for [(+)-epoxy]bacteriorhodopsin and 0.017 pH for [(−)-epoxy]bacteriorhodopsin. The pH change of [(+)-epoxy]bacteriorhodopsin was about 7% of bacteriorhodopsin-lipid vesicles and could be regarded that it was larger than that of control experiment as significant. Proton uptake of [(−)-epoxy]bacteriorhodopsin was comparable to that of control speci-

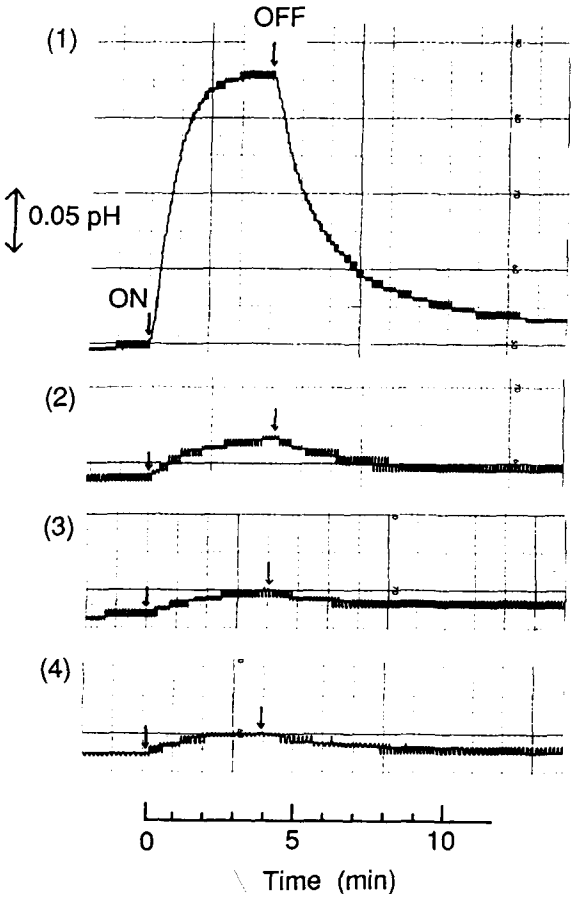

Fig. 11. Light-induced pH change of pigment-lipid vesicles. Bacteriorhodopsin-lipid vesicles (1), [(+)-epoxy]bacteriorhodopsin-lipid vesicles (2), [(−)-epoxy]bacteriorhodopsin-lipid (3) vesicles and control (4). The control specimen was prepared by incorporation of apomembranes into lipid vesicles. Initial pH of the suspension was adjusted with HCl to 5.8–5.9 before measurement. The light source was the same as described in Fig. 9. The concentration of valinomycin was 0.75 μM. The temperature was maintained within 25 ± 1° C.

men, indicating that the activity of [(−)-epoxy] bacteriorhodopsin is smaller than that of [(+)-epoxy]bacteriorhodopsin. It was concluded that the bacteriorhodopsin analogues containing 5,6-epoxyretinal enantiomers as chromophore, at least one of them, have a light-driven proton-pump activity with a rather low yield.

Chiral discrimination of chromophores

When a racemic mixture of epoxyretinal was added to apomembranes, it was investigated whether the chiral discrimination of chromophores was observed in the course of pigment regeneration. Curve 1 in Fig. 12a is the absorption spectrum of apomembranes. The peak around 360 nm is due to the retinaloxime remained in apomembranes. Curve 2 indicates the absorption spectrum of membranes regenerated by addition of two-fold molar excess of epoxyretinal, a racemic mixture, to apomembranes. Regenerated pigments showed the absorption peaks around 440 nm and 500 nm. The absorption of excess epoxyretinal was overlapped on the peak of retinaloxime around 360 nm. Fig. 12b is the CD spectrum of regenerated membranes. Negative CD peak around 500 nm and positive peaks around 430 and 350 nm were observed.

Nonbonding, excess, epoxyretinal in reconstituted membranes was removed by washing with 5% cholic acid prior to extraction of chromophores. Fig. 12c is the absorption spectrum of the cholate-treated specimen. The absorption maximum was around 466 nm and the peak around 360 nm, observed in (a), almost disappeared. Regenerated pigments were remained in membranes without extraction, but most of non-bound epoxyretinal and retinaloxime were removed by washing with cholic acid.

Fig. 12d is the CD spectrum of cholate-treated membranes. The positive and negative bands remained around 500 and 440 nm after washing, but the positive CD around 350 nm which was observed in (b) diminished. The 350-nm CD peak is also observed in apomembranes. Therefore, the CD extremum near 350 nm is due to the retinaloxime formed in apomembranes. Crystalline packing of regenerated pigments and bilobed CD spectrum were observed for [(+)-epoxy]bacteriorhodopsin as described above (Figs. 6 and 7). It was expected that one of enantiomers, (+)-epoxyretinal, was preferentially bound to bacterioopsin when a racemate was added and caused the exciton coupling of chromophores in the crystalline-packed pigments. Therefore, the chromophore of regenerated membranes was extracted after washing with cholic acid and the ratio of enantiomers, (+)-/(−)-epoxyretinal, was analyzed using a chiral column.

Fig. 13a shows the elution profile of extracted chromophores through Zorbax SIL column. The

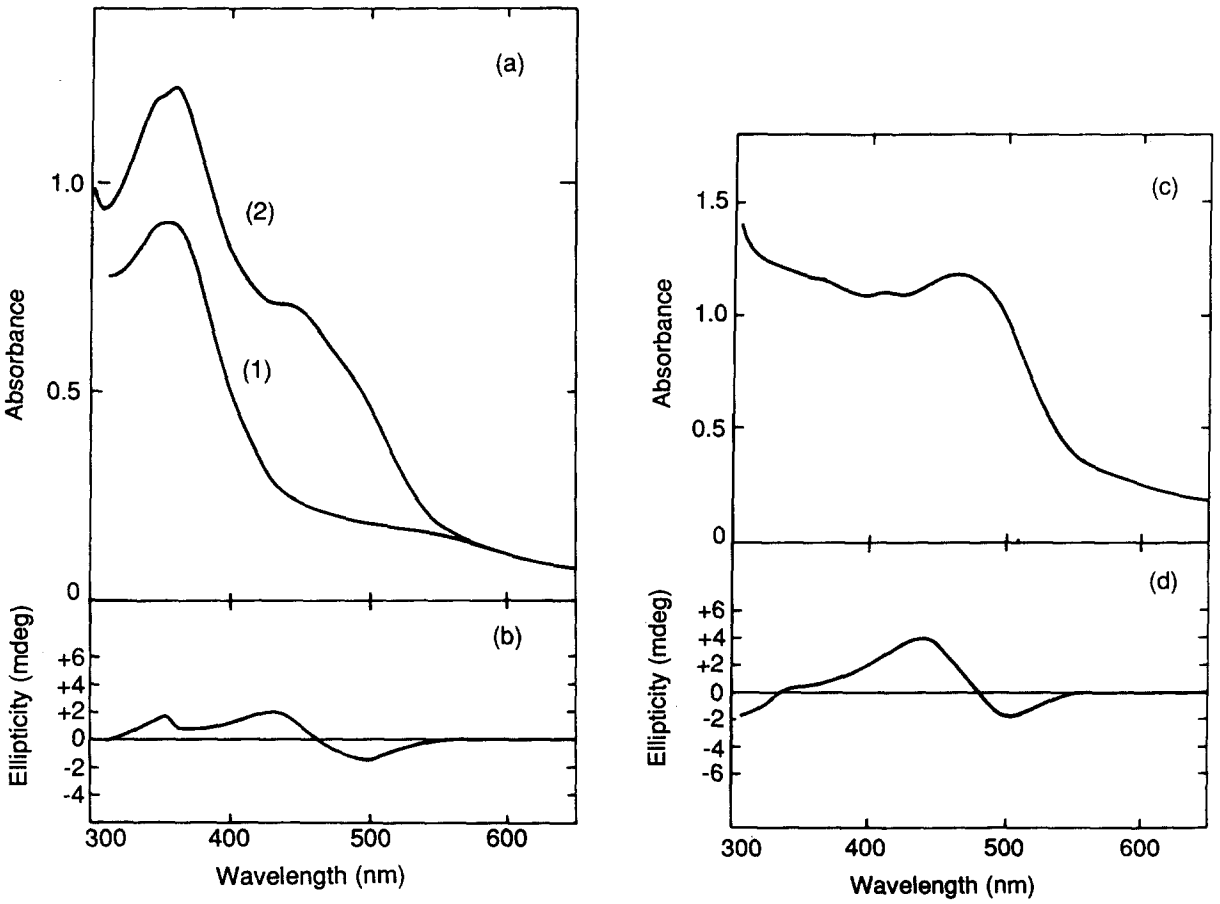

Fig. 12. Absorption and CD spectrum of pigments regenerated by the addition of a racemate of epoxyretinal to apomembranes. (a) Absorption spectrum: before (1) and after (2) addition of 2-fold molar excess of epoxyretinal. The absorption spectrum was recorded against distilled water in the reference. Apomembranes were used without removal of retinaloxime. (b) CD spectrum of regenerated membranes. Excess epoxyretinal and retinaloxime in reconstituted membranes were removed by washing with cholic acid. The absorption and CD spectrum after cholate treatment are shown in (c) and (d), respectively.

all-*trans* isomer of epoxyretinal was collected and applied to Chiralcel OK column to investigate the optical purity. The elution profile is shown in Fig. 13b. Each of the optically resolved enantiomers, (+)- and (−)-epoxyretinal, was respectively applied to a chiral column and subsequently eluted under the same conditions. The elution profile of recovered chromophores was divided into two areas by fitting the elution profile of each enantiomer assuming that the front half of the first peak in the elution profile consisted of pure (+)-epoxyretinal. The area ratio, the optical purity, for (+)-/(−)-epoxyretinal was approx. 73 : 27. When a racemate of epoxyretinal was added to apomembranes, some chiral discrimination of chromophores was observed during the pigment regeneration.

It was investigated whether the retinaloxime remained in apomembranes caused the chiral discrimination of chromophores. The retinaloxime-removed apomembrane was prepared by washing with bovine serum albumin. Subsequently, the pigments regeneration, the washing with cholate, the extraction of chromophores and the analysis of chiral column elution were performed as described above. The optical purity of recovered all-*trans* epoxyretinal chromophores for (+)-/(−)-epoxyretinal was approx. 77 : 23. The similar value of optical purity was derived despite the presence or absence of the retinaloxime, indicating that reti-

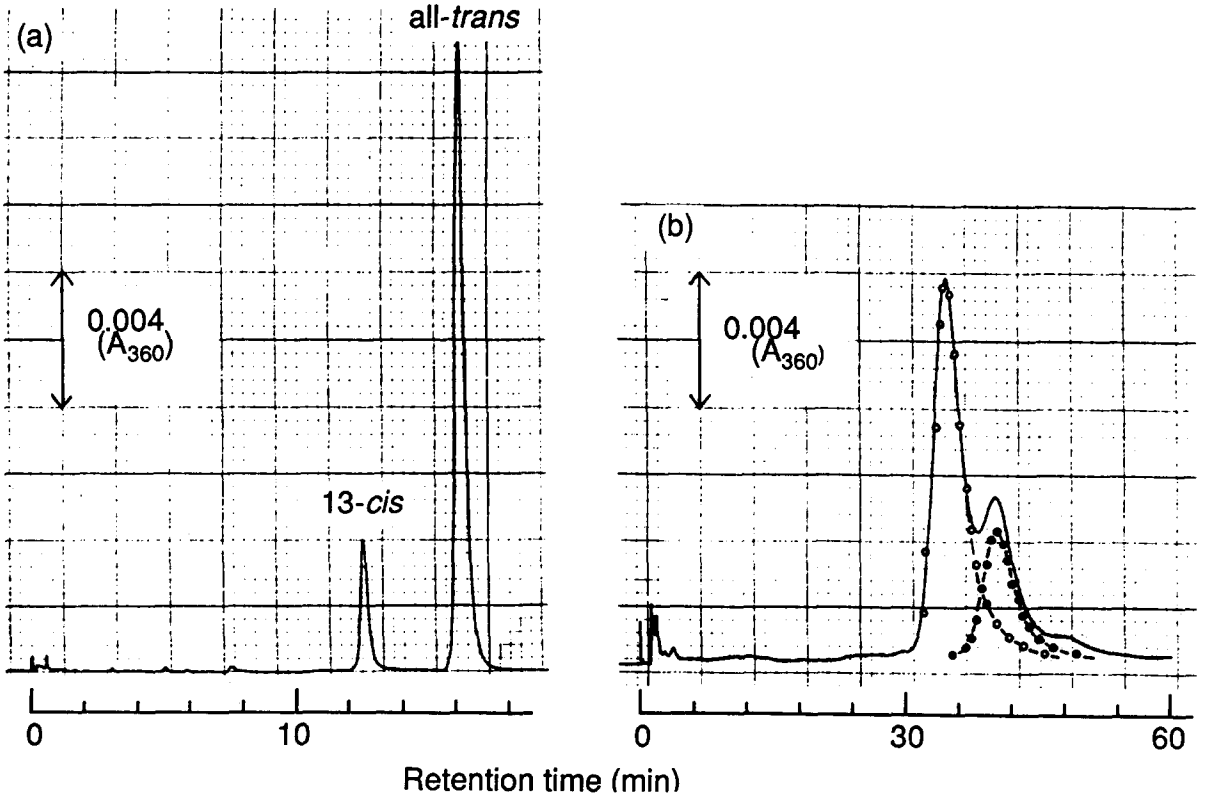

Fig. 13. HPLC of chromophores extracted from the pigments regenerated by addition of a racemate of epoxyretinal. (a) After removal of excess epoxyretinal and retinaloxime, the chromophore of regenerated pigments was extracted and applied to Zorbax SIL column. The movile phase for diethylether/ethanol/hexane was 8:0.5:91.5 (v/v) and the flow rate was 2 ml/min. Elution profile was detected by the absorbance at 360 nm. '13-*cis*' and 'all-*trans*' in Fig. 13 a show the positions of standard 13-*cis* and all-*trans* epoxyretinal elutions. (b) Recovered all-*trans* isomer eluted from silica gel column was applied to a chiral column to analyze the ratio of enantiomers. Movile phase for CH_3CN/H_2O was 45:55 (v/v). The elution was performed with a flow rate of 1.5 ml/min and the profile was detected by the absorbance at 360 nm. The elution profile was divided into (+)- (○) and (−)-epoxyretinal area (●) by fitting the elution profile of each standard enantiomer.

naloxime remained in apomembranes did not cause the chiral discrimination.

It was investigated whether the chromophore was displaced when (+)-epoxyretinal and (−)-epoxyretinal were respectively added to the fully regenerated [(−)-epoxy]bacteriorhodopsin and to [(+)-epoxy]bacteriorhodopsin. Following the addition of enantiomers, the same amount of bacterioopsin content, each specimen was stored in the dark at room temperature (about 25°C) for 2 weeks. The all-*trans* isomer extracted from each specimen was analyzed with a chiral column. The ratio of enantiomers for (+)-/(−)-epoxyretinal was approx. 7/3 for both specimens. This value was consisted with the optical purity of the specimen regenerated by addition of a racemate.

Discussions

Enantiomers of all-*trans*- and 13-*cis*-5,6-epoxyretinal, (5S, 6R) and (5R, 6S), were optically resolved using a chiral column. In the case of the all-*trans* isomer, one of the enantiomers were not determined. Enantiomers showed the positive CD peak around 245 nm and the weak negative peak around 355 nm. It was confirmed that the other enantiomer showed the symmetrical positive and negative CD extrema at the same wavelengths. In this study, absolute configurations of optically resolved enantiomers were not determined. Enantiomers showing the positive and the negative CD peak at 245 nm are termed as (+)-epoxyretinal and (−)-epoxyretinal, respectively. The all-*trans*-

retinal in the solution has been considered to be a distorted 6-s-*cis* conformation with the tortional angle in the range 30°–70° [32]. If 5,6-epoxyretinal in solution has similar s-*cis* conformation about the $C_6 - C_7$ single bond and the CD peak around 245 nm in ethanol is mainly induced by the transition moment of $-O-C_6-C_7=C_8-$ bonding, the allylic bond polarization model [33] predicts the sign of CD band at 245 nm that (5S, 6R)-epoxyretinal is positive and (5R, 6S) is negative.

Ultraviolet light for measurement of CD spectrum would cause the isomerization of epoxyretinal. Therefore, the absorption spectrum and the isomer composition were checked before and after the CD measurement. In the case of all-*trans* enantiomers, the absorption spectrum was hardly changed and the content of 13-*cis* isomer, which was formed by isomerization, was negligibly small after CD measurement. On the other hand, the absorption maximum of 13-*cis* enantiomer demonstrated a slight red shift after CD measurement. The ratio of 13-*cis*/all-*trans* isomer for (+)-epoxyretinal was 95 : 5 before CD measurement and changed to 92 : 8 after measurement. For (−)-epoxyretinal, 13-*cis*/all-*trans* was 96 : 4 and 94 : 6 before and after CD measurement, respectively. In ethanol, all-*trans* isomer of epoxyretinal was more stable comparing with 13-*cis* isomer.

Two enantiomers of all-*trans*-5,6-epoxyretinal, optically resolved with a chiral column, were respectively added to apomembranes. The regenerated bacteriorhodopsin analogues complexed with (+)-epoxyretinal and with (−)-epoxyretinal are termed as [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin, respectively. The opsin shift of [(+)-epoxy]bacteriorhodopsin was 3100 cm^{-1}, indicating that this value was the same order as 2500 cm^{-1} of [5,6-dihydro]bacteriorhodopsin pigment [25,34,35]. The molecular arrangement of [(+)-epoxy]bacteriorhodopsin in the membrane was a hexagonal lattice with a 6.3 nm cell-edge length as observed in native purple membranes. On the other hand, the opsin shift of [(−)-epoxy]bacteriorhodopsin, 1300 cm^{-1}, was small compared with that of [(+)-epoxy]bacteriorhodopsin. The [(−)-epoxy]bacteriorhodopsin molecules in the membrane were aligned in paracrystalline as observed for the bacterioopsin arrangement in apomembranes. Addition of retinal to apomembranes results in appearance of the absorbance band characteristic to bacteriorhodopsin [16] and the regenerated bacteriorhodopsin aggregates spontaneously to form a two-dimensional hexagonal array which gives the X-ray diffraction pattern as observed in native purple membranes [29,30]. It has been known that some structural differences exist between bacterioopsin and bacteriorhodopsin. The helical axes of the bacterioopsin polypeptide segments are significantly tilted in respect to the normal to the membrane plane while those of bacteriorhodopsin are nearly parallel to it [36]. The isoelectric point of bacterioopsin is lower than that of bacteriorhodopsin (Hiraki, K., Hamanaka, T. and Kito, Y., unpublished data). The dissociation equilibria of at least two amino acid residues of chromophoric site are changed during the pigment regeneration [37]. The β-ionone ring and the side chain of retinal are forced to assume a planar conformation at the early stage of bacteriorhodopsin regeneration [38,39] and the chromophore conformation is changed to 6-s-*trans* in the pigment from the 6-s-*cis* conformation in solution [40,41]. Intramolecular conformational change of pigments in the course of regeneration alters the interaction between bacteriorhodopsin molecules and induces the self-aggregation to form the crystalline packing. The external point-charge model proposes that two negative protein residues are present near the cyclohexane ring and the Schiff base end of chromophore to explain how bacteriorhodopsin shifts the absorption maximum of its retinal protonated Schiff base chromophore to the red [25]. ^{13}C-NMR experiments have supported the initial proposal of protein charges near the cyclohexane ring [40]. The reduction of the opsin shift to about 2500 cm^{-1} in [5,6-dihydro]bacteriorhodopsin from 4800 cm^{-1} of native bacteriorhodopsin is caused by an altered interaction due to the shorter conjugated chain with protein charges of opposite sign in the vicinity of cyclohexane ring [35]. From the binding experiments with 7,8-dehydroretinal [35] and a series of linear polyene with no 6-membered ring [42], it has been considered that much of the bacteriorhodopsin-induced red shift must be due to the interaction of the protein with the Schiff-base end of the chromophore. Muradin-Szweykowska et al. [43] have pointed out that the

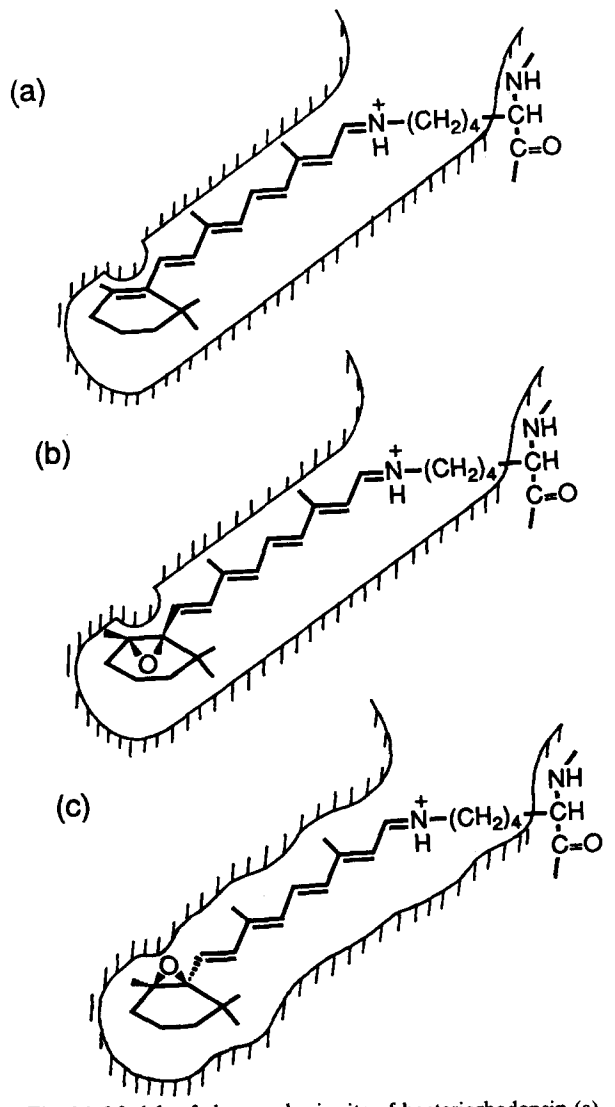

Fig. 14. Models of chromophoric site of bacteriorhodopsin (a), [(+)-epoxy]bacteriorhodopsin (b) and [(−)-epoxy]bacteriorhodopsin (c).

cyclohexane-ring part in chromophore ensures a proper distance between the interacting parts of the protein and the chromophore and thus the light-driven proton-pump activity. In this study, it was investigated whether the modification of cyclohexane-ring part in chromophore influenced the opsin shift and the proton-pumping activity. About 1800 cm^{-1} of opsin shift was found to be different between [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin.

Fig. 14 shows the models of chromophoric site of [(+)-epoxy]bacteriorhodopsin and [(−)-epoxy] bacteriorhodopsin. Cyclohexane ring of (+)-epoxyretinal could be accomodated in the chromophoric site by facing the epoxy group to the opposite direction of closely positioned protein residues. With increasing proximity of the ring to chromophoric site, the interaction with proposed protein-charged groups would result in a similar degree of opsin shift as [5,6]-dihydro]bacteriorhodopsin. [(+)-epoxy]bacteriorhodopsin restores the intramolecular structure similar to native bacteriorhodopsin and induces the spontaneous crystallization of pigments. In the case of (−)-epoxyretinal addition, the steric hindrance of epoxy group projecting to the opposite direction of (+)-epoxyretinal would prevent the access of protein residues. The conformational change of pigments as observed in the course of native bacteriorhodopsin, [5,6-dihydro]bacteriorhodopsin and [(+)-epoxy]bacteriorhodopsin regeneration would not take place and the disordered array of pigments, similar to the paracrystalline arrangement of bacterioopsin in apomembranes, remain. The interaction of the protonated Schiff base end of the chromophore with near electronegative groups in the protein is influenced by the conformation of cyclohexane ring, steric hindrance due to epoxy group.

CD spectrum of [(+)-epoxy]bacteriorhodopsin in membranes indicated the bilobed extrema due to exciton coupling of chromophores in the visible region (Fig. 6a). The first Cotton effect was negative and the second was positive. These signs of CD peaks were the same as those of purple membranes [44]. In X-ray diffractions of [(+)-epoxy] bacteriorhodopsin, it was shown that spacings of reflections were the same and the intensity distribution resembled very closely to native purple membranes. These observations suggest that the molecular arrangement of [(+)-epoxy]bacteriorhodopsin in the unit cell and the mutual orientation of neighbouring chromophore polyene chains were the same as in the purple membrane.

The absorption and CD spectrum of [(−)-epoxy]bacteriorhodopsin showed the shoulder around 490 nm (Figs. 4 and 6b). The isomer composition of extracted chromophores for all-*trans*/13-*cis* was approx. 95 : 5 (Table II), indicating that the thermal isomerization hardly took

place in the dark-adapted pigment. The 490-nm shoulder is not due to the appearance of the pigment having the chromophore other than the all-*trans* form. The racemization of (−)-epoxyretinal was not observed during the pigment regeneration. The absorption maximum was maintained at 445 nm regardless of the regeneration ratio (Fig. 4). The small shoulder at 490 nm in the absorpotion spectrum was definitely observed in CD spectrum (Fig. 6b). It is considered that part of (−)-epoxyretinal in the pigment assumes a twisted conformation.

Bacteriorhodopsin analogue regenerated by addition of 5,6-epoxyretinal in *Halobacterium halobium* cells does not mediate the photophosphorylation [12]. In this study, the proton-pumping activity was compared between [(+)-epoxy] bacteriorhodopsin and [(−)-epoxy]bacteriorhodopsin incorporated into lipid vesicles. Pigment-lipid vesicles having the same orientation were prepared as described in Materials and Methods. Light-induced alkalization of suspension was observed for all specimens. An extent of maximum proton uptake of [(+)-epoxy]bacteriorhodopsin was about 7% of unmodified bacteriorhodopsin-lipid vesicles (Fig. 11), but the proton uptake of [(−)-epoxy]bacteriorhodopsin was small and the similar degree as that of control, the proteoliposome reconstructed from apomembranes and lipid vesicles. Since absorption spectra of these specimens were different as shown in Fig. 10, however, the correction must be made for the dependence of the illuminated light-intensity on the wavelength. Incident photon energy was estimated from the emission spectrum of a tungsten lamp and the transmission of a cut-off filter and the sample chamber. After correction for the absorption spectrum of sample and the energy spectrum of the incident light, these proton uptakes were about 75% and 15% of bacteriorhodopsin-lipid vesicles, respectively. Bacteriorhodopsin analogues complexed with 5,6-epoxyretinal enantiomers, at least one of them, have a light-induced proton-pumping activity.

Chromophores extracted from the pigment which was regenerated by the addition of a racemate of epoxyretinal showed to be chiral. The optical purity of chromophores for (+)-/(−)-epoxyretinal was approx. 7 : 3. It was found that the chiral discrimination of chromophore took place in the course of the pigment regeneration. The retinaloxime formed in the apomembranes did not affect the chiral discrimination. It is possible to consider the difference of binding rate between (+)- and (−)-epoxyretinal to bacterioopsin causes the chiral discrimination. The half-time of pigment regeneration at 10 °C was 400 s for (+)-epoxyretinal and 48 s for (−)-epoxyretinal addition. The result of chiral discrimination could not be explained from the difference of the binding rate. It is considered that the interaction between the chromophoric site and the cyclohexane ring of epoxyretinal gives two stable energy minima and this defines the ratio of each enantiomer in the pigment.

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