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THE ABSORBANCE SPECTRUM OF THE BROWN HOLO-MEMBRANE AND THE COMPARISON OF p*I* VALUES OF BACTERIORHODOPSIN SOLUBILIZED FROM PURPLE MEMBRANE AND FROM BROWN HOLO-MEMBRANE

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Brown holo-membrane was prepared by the addition of all-*trans*-retinal to brown apo-membrane which was isolated from *Halobacterium halobium* grown in the presence of nicotine. The effects of pH and NaCl concentration on the absorbance spectrum of the brown holo-membrane were investigated in comparison with those of the purple membrane. The λ_{max} of the dark-adapted brown holo-membrane shifted from 560 to 600 nm by lowering pH. The p*K* value which was determined as the mid-point pH for the spectral red-shift was 5.8 in the absence of NaCl. It was lowered to 4.5 and 3.4 in 0.1 and 1 M NaCl solutions, respectively. The p*K* value for the brown holo-membrane was larger than the corresponding value for the purple membrane in the NaCl solution. Bacteriorhodopsins present in the purple membrane and in the brown holo-membrane were solubilized in the nonionic detergent, lauryl ester of sucrose. For both solubilized bacteriorhodopsins, the p*K* value of spectral red-shift was about 3.1 in water, and the p*I* value, determined by chromatofocusing, was about 4.6 at 22°C.

Introduction

Bacteriorhodopsin (BR), the only protein in the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump [1]. The apo-protein, bacterioopsin, consists of a single polypeptide of 248 amino acids [2,3] to which retinal is bound through a Schiff's base with the ε-amino-group of a lysine residue [4–6].

The cells grown in the presence of nicotine are inhibited from synthesizing retinal but produce the bacterioopsin [7]. Bacterioopsin molecules are localized in the differentiated domains of the cell membrane, which can be isolated as membrane patches. This membrane fraction is called the brown apo-membrane. Brown apo-membrane contains bacterioopsin as a major protein as well as other minor protein species, such as cytochrome *b*-type protein. Addition of all-*trans*-retinal to the brown apo-membrane results in appearance of the absorbance band characteristic to BR [8]. Also, the reconstituted BR aggregates spontaneously to form a two-dimensional crystal which gives the X-ray diffraction pattern as observed in native purple membrane [9,10]. The membrane specimen which is prepared from the brown apo-membrane by addition of retinal is called brown holo-membrane [11].

The absorption maximum (λ_{max}) of the dark-

Abbreviations: BR, bacteriorhodopsin; BR I, bacteriorhodopsin present in the purple membrane; BR II, bacteriorhodopsin present in the brown holo-membrane; L-1690, lauryl ester of sucrose.

adapted purple membrane in neutral pH is 560 nm and it shifts to 600 nm when pH is lowered to about 2. The value of pK which is determined as the mid-point pH of the spectral red-shift is 3.4 [12–19].

In the previous study [20], we reported that pK of the brown holo-membrane was larger than that of the purple membrane. In the present study, we have investigated this phenomenon in more detail, including the effects of NaCl concentration on pK. It seemed a possibility that some structural difference exists between BR in brown holo-membrane and the purple membrane and caused the difference in pK. We solubilized BR molecules in the brown holo-membrane and purple membrane, respectively, with nonionic detergent and examined their pK values and isoelectric points (pI). Chromatofocusing was found to be useful for the determination of the pI difference in solubilized membrane proteins. Results proved that there was no appreciable difference either between pK or between pI of the solubilized BR from the brown holo-membrane and the purple membrane.

Materials and Methods

Preparation of purple membrane and brown holo-membrane. H. halobium R_1 was kindly supplied by Dr. W. Stoeckenius. Cells were grown and purple membrane was isolated following the procedure by Oesterhelt and Stoeckenius [21]. Brown apo-membrane was purified from cells cultured in the presence of 1.5 mM nicotine [8]. Brown holo-membrane was prepared by addition of all-*trans*-retinal to the brown apo-membrane as described before [8,9]. In this study, BR molecules present in purple membrane and in brown holo-membrane are distinguished as BR I and BR II, respectively.

Removal of cations bound to the purple membrane. The cation-depleted purple membrane was prepared as described [22,23] by passing through a column (2.0 × 3.5 cm) of the cation-exchanger, Dowex 50W-X4 (Dow Chemical Co.). After application to the column, λ_{max} of the eluate was 600 nm and pH was 4–5.

Titration of cation-depleted purple membrane. The absorbance spectrum of the cation-depleted purple membrane was so sensitive to the salt concentration that KCl which leaked from the pH electrode during the measurement affected the λ_{max}. The titration was carried out as follows. The eluate from the cation-exchanger was divided into ten centrifuge tubes. Microliter quantities of HCl or ethanolamine were added to 3-ml aliquots monitoring λ_{max} of each specimen. After the record of absorbance spectra, each specimen was centrifuged at 100 000 × g for 1 h. The pH of each supernatant was measured as a whole pH.

Removal of cations in L-1690 solution. Cations contained in L-1690 solution were removed, as well as the purple membrane. Before application to a cation-exchanger column, Triton X-100 (final concentration of 0.06%) was added to 10% L-1690 solution as a marker. The concentration of L-1690 in the eluate was estimated by comparing the high absorbance at 280 nm of Triton X-100 before and after the treatment.

Solubilization of BR I and BR II. Nonionic detergents, L-1690 and Triton X-100, were used for solubilization. Purple membrane or brown holo-membrane containing about 200 nmol BR was dispersed in 20 ml 10% (w/v) L-1690 or 10% (v/v) Triton X-100 (pH 6.5) and incubated at 20 °C for 2 days in the dark. Then, the solution was centrifuged at 100 000 × g for 1 h and the supernatant was collected. The λ_{max} of BR II-L-1690 micelles in the supernatant was 556 nm at pH 5.6, which was identical to that observed for BR I-L-1690 micelles [24]. The detergent L-1690 was kindly synthesized by Dr. N. Kawase (Ryoto Co. Ltd.). Triton X-100 was purchased from Wako Pure Chemicals (Osaka).

Delipidation of BR solubilized from brown holo-membrane. Endogenous lipids in the BR II solution were removed following the method by Huang et al. [25], except that the initial solubilization of the brown holo-membrane was performed by the addition of L-1690, instead of Triton X-100. After solubilization, 0.5% of Triton X-100 was added as a marker. Then, the specimen was applied to a column (1.5 × 100 cm) of Bio-Gel A-0.5m. When the absorbance at 280 nm of the eluate was monitored, two peaks were observed: the first peak near the solvent front contained most of the BR, while the second peak contained a large amount of Triton X-100 micelles. The first peak fractions were collected and dialyzed against distilled water for 1 day in the dark at 4 °C. After dialysis, the

specimen was turbid. With the addition of L-1690 at a concentration of about 3%, turbidity was diminished, and λ_{max} was 556 nm at pH 5.6.

Chromatofocusing. Experimental runs of chromatofocusing were performed according to the literature [26] and to the manual by Pharmacia. The eluting solution was prepared as follows. Polybuffer (Pharmacia) was diluted 1 : 10 and L-1690 was added to a final concentration of 3%. The solution was adjusted to pH 3.9 with HCl and used as the eluent. The volume of the eluent was 100 ml for one experiment.

The specimens used for the p*I* determination were prepared as follows. BR I and BR II solubilized in L-1690 was adjusted to pH 6.0 by addition of histidine. The specimen was applied to a column (1.2 × 3.5 cm) of anion-exchanger, PBE 94 (Pharmacia), that had been equilibrated with 25 mM histidine-HCl buffer (pH 5.7). BR molecules adsorbed at the top of the column were eluted by 25 mM lactic acid/histidine solution (pH 3.5) containing 3% L-1690. The BR fractions were collected and dialyzed against distilled water overnight in the dark. After this treatment, the lipid content of the BR/L-1690 solution was reduced less than 4 lipid phosphorus/BR.

BR I/or BR II/L-1690 solutions, prepared as described above, were adjusted to pH 5.7 by addition of histidine. Then, the specimen (14–21 nmol BR) was applied to a water-jacketed column of PBE 94 (1.0 × 10 cm) that had been equilibrated with 25 mM histidine-HCl buffer (pH 5.7). The adsorbed BR was eluted at 22°C with the above Polybuffer solution. Fractions (1.7 ml) were collected at a flow-rate of 34–37 cm/h. Then the absorbance spectrum and pH were determined for each fraction. The recovery of BR was always greater than 86%. All procedures were performed under dim red light.

Concentration of BR. The concentration of BR present in the purple membrane and in the brown holo-membrane was calculated using a molar extinction coefficient of 63000 $M^{-1} \cdot cm^{-1}$ for the light-adapted purple membrane [27,28]. The concentration of BR I and of BR II in detergent micelles was estimated assuming a molar extinction coefficient of 49000 $M^{-1} \cdot cm^{-1}$ for the dark-adapted BR I in L-1690 micelles [24].

Measurement of absorbance spectrum and pH value. Absorbance spectra were recorded on either Union SM401 or Shimazu UV200 spectrophotometer. The value of pH was measured using a Horiba 6028-10T combination electrode with a Horiba F-7 pH meter.

Results

Absorbance spectrum of the brown holo-membrane

Fig. 1a indicates the absorbance spectra of the dark-adapted brown holo-membrane in water between pH 4.7 and 7.5. The λ_{max} was 558 nm at pH 7.5 and 598 nm at pH 4.7. Fig. 1b illustrates the plot of λ_{max} derived from Fig. 1a vs. pH. The p*K* for the spectral red-shift was about 5.8. This value was higher than that reported for the purple membrane (about 3.4) [12–19]. Absorbance spectra in

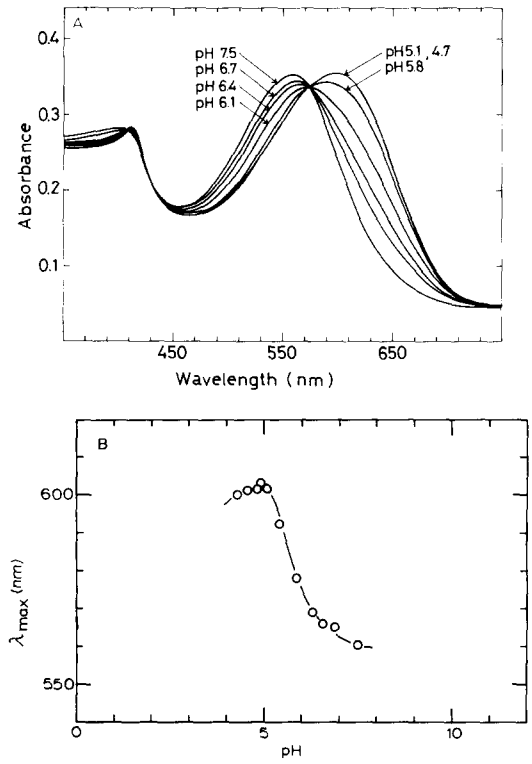

Fig. 1. (a) Absorbance spectrum of dark-adapted brown holo-membrane. Brown holo-membrane stored in 20 mM Tris-HCl (pH 7.4) was washed once with distilled water. Then, the pH was adjusted to 7.5 by addition of ethanolamine and titrated with HCl to acidic pH. Absorbance spectra were recorded against distilled water. The absorbance peak around 430 nm is the Soret band of cytochrome *b*-type protein. (b) Plot of λ_{max} derived from (a) vs. pH.

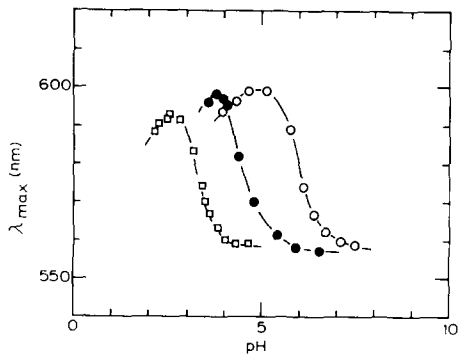

Fig. 2. The effect of NaCl on the absorbance spectrum of brown holo-membrane. The λ_{max} of the dark-adpated brown holo-membrane in water (○), in 0.1 (●) and in 1 M NaCl (□) were plotted against pH. When the specimen was titrated to the desired pH, absorbance spectrum was immediately recorded against distilled water.

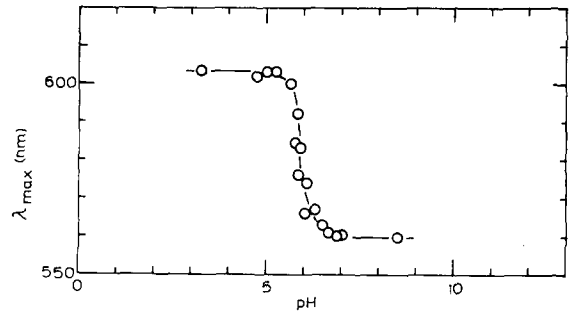

Fig. 3. The spectral titration of the cation-depleted purple membrane. Cations bound to the purple membrane were removed by passing through a cation-exchanger column [22,23]. The λ_{max} of the effluent was 600 nm between pH 4 and 5. The eluate of the column was divided. To a series of aliquots, HCl or ethanolamine was added until λ_{max} of each specimen became the desired value. After recording of the absorbance spectrum, each specimen was centrifuged. The pH of the supernatant was measured as a whole pH of the specimen.

Fig. 1a were recorded using distilled water as a reference. Therefore, λ_{max} of each absorbance spectrum in Fig. 1a shifted to shorter wavelength by 2 nm, comparing with the difference spectrum between brown holo-membranes and brown apo-membranes.

Fig. 2 indicates the plots of λ_{max} vs. pH under various concentrations of NaCl. The pK value of the brown holo-membrane was 4.5 in 0.1 M NaCl and was 3.4 in 1 M NaCl. The pK in 1 M NaCl was close to that of the purple membrane in distilled water.

Absorbance spectrum of cation-depleted purple membrane

Fig. 3 indicates the plot of λ_{max} of the cation-depleted purple membrane vs. pH. The cation-depleted purple membrane was prepared by passing through a column of cation-exchanger. Special caution was necessary when measuring pH of the cation-depleted purple membrane solution, because λ_{max} and pH were concurrently changed when the pH electrode was immersed into the specimen. This seems to be due to a replacement of the protons bound to the cation-depleted purple membrane with the potassium ions which leaked from the pH electrode. Therefore, pH of the supernatant obtained by centrifugation of a properly adjusted solution was measured as described in Materials and Methods. The relation between λ_{max} and pH is shown in Fig. 3. The pK value of the cation-depleted purple membrane for the spectral red-shift was about 5.9, indicating that it is higher than that of the untreated purple membrane.

The brown holo-membrane was treated with the cation-exchange gel as well as the purple membrane. Though pH of the eluate was 3.3, absorbance at about 450 nm rose like those of BR I-L-1690 micelles at pH 0.0 [24]. As the 450 nm product is not stable [24,29], titration of the cation-depleted brown holo-membrane was not performed.

The blue-shift of the absorbance spectrum of the cation-depleted purple membrane following addition of L-1690

It was found that λ_{max} of the cation-depleted purple membrane, being at 600 nm, shifted to shorter wavelength with the addition of the non-ionic detergent, L-1690. Fig. 4 shows the plot of λ_{max} vs. the molar ratio of added L-1690 to BR. When more than 1300 L-1690 in molar ratio was added, λ_{max} was 558 nm at pH 4.3. It was identical to that of BR I solubilized in L-1690 [24]. Cations were removed previously from the L-1690 solution as described in Materials and Methods.

Absorbance spectrum of BR II-L-1690 micelles

Fig. 5 indicates the results of the spectral titra-

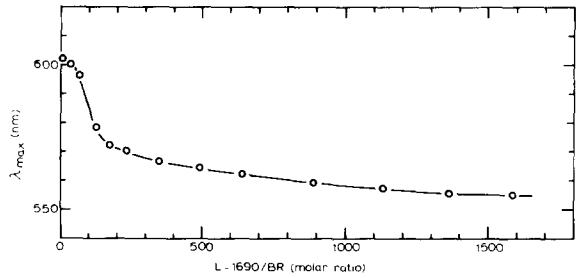

Fig. 4. The blue-shift of the absorbance spectrum of cation-depleted purple membrane following addition of L-1690. A definite volume of L-1690 was added to the cation-depleted purple membrane, its λ_{max} being 600 nm, and the absorbance spectrum was recorded after 1 h incubation. Then the procedure, addition of L-1690, incubation and the recording of absorbance spectrum, was repeated. The λ_{max} was plotted against the molar ratio of added L-1690. The pH of the specimen, measured after completion of the experiment, was 4.3.

tion for the nondelipidated and the delipidated BR II solubilized in L-1690. The pK value was 3.1 for both specimens. Polar lipids, which would affect the spectral shift, are fully replaced with detergent molecules by solubilization.

Table I indicates λ_{max} at the largest red-shift and pK for the spectral red-shift of nondelipidated BR II-L-1690 micelles under the various concentration of NaCl. Data in Table I were in good agreement with those of BR I dispersed in L-1690 [24]. These results indicate that the ionizable groups in BR related to the spectral shift are titrated in a

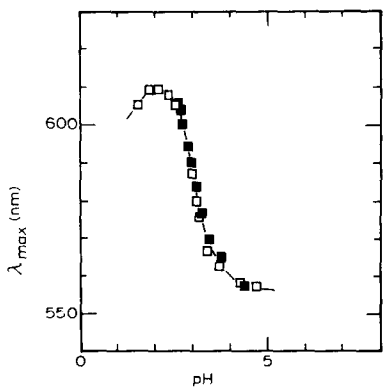

Fig. 5. The pH-dependence of the absorbance spectrum of solubilized BR II in L-1690 and of solubilized-delipidated BR II in L-1690. The λ_{max} of solubilized BR II (□) and solubilized-delipidated BR II (■) were plotted against pH.

TABLE I

THE λ_{max} AT THE LARGEST RED-SHIFT AND pK VALUES FOR THE SPECTRAL RED-SHIFT FROM 560 TO 600 nm OBSERVED FOR BR II SOLUBILIZED IN L-1690 UNDER VARIOUS CONCENTRATIONS OF NaCl

Added NaCl (mM)	λ_{max} (nm)	pK
0	609	3.1
10	608	3.2
100	604	3.4
1000	602	3.7

similar manner between BR I/ and BR II/L-1690 solutions.

The λ_{max} of the delipidated BR II was 535 nm at pH 8.0 in the deoxycholate solution and it shifted to 550 nm with the addition of L-1690. Lind et al. [30] have observed similar spectral shift

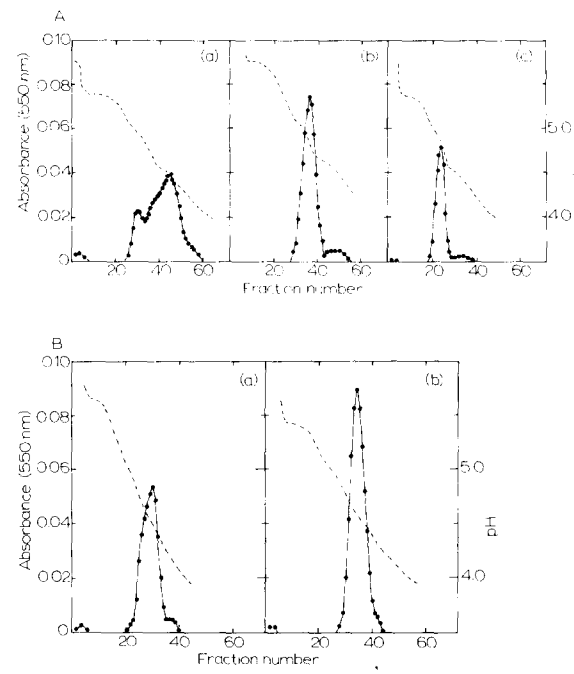

Fig. 6. The effect of the detergent concentration in the eluent on the elution profile of chromatofocusing. (A) BR I solubilized in Triton X-100 was chromatofocused under the condition of 1% (a), 3% (b) and 5% (c) Triton X-100 in the eluent. (B) BR I solubilized in L-1690 was chromatofocused under the condition of 1% (a) and 3% (b) L-1690 in the eluent. The absorbance at 550 nm of each fraction (●) and pH (------) are shown. The absorbance in front fractions is due to bacterioruberin.

for delipidated BR I: when the delipidated BR I in deoxycholate solution was recombined with endogenous lipids of *H. halobium*, λ_{max} shifted from 538 to 555 nm (pH is not described). Therefore, the detergent, L-1690, provides the hydrophobic microenvironment similar to that of endogenous lipids to the delipidated BR.

Chromatofocusing

Fig. 6A (a–c) shows the elution profiles of BR I dispersed in Triton X-100 when the concentration of Triton X-100 in the eluent is 1, 3 and 5%, respectively. In the case of 1%, BR was eluted in the range of pH 4–5. Results of 3 and 5% concentrations showed the main peak at pH 4.83 for both cases. The half-width of the main peak was 0.34 pH unit for 3% and 0.31 pH unit for 5%. The result of 1% concentration suggests that the specimen would be poly-dispersive due to the insufficient amounts of detergent.

A similar experiment was carried out using L-1690 in place of Triton X-100. Fig. 6B (a and b) indicates the results of chromatofocusing of BR I-L-1690 micelles when the concentration of L-1690 in the eluent is 1 and 3%, respectively. The elution profile of 1% showed the broadened peak, and its half-width was 0.39 pH unit. That of 3% gave a main peak at pH 4.62 and the half-width was 0.19 pH unit. Hereafter, the determination of p*I* values of BR I and BR II were carried out with the eluent containing 3% L-1690. Fig. 7 shows the elution profiles of both pigments at 22°C. The main peak around pH 4.6 was observed in the profiles of both specimens. Small subpeaks were observed around pH 5.0 and 4.3. The p*I* value and the half-width of the main peak are summarized in Table II. The averaged p*I* value at 22°C was 4.62 for BR I- and 4.64 for BR II-L-1690 micelles. The main p*I* values of both pigments were indistinguishable, suggesting that the surface charge was the same between both specimens.

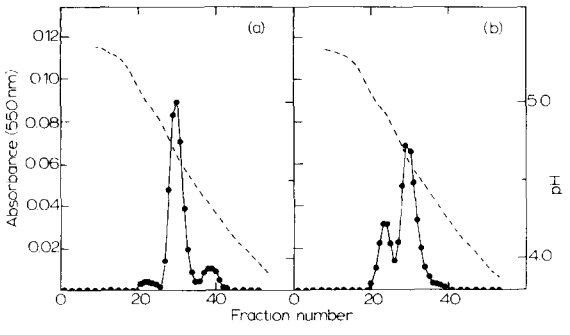

Fig. 7. Chromatofocusings of BR I- (a) and BR II-L-1690 micelles (b) at 22°C. The concentration of L-1690 in the eluent was 3% and the rate of elution was controlled in the range of 34–37 cm/h. Symbols are the same as in the legend of Fig. 6.

TABLE II

THE p*I* VALUE AND THE HALF-WIDTH OF THE MAIN PEAK OBSERVED IN THE ELUTION PROFILE OF THE CHROMATOFOCUSING OF BR I- AND BR II-L-1690 MICELLES AT 22°C

	p*I*	Half-width (pH unit)
BR I	4.55	0.18
	4.59	0.24
	4.62	0.19
	4.63 [a]	0.18 [a]
	4.65 [a]	0.17 [a]
	4.66 [a]	0.18 [a]
Averaged value	4.62 ± 0.04	0.19 ± 0.025
BR II	4.64 [a]	0.19 [a]
	4.64 [a]	0.20 [a]
	4.65 [a]	0.20 [a]
Averaged value	4.64 ± 0.005	0.19 ± 0.006

[a] Chromatofocusing of both proteins was performed alternately using the same column.

Discussion

Preliminary chromatofocusing often gave different p*I* values for both pigments, such as 4.4 for BR II- and 4.6 for BR I-L-1690 micelles. At that stage, experimental conditions, such as solubilization of the specimen and/or stacking of PBE 94 gel in the column, were not rigorously controlled. We designed to perform the experimental run repeatedly under identical conditions. Cares in runs were as follows. At first, in order to reduce lipid content in BR/L-1690 solutions and obtain homogeneous BR-L-1690 complexes, the specimen was treated with a PBE 94 gel prior to use in chromatofocusing as described in Materials and Methods. The molar ratio of L-1690 to BR was about 19000, that was much larger than 2900 which was

used to prepare the BR I monomer [24]. Although the native purple membrane contains 12 lipid phosphorus per BR, estimated from the literature [31], and the buoyant density of the brown holo-membrane indicates that the brown holo-membrane has a higher lipid-to-protein ratio than the purple membrane, the number of lipid phosphorus per BR was reduced to below 4 for both pigment solutions after the treatment. Secondly, chromatofocusings of both pigments were performed alternately using the same column. Under these conditions, the same p*I* value, 4.6 at 22°C, was derived for BR I- and BR II-L-1690 micelles.

The agreement of p*I* values for both pigments was examined in the different way. The brown holo-membrane was prepared by addition of all-*trans*-3-dehydroretinal (retinal$_2$). BR II-containing retinal$_2$ was solubilized in L-1690 and mixed with BR I/L-1690 solution, and chromatofocused. The chromophore composition in the eluate was examined with high-performance liquid chromatography. The ratios of retinal$_1$ to retinal$_2$ for each fraction near the main peak of BR agreed within $\pm$ 5%. This result supports the conclusion described above, on the assumption that the surface charge of BR II did not significantly change by the replacement of retinal$_1$ with retinal$_2$.

The chromatofocusing of BR I showed two small subpeaks around pH 5.0 and 4.3, in addition to the main peak around pH 4.6 (Fig. 7a). The subpeak around pH 5.0 was also observed in the elution profile of BR II (Fig. 7b). Although the subpeak around pH 4.3 was not seen in Fig. 7b, it was observed in the other run of BR II.

Plantner and Kean [32] have performed isoelectric focusing of BR I solubilized in Nonidet P-40 at 4°C. They observed four forms of BR having p*I* values of 3.93, 4.34, 5.03 and 5.49. They also reported that forms of 3.93, 4.34 and 5.49 were transient and the form of 5.03 was stable. The results of Platner and Kean and of this study cannot be directly compared, because the temperature of the run and the detergent used for experiments are not identical. It was reported that the pH stability, thermal stability and λ_{max} of the solubilized BR were different between Triton X-100 and L-1690 solutions [24]. Also, the p*I* value was dependent on the kind of detergent used for solubilization. The derived main p*I* of BR I-Triton X-100 was 4.83 and of BR I-L-1690 was 4.62 (Fig. 6, Table II). The conformation of the membranous protein is more or less affected by the interaction with detergent molecules. The polar headgroups of detergent are different. These differences would cause the variation of the surface charge of the protein in detergent micelles and the p*I* value of the solubilized protein would be dependent on detergent.

Sluyterman and co-workers [33,34] have indicated that the p*I* value derived from chromatofocusing is not the absolute value. Minute differences in the surface charge of the protein, however, can be distinguished sensitively by chromatofocusing [35]. Therefore, it is meaningful that the surface charge of BR I and of BR II are compared by using p*I* derived from chromatofocusing.

Upon acidification, λ_{max} of the brown holo-membrane was red-shifted by approx. 50 nm, similar to the spectral red-shift reported for the purple membrane. The p*K* of the spectral red-shift was about 5.8 for the brown holo-membrane washed once with distilled water, which was larger than 3.4 for the purple membrane in water. The purple membrane contained 8.4 mol Na^+, 0.2 mol K^+, 1.0 mol Ca^{2+} and 2.8 mol Mg^{2+} per mol BR in water solution, which was estimated by atomic absorption spectrometry. However, λ_{max} of purple membrane which was dispersed in 10 mM EDTA (pH 8.0) and washed three times in distilled water, shifted to 600 nm at pH 4.5. Then, the specimen contained 4.0 mol Na^+, 0.4 mol K^+, 0.5 mol Ca^{2+} and 0.1 mol Mg^{2+} per mol BR. This result indicates that the divalent cations, which bind to the membrane more strongly than monovalent cations, are effective for lowering p*K* of the spectral shift. Cation-depleted purple membrane can also be easily prepared through a cation-exchanger and this method was employed in obtaining the specimen for titration experiments of the purple membrane (Fig. 3). As shown in Fig. 2, p*K* for spectral shift of the brown holo-membrane was lowered upon increasing NaCl concentration. In the case of the purple membrane, Fischer and Oesterhelt [15] reported that p*K* for the spectral red-shift became below 3.4 with increasing the ionic strength. Though p*K* values for spectral shift of both the purple membrane and brown holo-mem-

brane were dependent at any rate on the concentration of cations, the ionic strength to give the same pK value was different between the brown holo-membrane and the purple membrane. These facts suggest that the surface charge of the brown holo-membrane was more negatively charged than that of the purple membrane. It is known that the polar lipids of purple membrane have acidic residues in headgroups [36], and the lipid composition of brown holo-membrane is similar to that of the purple membrane [10]. Therefore, the negative charge of membrane surface is consisted of the surface charge of BR and polar lipid headgroups.

The relation between pK for the spectral shift and the surface charge of BR itself has been reported by several authors [17,19]. A modification of carboxyl groups of BR in purple membrane with carbodiimide, a water-soluble reagent, lowers the pK value from 3.4 to 2.6 [17]. The acetylated purple membrane, in which all lysine residues of BR are acetylated, raises the pK value from 3.4 to 4.8 [19]. These results indicate that the increase of negative charge on the water-accesible surface of BR raises the pK value for spectral red-shift.

Our interest is why the pK of brown holo-membrane is larger than that of the purple membrane. It is considered to be the reason why the surface charge of BR present in both membranes is different and/or the distribution of polar acidic lipids around BR is not the same. In this study, as a first step, we examined if the surface charge of BR was different between the purple membrane and the brown holo-membrane. The molecules of BR present in both membranes were solubilized respectively and pI values were compared. The spectral titration was also performed for the solubilized pigments. Values of pK and pI for both pigments were in good agreement (Tables I and II). These results indicate that the surface charge is the same for both pigments and the anionic groups which are concerned with the red-shift of the absorbance spectrum [15] are titrated in the same way for both BR molecules. Therefore, we consider that BR molecules in purple membrane and in brown holo-membrane are common to each other.

The λ_{max} of the deionized purple membrane, being at 600 nm, was blue-shifted by solubilization (Fig. 4). This result implies that the interaction between BR and acidic polar lipids around BR was weakened by solubilization, resulting in the shift of pK to the lower value. In the model Schiff's base and the visual pigment, pK of the protonation of the Schiff's base is strongly affected by the charge of membrane lipids and detergent surrounding the model Sciff's base or cepharopod metarhodopsin [37,38]. It has also been reported that pK for spectral red-shift depends on the lipid distribution around BR [30,39]. The difference of pK between the purple membrane and the brown holo-membrane might arise from differences in the distribution of acidic lipids around BR of both membranes.

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