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RETINAL-PROTEIN INTERACTIONS IN BACTERIORHODOPSIN MONOMERS, DISPERSED IN THE DETERGENT L-1690

TAKAYUKI NAITO [a], YUJI KITO [a], MASAMICHI KOBAYASHI [b], KENJI HIRAKI [c] and TOSHIAKI HAMANAKA [c]

Departments of [a] Biology and [b] Polymer Science, Faculty of Science and [c] Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560 (Japan)

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Bacteriorhodopsin monomer dispersed in a solution of the detergent L-1690 could maintain the specific interaction between retinal and protein in the pH range 9.0–0.0 at 25°C. λ_{max} of the absorbance spectrum was 550 nm at pH 9.0, 556 nm at pH 5.5, 609 nm at pH 2.1 and 570 nm at pH 0.0. Increasing the NaCl concentration in the solution promoted formation of the 609 nm product at pH 5.0–3.0 and also its transition to the 570 nm product at pH 2.5–1.0. Retinal isomer analysis gave a ratio of 13-*cis*- to all-*trans*-retinal of 53 : 47 at pH 5.5. When the pH of the solution was reduced, the relative content of all-*trans*-retinal increased and the ratio of 13-*cis*- to all-*trans*-retinal was 14 : 86 at pH 0.0. Illumination of the solution at pH 7.2 yielded a product containing 9-*cis*-retinal or 9-*cis*, 13-*cis*-retinal, which may be due to a reaction other than the photoreaction cycle.

Introduction

Bacteriorhodopsin found in *Halobacterium halobium* is a chromoprotein containing retinal as its chromophore, like visual pigments of higher animals [1]. This molecule forms a two-dimensional hexagonal crystalline lattice in the purple membrane [2,3]. Bacteriorhodopsin functions not only as a sensory receptor molecule for phototactic behavior, but also as a light-driven proton pump for various energy-dependent processes occurring inside the cell [4–6].

Absorption of light by visual pigment brings about the isomerization of its chromophoric retinal accompanied by successive changes in the retinal-protein interaction which may be recognized as several intermediates of different absorbance spectra [7]. Changes in protein conformation involving retinal-protein interactions may trigger the visual transduction process.

Photoreaction of bacteriorhodopsin constitutes a cycle made up of several intermediate stages [8], possibly involving photoisomerization and thermal isomerization of retinal [9] and concomitant changes in protein conformation. Purple membranes contain all-*trans*- and 13-*cis*-retinal in an equimolar ratio in the dark at neutral pH [10,11]. Light changes the molar ratio towards a predominance of all-*trans*-retinal [10,11] and the equilibrium is recovered by thermal reaction in the dark. On the other hand, the absorbance spectrum of bacteriorhodopsin is altered depending on pH, and concomitantly its chromophore composition is varied [12,13]. A close correlation between the protein structure and configuration of retinal can be seen in bacteriorhodopsin. Thus, bacteriorhodopsin is an interesting material and knowledge about this molecule will give significant information to help understand the retinal-protein interactions in visual pigment and its intermediates.

In the purple membrane, there may be some contribution from the crystalline structure to retinal-protein interactions [14,15]. Investigation of the individual bacteriorhodopsin molecule (monomer) should provide clues as to the contribution from the crystalline structure.

The bacteriorhodopsin monomer is obtainable by solubilizing the purple membrane with detergents

such as Triton X-100 [16–21] and octyl glucoside [19]. We used the detergent L-1690 which is effective for dispersing other photoreceptor membranes [22]. In this paper, pH-dependent spectral changes of solubilized bacteriorhodopsin were examined together with retinal isomer compositions.

Materials and Methods

Purple membrane was prepared from *H. halobium* R_1M_1 according to the method of Oesterhelt and Stoeckenius [23]. Detergents used for solubilizing purple membranes were L-1690 (lauryl ester of sucrose, monoester 90%) and Triton X-100. Dark-adapted purple membrane was dispersed in 2% detergent solution (1 ml/mg purple membrane) at pH 5–6 and incubated at room temperature for 2 days in the dark. Then the solution was centrifuged at 100 000 × *g* for 1 h and the supernatant was collected. λ_{max} of the absorbance spectrum of the supernatant was 556 nm in L-1690 solution and 550 nm in Triton X-100 solution at pH 5.5, shorter than that of the purple membrane (560 nm), It is known that bacteriorhodopsin in the monomeric state does not show the negative CD couplet in the visible region characteristic of the purple membrane [16,17]. The supernatant obtained did not show the negative CD couplet but a weak positive CD in the visible region, indicative of the monomeric state [16,17]. The bacteriorhodopsin solubilized in L-1690 solution was stable for at least 1 month at pH 5.5 and 4°C. It denatured at 60°C with a half-life of 250 min.

pH and salt dependence of the absorbance spectrum were examined on the dark-adapted solubilized bacteriorhodopsin. The solubilized bacteriorhodopsin was illuminated at 4°C and at pH 7.2 by light from a tungsten lamp (500 W) which had passed through glass filters (Toshiba) (see legend to Fig. 4).

Retinal isomer composition was analyzed by the method of Pettei et al. [11] with a slight modification. The bacteriorhodopsin solution (1 ml) was mixed with 2 ml of dichloromethane followed by shaking. The pH of the mixture was adjusted to neutral. The mixture was allowed to stand for 5 min at 0°C, shaken with 20 ml of diethyl ether and centrifuged at 5 000 rev./min for 5 min. The upper layer of the supernatant was collected and dehydrated by adding anhydrous Na_2SO_4 and evaporated to 10 µl. This sample was applied to an HPLC system (Yanaco L-2 000) equipped with a silica gel column (Merckosorb SI 60, 4 × 500 mm). The elution solvent was a mixture of hexane, diethyl ether (12%) and acetone (0.25%). The flow rate was set at 1.0 ml/min and each isomer was detected by the absorbance at 375 nm. The relative content of each isomer was determined after correcting for the difference in molar extinction of each isomer in hexane [24]. The ratio of the molar extinction coefficient of each isomer in our solvent system was estimated to be the same as in hexane within the experimental error of ±2%.

Absorbance spectra were determined with a Union SM-401 spectrophotometer (scanning rate 10 nm/s). CD spectra were measured with Jovin-Yvon Dichrograph Mark III-J. The temperature of the cell-holders was controlled by circulating water of a constant temperature.

The detergent L-1690 was kindly synthesized by Ryoto Co. Ltd. (Tokyo). Other chemicals were purchased from Wako Pure Chemicals (Osaka). *H. halobium* R_1M_1 was generously supplied by Dr. Stoeckenius.

Results

Fig. 1 shows the absorbance spectra of bacteriorhodopsin solubilized in L-1690 solution at various pH values. When the pH of the solution was lowered from 5.5 to 2.1 by the addition of HCl, λ_{max} shifted towards longer wavelengths from 556 nm (curve 1) to a limit of 609 nm (curve 2). Absorbance spectra measured in the pH range 5.5–2.1 did not show a definite isosbestic point and intersected in the wavelength region 572–575 nm. With further lowering of pH, λ_{max} returned from 609 nm to shorter wavelengths: 602 nm at pH 1.4 and 592 nm at pH 0.9. When the solution was kept below pH 2.0 for a while in the dark, A_{max} gradually decreased and the absorbance at about 450 nm rose (half-time of the reaction: 7 h at pH 1.5 and 25°C). In contrast, in the presence of 1 M NaCl the formation of the 450 nm product mentioned above did not occur below pH 2.0. Curve 3 shows the absorbance spectrum at pH 0.0 in 1 M NaCl (λ_{max} 570 nm). The solution, being kept for more than 1 h at pH 0.0, became turbid. This was due to the hydrolysis of L-1690. In a paral-

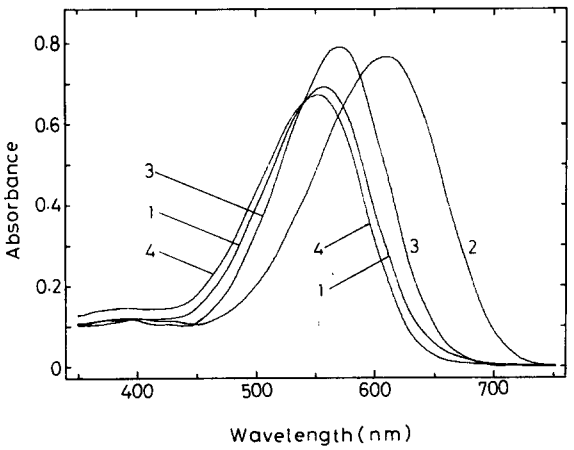


Fig. 1. Absorbance spectra of solubilized bacteriorhodopsin in L-1690 solution at various pH values. The pH of the bacteriorhodopsin solution (1.5% L-1690) was adjusted by adding HCl or NaOH. Spectra measured at 25°C, with a 1 cm light-path cell. (Curve 1) pH 5.5, λ_{max} = 556 nm; (curve 2) pH 2.1, λ_{max} = 609 nm; (curve 3) pH 0.0, λ_{max} = 570 nm, in the presence of 1 M NaCl; (curve 4) pH 9.0, λ_{max} = 550 nm. Curves 1, 2, and 4 were measured in the absence of NaCl.

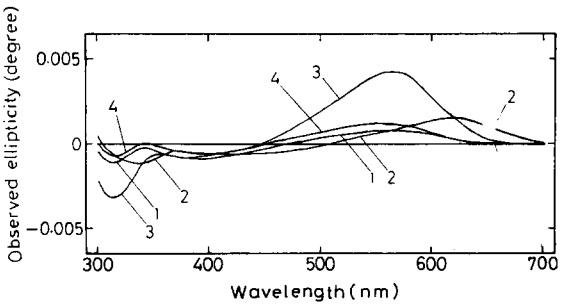


Fig. 2. The CD spectra of solubilized bacteriorhodopsin corresponding to the absorbance spectra in Fig. 1. (Curve 1) pH 5.5, (curve 2) pH 2.1, (curve 3) pH 0.0, in the presence of 1 M NaCl, (curve 4) pH 9.0. Spectra were measured at 25°C with a 1 cm light-path cell.

lel experiment, bacteriorhodopsin solubilized in Triton X-100 solution was converted below pH 4.0 to the product having a λ_{max} of 450 nm even in the presence of 1 M NaCl. The 450 nm product was further converted at pH 3.0 and 25°C with a half-life of 5 min to a product having a λ_{max} of 385 nm. The 385 nm product did not show CD in the wavelength region 300–600 nm, and the addition of 20 mM NH_2OH to the solution containing the 385 nm product resulted in the formation of retinal oxime. This fact suggests that the specific interaction between chromophoric retinal and protein disappeared in the Triton-solubilized bacteriorhodopsin.

The absorbance spectrum of the purple membrane is not altered in the pH range 5.0–8.5 [25]. When bacteriorhodopsin solubilized in L-1690 solution was brought from pH 5.5 to a weak alkaline pH value, λ_{max} shifted to shorter wavelengths and A_{max} slightly decreased. Curve 4 in Fig. 1 shows the spectrum at pH 9.0 (λ_{max} 550 nm). Absorbance spectra measured in the pH range 5.5–9.0 intersected at about 540 nm.

Fig. 2 shows the CD spectra of solubilized bacteriorhodopsin corresponding to the absorbance spectra in Fig. 1. The spectra do not show the negative CD couplet but do show positive CD corresponding to the respective α-bands. The CD magnitude is particularly large at pH 0.0 (curve 3). Another feature which is shown is that the CD spectrum at pH 2.1 (curve 2) has a λ_{max} of 620 nm, being different from λ_{max} of the absorbance spectrum (609 nm).

Table I shows the retinal isomer composition of bacteriorhodopsin solubilized in L-1690 solution at various pH values in the dark. Among the isomers, only 13-*cis*- and all-*trans*-retinal were detected. The ratio of 13-*cis*- to all-*trans*-retinal was 53 : 47 at pH 5.5. On lowering the pH of the solution, the relative content of all-*trans*-retinal increased below pH 5.5. The ratio did not vary significantly in the pH range 5.5–9.0. In order to determine the molar extinction coefficient of solubilized bacteriorhodopsin, the bacteriorhodopsin was denatured with 2% sodium dodecyl sulfate in the presence of 100 mM NH_2OH

TABLE I

THE RETINAL ISOMER COMPOSITION OF SOLUBILIZED BACTERIORHODOPSIN IN L-1690 SOLUTION AT VARIOUS pH VALUES IN THE DARK

Each experiment was performed at least twice. The experiment at pH 0.0 was carried out in the presence of 1 M NaCl.

pH	% composition	
	13-*cis*-Retinal	all-*trans*-Retinal
0.0	14 ± 2	86 ± 2
2.2	36 ± 2	64 ± 2
5.5	53 ± 1	47 ± 1
7.0	57 ± 1	43 ± 1
9.0	52 ± 3	48 ± 3

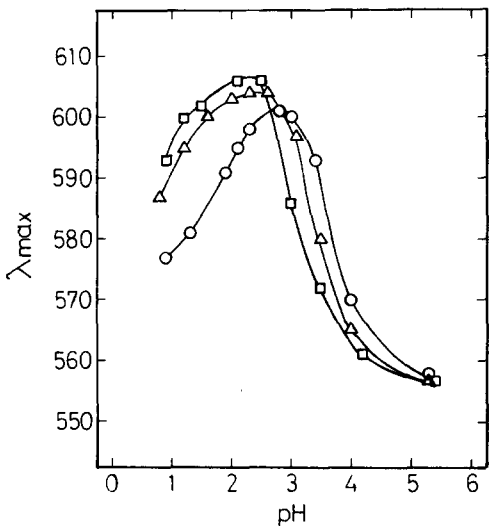


Fig. 3. pH dependence of λ_{max} of the absorbance spectrum of solubilized bacteriorhodopsin at different concentrations of NaCl. Bacteriorhodopsin was solubilized into L-1690 solutions containing 10 mM NaCl (□), 100 mM NaCl (△), and 1 M NaCl (○). By the addition of HCl, the pH of each solution was changed at 25°C.

at pH 5.5. A_{max} of the retinal oxime formed was equal to A_{max} of the original bacteriorhodopsin. The molar extinction coefficient of the dark-adapted bacteriorhodopsin solubilized in L-1690 solution at 556 nm (pH 5.5) was estimated at 49 000 ± 1 000 $M^{-1} \cdot cm^{-1}$, using the values of 51 600 $M^{-1} \cdot cm^{-1}$ for all-*trans*-retinal oxime [26] and 46 600 $M^{-1} \cdot cm^{-1}$ for 13-*cis*-retinal oxime.

The pH dependence of the absorbance spectrum of solubilized bacteriorhodopsin was examined also in the presence of NaCl at various concentrations. λ_{max} values of the spectra in 10 mM, 100 mM and 1 M NaCl were plotted against pH in Fig. 3. In the sequence 10 mM, 100 mM, 1 M NaCl, λ_{max} became longer at pH 5.0–3.0, while it became shorter at pH 2.5–1.0. This means that in the presence of 1 M NaCl

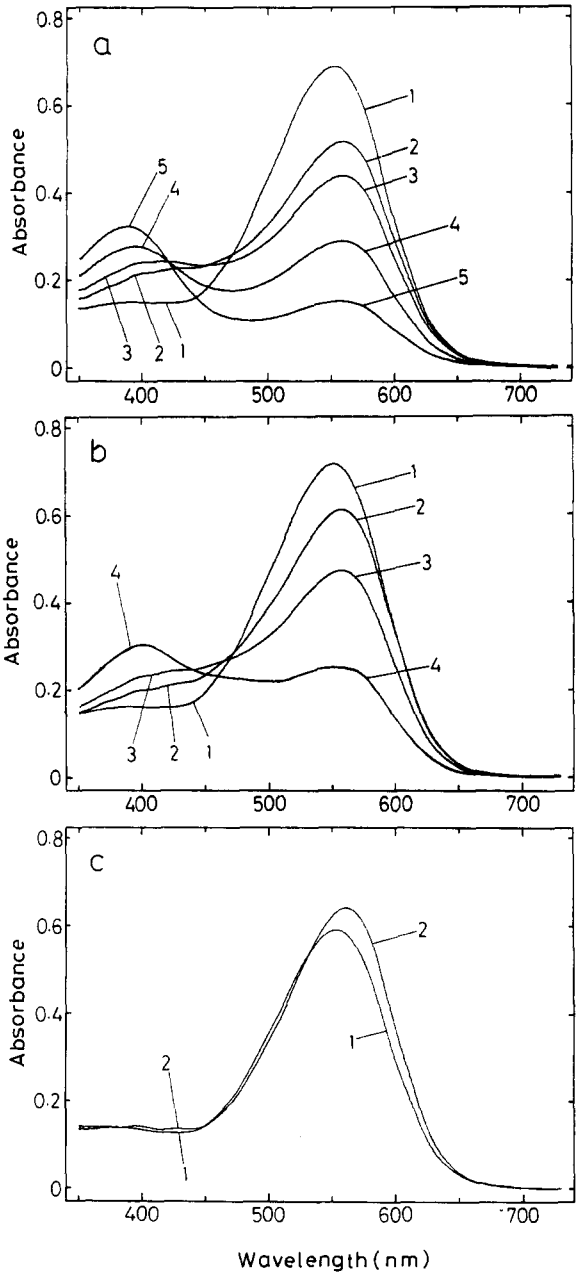


Fig. 4. The absorbance spectra of solubilized bacteriorhodopsin illuminated with light of various wavelengths. (a) Bacteriorhodopsin in 0.5% L-1690, 10 mM Tris-HCl (pH 7.2) solution was illuminated at 4°C with light ($\lambda > 600$ nm) passed through a glass filter (Toshiba VR-60). (Curve 1) The absorbance spectrum of solubilized bacteriorhodopsin (curve 2) illuminated for 15 min, (curve 3) illuminated for 30 min, (curve 4) illuminated for 90 min, (curve 5) illuminated for 210 min. (b) The same as a except illumination with light of $\lambda > 640$ nm passed through a glass filter (Toshiba VR-64). (Curve 1) The absorbance spectrum of solubilized bacteriorhodopsin, (curve 2) illuminated for 40 min, (curve 3) illuminated for 120 min, (curve 4) illuminated for 300 min. (c) Bacteriorhodopsin in 1.5% L-1690, 10 mM Tris-HCl (pH 7.2) solution was illuminated at 4°C with violet light passed through a glass filter (Toshiba VV-40, T_{max} = 400 nm, half-bandwidth = 106 nm). (Curve 1) The absorbance spectrum of solubilized bacteriorhodopsin, (curve 2) illuminated for 20 min.

the formation of the 609 nm product and its transition to the 570 nm product occurred at higher pH than in 10 mM and 100 mM NaCl.

Fig. 4 shows the photoreactions of solubilized bacteriorhodopsin and Fig. 5 illustrates the concomitant chromophore analysis of illuminated bacteriorhodopsin on HPLC. Fig. 4a shows the absorbance spectra of the solution illuminated at pH 7.2 and 4°C with red light ($\lambda > 600$ nm): λ_{max} shifted from 552 nm (curve 1) to 559 nm (curve 2) after 15 min illumination and simultaneously A_{max} fell and the absorbance at about 440 nm rose. Further illumination promoted this tendency and finally after 210 min illumination, the product having a λ_{max} of 390 nm appeared. The HPLC pattern shows that the solution illuminated for 30 min contains a considerable amount of 9-*cis*-retinal and a small amount of 9-*cis*, 13-*cis*-retinal compared to all-*trans*- and 13-*cis*-retinal (Fig. 5a-1). Further illumination increased the relative content of 9-*cis*, 13-*cis*-retinal (Fig. 5a-2 and a-3). At first sight, formation of the 390 nm product seemed to be parallel to the formation of 9-*cis*, 13-*cis*-retinal, but this was not true. When the photoproduct of curve 3 in Fig. 4a was allowed to stand at 25°C in the dark, the absorbance at about 440 nm decreased and that at 390 nm increased. Another observation is shown in Figs. 4b and 5b. When solubilized bacteriorhodopsin was illuminated with light of $\lambda > 640$ nm, the spectral change occurred in a manner similar to that in the case of illumination with light of $\lambda > 600$ nm. However, the HPLC pattern of the 390 nm product (curve 4 in Fig. 4b) shows the formation of 9-*cis*-retinal and a trace amount of 9-*cis*, 13-*cis*-retinal (Fig. 5b).

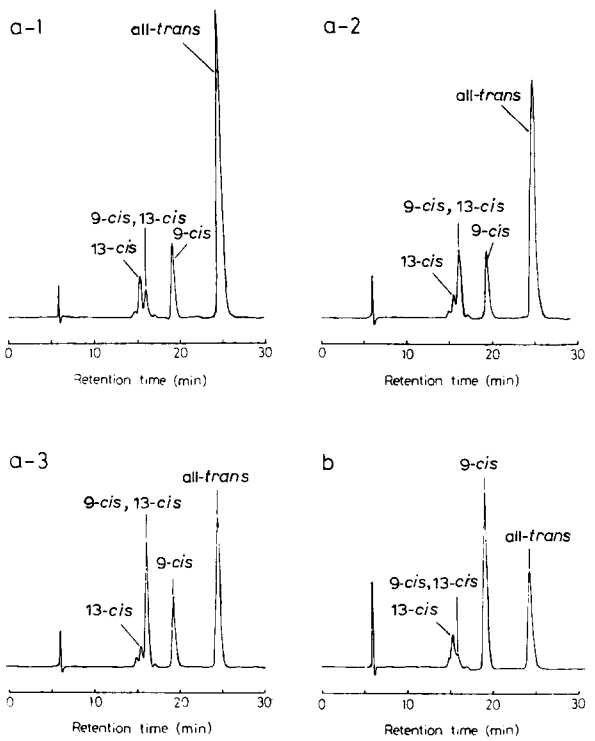
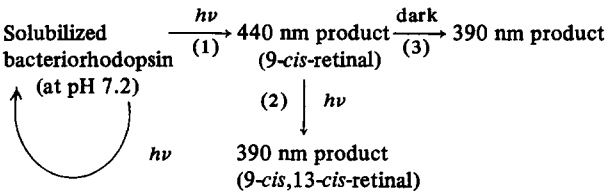


Fig. 5. HPLC patterns of retinal extracted from solubilized bacteriorhodopsin. (a-1) Illuminated with light ($\lambda > 600$ nm) for 30 min, corresponding to curve 3 in Fig. 4a. (a-2) Illuminated for 90 min (curve 4 in Fig. 4a). (a-3) Illuminated for 210 min (curve 5 in Fig. 4a). (b) Illuminated with light ($\lambda > 640$ nm) for 300 min (curve 4 in Fig. 4b).

This fact (the formation of the 390 nm product) may be explained using the following scheme:



The solubilized bacteriorhodopsin is converted to the 440 nm product by reaction 1, being different from the photoreaction cycle of bacteriorhodopsin known in the purple membrane. This is transformed further to the 390 nm product through reaction 3 in the dark, or reisomerized by light (reaction 2) to the 390 nm product containing 9-*cis*, 13-*cis*-retinal. Red light ($\lambda > 640$ nm) is not absorbed by the 440 nm product and cannot promote reaction 2. The curved arrow shows the main photoreaction cycle (see Discussion).

Fig. 4c shows the spectral change caused by violet light ($\lambda_{max} = 400$ nm) where λ_{max} (552 nm) in the dark shifted to 560 nm. After 30 min illumination, 91% of the total chromophore was all-*trans*-retinal and the rest 13-*cis*-retinal. In this case, the 440 nm product and the 390 nm product, if any, could absorb the violet light to be reconverted to the 560 nm product. In fact, the 440 nm product and the 390 nm product of curves 2 and 5 in Fig. 4a could be reconverted by the 400 nm light to the original pigment. In the parallel experiment, the purple membrane was illuminated with red light ($\lambda > 600$ nm) or violet light ($\lambda_{max} = 400$ nm) for 30 min at pH 7.2.

Retinal isomer analysis revealed that the ratio of 13-*cis*- to all-*trans*-retinal was 5 : 95, independent of the wavelength of the illuminating light.

After bacteriorhodopsin was illuminated with violet light for 15 min, the light-dark adaptation in L-1690 solution proceeded about 4-times faster in terms of the half-time of the reaction at pH 7.2 and 25°C than in the purple membrane.

Discussion

As demonstrated in Fig. 1, the bacteriorhodopsin monomer in L-1690 solution showed spectral changes depending on pH in the pH range below pH 5.5 in a manner essentially similar to that in purple membranes [12,13,25]. On the other hand, the Triton X-100-solubilized bacteriorhodopsin was relatively unstable and did not maintain the specific interaction between retinal and protein at such a low pH as shown in purple membranes and in L-1690 solution. In the study of cephalopod metarhodopsin [27], the p*K* of protonation of the retinal Schiff base was largely affected by the environmental phospholipids and detergents. L-1690 acted on the rhodopsin similarly to phospholipids.

Absorbance spectra of visual pigments are determined by the configuration of retinal, the degree of protonation to the Schiff base, and the solvent effect of the protein microenvironment [28,29]. This explanation could also be applicable to bacteriorhodopsin. From investigations on purple membranes, Mowery et al. [12] reported that bR_{605}^{acid} and bR_{565}^{acid} (following their nomenclature) were formed depending on pH, and Fischer et al. [13] reported that BR-605 was formed from the purple complex by binding of proton, and $PC_a^{L^-}$ arose from BR-605 by selective binding of anions ($PC_a^{L^-}$ and BR-605 according to the nomenclature of Fischer et al. [13]). We observed similar spectral changes in the solubilized bacteriorhodopsin. The 609 nm product had a CD peak of 620 nm and the 556 nm product had a weak CD (Fig. 2). Thus, the 609 nm product might contain the product having a λ_{max} longer than 609 nm as a major component and the 556 nm product as a minor component. The transition of the 556 nm product (pH 5.5) to the 609 nm product was pH dependent and affected by NaCl concentration as shown in Fig. 3. This might be explained by the shift of the p*K* value of the transition. The p*K* may be estimated to be between 3.0 and 4.0 and this is suggestive of the ionization of carboxyl groups of the protein. Fischer et al. [13] demonstrated in purple membranes that the anion-induced blue shift of λ_{max} due to the formation of $PC_a^{L^-}$ became larger at pH 2.0 in the order ClO_4^-, I^-, Br^-, Cl^-, of decreasing anion radius. We examined the formation of the 570 nm product at pH 1.0 in the presence of 1 M NaCl, NaBr and NaI, and ascertained that the blue shift of λ_{max} became larger in the order I^-, Br^-, Cl^-. When bacteriorhodopsin solubilized in L-1690 solution was brought to pH 0.0 by the addition of H_2SO_4, a product having a λ_{max} of 601 nm was formed and the product converted to the 450 nm product with a half-life of 30 min at 25°C. Even at the same pH, λ_{max} and the stability of the product were different depending on the anion species. Anions such as I^- and SO_4^{2-} could not resist the formation of the 450 nm product so effectively as Cl^- or Br^-. Thus, pH and salt concentration affected the structure of bacteriorhodopsin protein, i.e., the microenvironment around the retinal Schiff base, and possibly altered the spatial arrangement of the Schiff base in the protein (the degree of protonation). The retinal configuration in bacteriorhodopson changed dpending on pH, as shown in Table I, like in the purple membrane [12,13]. This isomerization of retinal was induced in the dark by the pH-sensitive structural change in protein and was related to the spectral change of bacteriorhodopsin.

The absorbance spectrum of bacteriorhodopsin in the purple membrane did not alter in the pH range 5.5–9.0, but did so in the detergent solution (curves 1 and 4 in Fig. 1). At pH 7.2, photoreaction of solubilized bacteriorhodopsin resulted in the formation of a product containing 9-*cis*- and 9-*cis*, 13-*cis*-retinal (Figs. 4 and 5). This was not observed in the purple membrane and may be due to a reaction other than the well known photoreaction cycle of bacteriorhodopsin. Since we observed the M intermediate by means of laser Raman spectroscopy (unpublished data) and the so-called dark- and light-adapted spectrum was clearly distinguishable as well as in the purple membrane, the main photoreaction of the solubilized bacteriorhodopsin should be similar to that of the purple membrane. The formation of the 390 nm product containing 9-*cis*-retinal seems to result from the inability of 9-*cis*-retinal to accommodate to the binding site of bacteriorhodopsin. A

reconstitution experiment demonstrated the inability of 9-*cis*-retinal to accommodate to the binding site of bacteriorhodopsin [30]. The formation of 9-*cis*-retinal may be due to the altered conformation of bacteriorhodopsin protein in detergent solution. The product containing 9-*cis*-retinal was observed in purple membranes illuminated under acidic conditions [31].

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