Properties of an Analogue Pigment of Bacteriorhodopsin Synthesized with Naphthylretinal†

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ABSTRACT: The artificial pigment of bacteriorhodopsin was synthesized from *all-trans*-naphthylretinal [(*all-E*)-3-methyl-7-(2-naphthyl)-2,4,6-octatrienal] and bacterioopsin in membranes. Two kinds of pigments were formed with *all-trans*-naphthylretinal; one has its absorption maximum at 503 nm (Np-bR_{503}) and another, at 442 nm (Np-bR_{442}). When the retinal/protein ratio on mixing is smaller, the larger amount of Np-bR_{503} is formed. The equimolar mixture produces Np-bR_{442}. Successive addition of small amounts of naphthylretinal yields almost 100% of Np-bR_{503}. The all-trans isomer of naphthylretinal was extracted from both pigments. After reduction of the pigments with sodium borohydride, addition of *all-trans*-retinal did not produce bacteriorhodopsin. This indicates that the binding site of naphthylretinal in both pigments is the same as that of *trans*-bR. But the CD spectra of both pigments were different from each other; that of Np-bR_{503} represents characteristic exciton CD bands, while that of Np-bR_{442} shows only a positive one. When incorporated into lipid vesicles, both pigments showed a light-dependent proton pumping activity.

Bacteriorhodopsin (bR),[1] the sole protein component of purple membrane of *Halobacterium halobium*, transports protons across the membrane upon absorbing light (Stoeckenius et al., 1979). The color of the protein results from a retinal moiety bound to Lys-216 of the apoprotein via a protonated Schiff-base linkage (Lemke & Oesterhelt, 1981; Bayley et al., 1981a). Two different configurations of the chromophore were isolated from bacteriorhodopsin; all-trans and 13-cis (Maeda et al., 1977; Pettei et al., 1977). Only the pigment having *all-trans*-retinal as its chromophore (*trans*-bR) has the ability to transport protons (Yoshida et al., 1977). Upon absorption of light, *trans*-bR undergoes a cyclic photoreaction through K, L, and M intermediates, which were investigated by several authors (Stoeckenius & Lozier, 1974; Lozier et al., 1975; Dencher & Wilms, 1975) and us (Tokunaga et al., 1976; Iwasa et al., 1979, 1980).

The use of retinal analogue as a probe for the microenvironment of the chromophore binding site was one of possible approaches for such studies. Tokunaga et al. (1981a) showed that retinal interacts with the protein at its polyene chain from reconstitution experiments with C_{17} aldehyde. Also, Towner et al. (1981) concluded that the specific binding site for the β-ionone ring does not exist on the basis of the reconstitution studies using β-ionone. It would be interesting to see whether or not the retinal analogues with a larger ring than that of ionone form a pigment and transport protons. In the present study we use 3-methyl-7-(2-naphthyl)-2,4,6-octatrienal (Np-retinal) as a probe for investigating the interaction of its chromophore with the protein.

Addition of Np-retinal to a bacterioopsin suspension resulted in the formation of two different complexes depending on the mixing ratio of protein and the chromophore; the absorption of one had its λ_{max} at 442 nm and the other, at 503 nm, although both pigments have *all-trans*-Np-retinal binding at the same region of the protein. They were different not only in color but also in their CD spectra, even though both pigments exhibited proton pumping activity.

Materials and Methods

Preparation of Purple Membrane and Bleached Membrane. Purple membrane was prepared according to the standard methods with a slight modification previously described by Iwasa et al. (1980). The purified purple membrane was suspended in 10 mM sodium phosphate buffer (pH 6.8) or 10 mM HEPES buffer (pH 6.8). Bleached purple membrane was prepared as described by Tokunaga & Ebrey (1978). After addition of hydroxylamine (pH 6.8; final concentration 0.75 M), purple membrane suspension in a water bath (20 °C) was illuminated with a 1-KW projector lamp (Master Hi-Lux) through a long-pass filter, Toshiba 0-53 ($\lambda > 510$ nm). When the suspension became colorless, the bleached membrane was collected by centrifugation (17000*g* for 20 min) and lyophilized. Retinal oxime was extracted from the bleached membrane by sonicating in hexane with Ultra Sonic Generator T-A-4280 (Kaijo Denki Co.). Until absorbance at 380 nm in the organic layer was not observed, the extraction was repeated. After almost complete removal of retinal oxime, the bleached membrane was dried up under a gentle stream of N_2 gas.

The bleached membrane was suspended in 10 mM sodium phosphate buffer (pH 6.8) or 10 mM HEPES buffer (pH 6.8) and sonicated for a few minutes to decrease light scattering. The bleached sample thus obtained was designated as bPM (bleached purple membrane). The concentration of bPM solution was determined by regenerating *trans*-bR with an excess of *all-trans*-retinal and measuring the absorption spectrum of its light-adapted form ($\epsilon_{570} = 6.3 \times 10^4$).

Measurements of Absorption and CD Spectra. Absorption spectra were measured by a conventional spectrophotometer (Shimadzu MPS-5000 or Shimadzu UV-300). For some

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[1] Abbreviations: bR, bacteriorhodopsin; *trans*-bR, bR having *all-trans*-retinal as its chromophore; 13-*cis*-bR, bR having 13-*cis*-retinal as its chromophore; λ_{max}, the wavelength of maximum absorbance; Np-retinal, (*all-E*)-3-methyl-7-(2-naphthyl)-2,4,6-octatrienal; Np-bR, the pigment reconstituted from Np-retinal and bacterioopsin; Np-bR_{503}, Np-bR showing its λ_{max} at 503 nm; Np-bR_{442}, Np-bR showing its λ_{max} at 442 nm; bPM, the bleached purple membrane; DMPC, dimyristoylphosphatidylcholine; HPLC, high-performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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measurements, the bPM solution was put in a reference cuvette in order to subtract a contribution from light scattering.

For measurements of CD spectra, samples were resuspended in 50% sucrose solution (10 mM HEPES buffer, pH 6.8) to decrease the contribution from light scattering. Absorbances of the samples at their λ_{max}'s were adjusted to about 1 OD in a cell with a 2-mm light path. CD spectra were measured by JASCO J-40A polarimeter and represented as an average of four scans (JASCO J-DPZ), or by a JASCO J-50 instrument.

Retinal Purification and Extraction. Naphthylretinal was synthesized as follows. A suspension of sodium hydride (0.106 g) (50% oil dispersion) in dimethoxyethane (70 mL) was added to the triethyl phosphonosenecionate (0.591 g). The temperature was allowed to rise to 55 °C for 2 h and then cool down to 0 °C. A solution of 3-(2-naphthyl)-2-butenal was added to the clear solution obtained above. Stirring was continued for 5 h. The temperature was raised to room temperature, and the solvent was removed in vacuo. Ether was added to the residue, and the extract was chromatographed on silica gel to give ethyl 3-methyl-7-(2-naphthyl)-2,4,6-octatrienoate (0.362 g) in 95.6% yield. Naphthylretinal was obtained by hydrolysis with KOH. The Np-retinal thus obtained was checked by mass spectroscopy.

The all-trans form of Np-retinal was purified by high-performance liquid chromatography (HPLC) (JASCO Tri-Rotar II, porous silica gel SS-05; solvent 15% diethyl ether and 85% petroleum ether; flow rate 1.5 mL/min). The isomeric configuration of isolated Np-retinal was confirmed with ^{1}H NMR spectrum (360 or 270 MHz). The fraction with a retention time at 17.5 min was isolated. Other isomers in the purified *all-trans*-Np-retinal were less than 1% judged from the chromatogram. As only the all-trans form was used in this study, "Np-retinal" means the all-trans isomer hereafter.

The extraction of the chromophoric retinal from Np-bR was performed according to the methods described by Tsuda et al. (1980). All procedures were carried out at 0 °C under dim red light. The sample (0.5 mL, nearly 1 OD) was mixed with an equal volume of CH_2Cl_2, and the mixture was sonicated for a total of 5 min in an ice bath. Petroleum ether (3 mL) was added to it. After separation of the layers, less than 15 μL of the upper phase was injected to the HPLC system. This extraction was repeated more than 3 times. The isomer was identified by comparing the retention time with that of an authentic sample. The recovery of retinals after extraction by this procedure was usually about 40%.

Measurements for Proton Pumping Activity. Naphthyl-bR in buffer was collected by centrifugation at 50000*g* for 25 min and resuspended in 150 mM KCl (pH 8.0). The pelleting and resuspension were repeated 3 times. Dimyristoylphosphatidylcholine (DMPC) (Sigma) was used for vesicle formation without further purification. The DMPC solution in chloroform was dried under N_2 gas and sonicated for about 10 min after addition of the Np-bR suspension (100 mol of DMPC/mol of Np-bR). During sonication under a gentle stream of N_2 gas the temperature was kept higher than 25 °C. After centrifugation at 50000*g* for 25 min, vesicles containing the pigment were separated from free membranes in the pellet and nonpigment-containing vesicles at the top.

The proton pumping activity of the vesicles was measured with a glass electrode (Beckman 39905) connected to a pH meter, Beckman 3605. The vesicle suspension in a glass cell with a circulated water jacket was irradiated with a 1-KW projector lamp (Master Hi-Lux). Wavelengths of light were selected by a water layer (10 cm), a long-pass filter [Toshiba L-39 ($\lambda > 370$ nm)], and/or an interference filter [Toshiba KL 44 ($\lambda = 442$ nm) or KL 56 ($\lambda = 560$ nm)].

When hydroxylamine was added to the vesicle suspension, the change of the spectrum was monitored by the spectrophotometer (Shimadzu UV-300). After further changes in the spectrum ceased, the mixture was washed with 150 mM KCl solution (pH 8.0) several times to remove the residual hydroxylamine. The energy of the irradiation light was measured with a calibrated photodiode (Hamamatsu TV, S-1133).

Results

Formation of Analogue Pigments, Np-bR$_{503}$ and Np-bR$_{442}$. Various amounts of Np-retinal were added to a given amount of bPM (0.16 mg, 0.7 mL) and the absorption spectra measured after incubation for 2 h at 20 °C. The absorption maxima of the products were different depending on the amount of the added Np-retinal (Figure 1a). When the amount of added Np-retinal was less than a half of bacterioopsin, the products showed broad absorption spectra between 450 and 530 nm. When the amount of added Np-retinal reached that of bacterioopsin, the λ_{max}'s shifted toward 442 nm (curves 4–6 in Figure 1a) due to formation of the different pigment.

On the other hand, a different phenomenon was observed when a small amount of Np-retinal was added to a larger amount of bPM at 2-h intervals (Figure 1b): Small amounts of Np-retinal (0.5 μL, $A_{370} = 75$ OD) were added successively to bPM (0.24 mg, 0.7 mL). When the amount of Np-retinal was below one-third saturated, the λ_{max} was at 503 nm (curves 1–4 in Figure 1b). Even until the amount of added Np-retinal reached saturation, the absorbance around 500 nm increased.

In Figure 1c, the absorbance increase at 540 nm [A_{540} (○)] in Figure 1b was plotted vs. the amount of Np-retinal. Until the amount of Np-retinal was less than one-third saturated, the absorbance at 540 nm increased linearly. This indicates that one species of the pigment was formed which we call Np-bR$_{503}$. When the amount of Np-retinal added was over a third saturated, the absorbance increase at 540 nm did not follow the previous phase. The absorption spectra of the products (curves 5–9 in Figure 1b) were not the same as those of Np-bR$_{503}$ (curves 1–4 in Figure 1b). The newly formed product showed its absorption maximum around 440 nm. The absorbance increase at 460 nm due to newly formed product was estimated as follows:

$$A_{460} = A^{n}_{460} - A^{\overline{1-4}}_{460} A^{n}_{540} / A^{\overline{1-4}}_{540}$$

where n and $\overline{1-4}$ mean the absorbance estimated from curve n and the mean value estimated from curves 1 to 4, respectively. From Figure 1c, A_{460} was zero until the added Np-retinal was about a third saturated. This was consistent with the results shown in Figure 1a; the wavelength of the maximum absorption shifted to 440 nm as the amount of added Np-retinal increased.

With a further addition of Np-retinal, the absorbance at 380 nm increased due to the excess chromophore. In Figure 1c, A_{360} was also shown. The value of A_{360} was estimated as follows:

$$A_{360} = A^{n}_{360} - A^{\overline{1-4}}_{360} A^{n}_{540} / A^{\overline{1-4}}_{540}$$

From the above results, it was concluded that two different types of pigments were formed from Np-retinal and bPM. The wavelength for the maximum absorbance of the product mixture depended on the molar ratio of added Np-retinal to bacterioopsin on mixing. The addition of the small amount of Np-retinal (less than one-tenth of bacterioopsin) furnished the pigment showing its λ_{max} at 503 ± 3 nm (Np-bR$_{503}$).

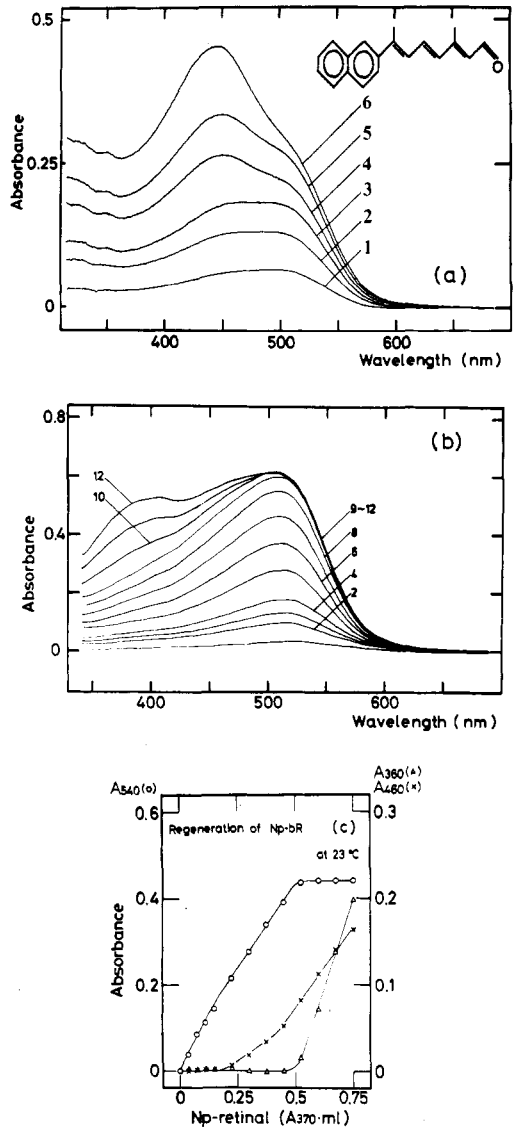

FIGURE 1: Formation of two different pigments from Np-retinal and bacterioopsin. (a) The formation of the pigments after addition of Np-retinal in different retinal/protein ratios (1/12 to 1/1). (Curves 1–6) the products from bPM (0.7 mL, 12 μM) after addition of Np-retinal (A_{370} = 60 OD) of 0.5, 1.0, 1.5, 3.0, 5.0, and 6.0 μL, respectively. (Inset) The chemical structure of *all-trans*-Np-retinal. (b) The formation of the pigments after successive addition of *all-trans*-Np-retinal. The absorption due to bPM (0.7 mL, 13 μM) was used as a base line. (Curves 1–12) The products after successive addition of 0.5 μL of Np-retinal (A_{370} = 75 OD) to bPM (curves 1–4) or 1 μL (curves 5–12). The absorption spectra were measured after a 2-h incubation in the dark. (c) The absorbance intensities at 540 (○), 460 (×), and 360 nm (△) were taken from (b) and plotted vs. the amount of retinal added. Details are described in the text.

When the equimolar amount of Np-retinal was added to bacterioopsin, the absorption spectrum showed λ_{max} at 442 ± 3 nm (Np-bR_{442}) together with a very small shoulder around 505 nm probably due to the concomitant production of the pigment mentioned above. Although further incubation at room temperatures in the dark caused a small increase in the absorbance at 503 nm, the λ_{max} of the product was still at 442 nm after overnight incubation in the dark.

The processes for the formation of both pigments were studied under two different conditions with varied molar ratios of the retinal and the protein. When a small amount of Np-retinal was added to bPM, the absorbance increase at 400 nm was observed (within 12 s) after addition of Np-retinal. Since the free Np-retinal showed its λ_{max} around 380 nm (curves 10–12 in Figure 1b), the absorbance increase at 400 nm may be due to an intermediate in the formation of Np-bR_{503}. This intermediate gradually changed to Np-bR_{503} with an isosbestic point at 440 nm.

In the case of pigment formation using a larger amount of Np-retinal, the absorbance increase at 400 nm was not observed even at 26 s after addition of Np-retinal. The intermediate that appears in the formation step of Np-bR_{503} was not observed. Absorbance at 442 nm increased with concomitant decreases in absorbances over a range of wavelengths shorter than 410 nm.

The values of λ_{max} of the synthesized pigments did not change within a day in the dark at room temperature. This contrasts with *all-trans*- or 13-*cis*-retinal, where the λ_{max} of the regenerated pigment (570 nm for *trans*-bR or 550 nm for 13-*cis*-bR) (Iwasa et al., 1981) shifted to that of the dark-adapted form (560 nm) within several hours in the dark at room temperature (Maeda et al., 1977).

Reaction with Hydroxylamine. Hydroxylamine is a reagent which attacks the retinal–Schiff base and forms retinal oxime. In the case of bR and cattle rhodopsin, the retinal–Schiff base is inaccessible to hydroxylamine in the dark. Both of the pigments generated from Np-retinal and bacterioopsin slowly react with hydroxylamine in the dark.

Parts a and b of Figure 2 show the reaction of Np-bR_{503} and Np-bR_{442} with an excess amount of hydroxylamine. Upon addition of hydroxylamine, the absorbance of the pigments slowly decreased with an isosbestic point at 400 nm (decay of Np-bR_{503}; Figure 2a) or at 395 nm (decay of Np-bR_{442}; Figure 2b), and the absorbance at shorter wavelengths increased. The decays of both pigments were a single exponential (Figure 2c). These spectral changes had different time courses (Figure 2c), but the UV spectra of both products had their λ_{max}'s at 365 nm with shoulders at 350 and 385 nm, suggesting that the product is Np-retinal oxime. The difference in the reaction rate ($\tau_{1/2}$ of Np-bR_{442} = 40 min; $\tau_{1/2}$ of Np-bR_{503} = 200 min) was due to a difference in environment around the Schiff-base portion of the chromophore.

Extraction of the Chromophore from Np-bR's. The isomeric composition of Np-retinal in both Np-bR_{503} and Np-bR_{442} was analyzed by use of HPLC. Both pigments gave mainly *all-trans*-Np-retinal (87 ± 3% for Np-bR_{503} and 88 ± 2% for Np-bR_{442}, assuming that both *all-trans*- and 13-*cis*-Np-retinal[2] have the same extinction coefficient at 350 nm) (data not shown). The small amount of 13-*cis*-Np-retinal may arise from thermal isomerization during extraction. No other isomers of Np-retinal were found. Thus, the isomeric form of the chromophore of both Np-bR_{442} and Np-bR_{503} was mainly all-trans.

Dark isomerization of the chromophore of both Np-bR's should be forbidden, since the same amount of *all-trans*-Np-retinal was extracted from the pigments kept in the dark at room temperature even for a quite long time. This notion is consistent with the observation that the λ_{max}'s of Np-bR's did not change during the regeneration process in the dark at room temperature.

Binding Site of Np-retinal in Bacterioopsin. In order to clarify the possibility that the binding sites of both pigments are different, both pigment suspensions were treated with sodium borohydride. Addition of sodium borohydride under illumination shifted λ_{max}'s of both pigment suspensions to shorter wavelengths. The main band due to Np-bR_{503} or

[2] 2-*cis*-3-Methyl-7-(2-naphthyl)-2,4,6-octatrienal. Since the position of the cis bond is corresponding to 13-*cis*-retinal, we designate it as 13-*cis*-Np-retinal.

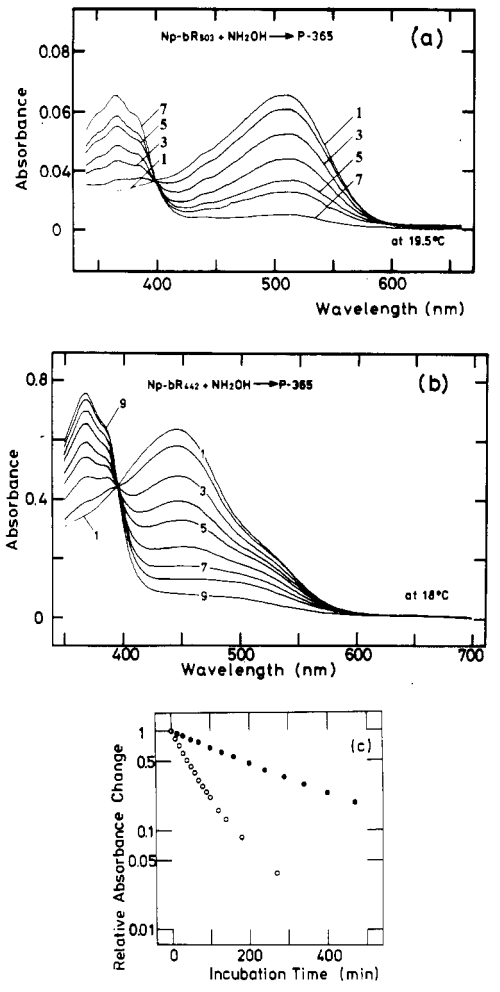

FIGURE 2: Reaction with hydroxylamine. (a) After Np-bR$_{503}$ was formed from 0.5 μL of Np-retinal (A_{370} = 115 OD) and bPM (4.3 nmol) and kept in the dark for 16 h (curve 1), 3 μL of hydroxylamine solution (4 M, neutralized with NaOH) was added to it. The absorption spectra were measured at 30, 100, 200, 340, and 470 min after the injection of hydroxylamine (curves 2–6, respectively). After an overnight dark incubation, the absorption spectrum (curve 7) was measured. (b) Np-bR$_{442}$ was formed from 4 μL of Np-retinal (A_{370} = 115 OD) and bPM (4.3 nmol). After an overnight dark incubation (curve 1), 3 μL of neutralized hydroxylamine (4 M solution) was added. The absorption spectra were measured at 6, 20, 35, 50, 80, 120, and 180 min after an addition of hydroxylamine (curves 2–8, respectively). After an overnight dark incubation, the final spectrum (curve 9) was measured. (c) The time course of the relative absorbance changes at 510 nm in (a) (●) and 440 nm in (b) (○), $(A_t - A_0)/(A_\infty - A_0)$, were plotted vs. the incubation time in logarithmic scale.

Np-bR$_{442}$ disappeared and the sharp peak at 343 nm having two shoulders at 325 and 360 nm appeared (curve 2 in Figure 3a,b). The shape of the spectra and wavelengths of the new peaks were the same, indicating that the chromophores of both products were identical. Then the sodium borohydride treated protein was washed several times to remove residual sodium borohydride, and *all-trans*-retinal was added. If Np-retinal binds to a different site from Lys-216, absorbance due to *trans*-bR would be expected to increase. But no significant absorbance increase was observed over a range of 500–600 nm after a 2-h dark incubation (curve 3 in Figure 3a,b). When a similar experiment was done with bacterioopsin without preaddition of Np-retinal, absorbance increase due to *trans*-bR was observed after addition of *all-trans*-retinal. These results strongly suggest that the binding sites of Np-retinal in both pigments were the same as those of *trans*-bR.

Another kind of experiment was performed to obtain information about the binding site of Np-retinal. When *all-*

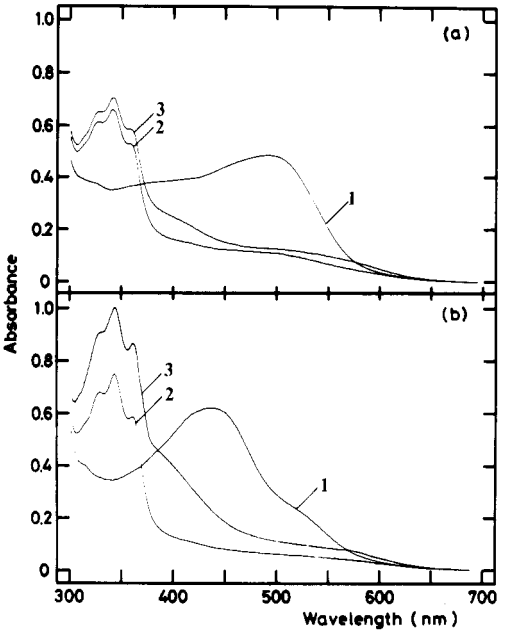

FIGURE 3: Effects of reduction with sodium borohydride on Np-bR$_{503}$ (a) and Np-bR$_{442}$ (b). (Curve 1) Np-bR$_{503}$ (a) or Np-bR$_{442}$ (b) was formed from a nearly equimolar mixture of bacterioopsin and *all-trans*-Np-retinal. (Curve 2) A small amount of $NaBH_4$ (final concentration of 1%, w/v) was added to Np-bR$_{503}$ (a) or Np-bR$_{442}$ (b). Light at 500 (a) or 440 nm (b) was irradiated for 1 h. (Curve 3) After the residual $NaBH_4$ was washed out with distilled water 3 times and precipitate was resuspended with 10 mM HEPES buffer (pH 6.8), an equimolar amount (a) or 1.4-fold (b) of *trans*-retinal was added to it. The absorption spectrum was measured after 1-h incubation in the dark.

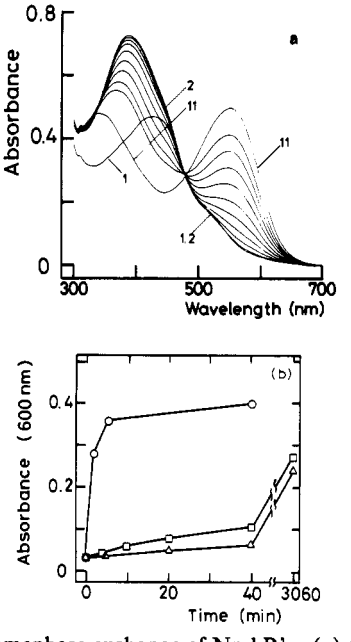

FIGURE 4: Chromophore exchange of Np-bR's. (a) (Curve 1) Np-bR$_{442}$. (Curves 2–11) The products of adding *all-trans*-retinal (7.3 nmol) to Np-bR$_{442}$ (curve 1). The absorption spectra were measured at 1, 4, 10, 20, 40, 80, 160, 330, 600, and 3060 min after the addition of *all-trans*-retinal, respectively. (b) A time course of the formation of *trans*-bR with or without Np-retinal. (○) The absorbance change at 540 nm after mixing equimolar *all-trans*-retinal with bacterioopsin. (△) The absorbance change at 600 nm after the addition of the excess amount (1.2-fold) of *all-trans*-retinal to fully regenerated Np-bR$_{503}$. (□) The absorbance change at 600 nm after the addition of an equimolar amount of *all-trans*-retinal to Np-bR$_{442}$.

trans-retinal was added to the suspension of fully synthesized Np-bR$_{442}$ (curve 1 in Figure 4a) or Np-bR$_{503}$ without sodium borohydride treatment, chromophore exchange took place; the

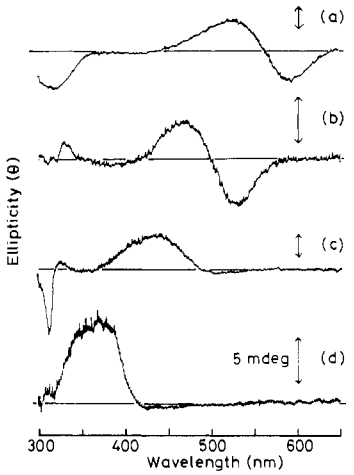

FIGURE 5: Circular dichroic spectra of the following samples in 50% sucrose solution (w/v). (a) *trans*-bR (A_{570} = 1.24 OD). (b) Np-bR_{503} (A_{503} = 0.86 OD). (c) Np-bR_{442} (A_{442} = 1.15 OD). (d) The product of Np-bR_{503} with addition of 5 μL of 2 M neutralized hydroxylamine solution. The bars on the right side of the figures represent the ellipticity (5 mdeg).

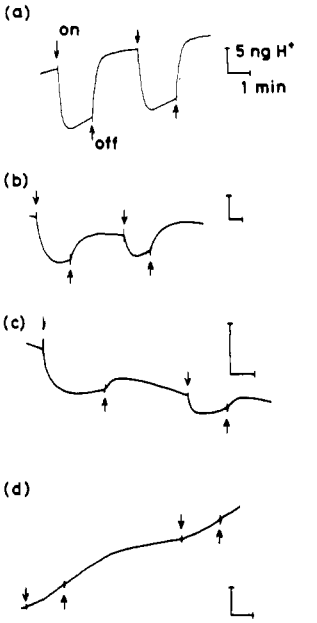

FIGURE 6: Light-dependent proton pumping activity of vesicles containing the following pigments: (a) bacteriorhodopsin, (b) Np-bR_{442}, (c) Np-bR_{503}, and (d) Np-bR_{442} with added hydroxylamine. Proton pumping activity of vesicles was measured in 150 mM KCl solution at 25 °C with white light illumination. Light on and light off were represented by the arrows (↑) and (↓), respectively. The bars on the right side of the figures represent the amount of protons (5 ng; vertical bar) and time (1 min; horizontal bar), respectively.

absorbance of Np-bR decreased as the absorbance of *trans*-bR increased (curves 2–11 in Figure 4a). The absorbance around 440 nm due to Np-bR_{442} (curve 1 in Figure 4a) disappeared (curve 11 in Figure 4a). The exchange rate was much slower than the regeneration rate of *trans*-bR (Figure 4b). This observation also suggests that the Np-retinal in Np-bR's and retinal in *trans*-bR occupy the same site of the bacterioopsin.

Circular Dichroic Spectra of Np-bR's. The CD spectrum of bacteriorhodopsin in purple membrane consists of oppositely directed two bands due to an exciton interaction between the trimerically arranged chromophores (Figure 5a; Ebrey et al., 1977). No CD signal was observed in bPM. After addition of Np-retinal to the suspension, the CD bands appeared, but their spectral shapes of the two pigments were different; the CD spectrum of Np-bR_{503} was typical of exciton-coupled chromophores (Figure 5b). But only a positive peak was observed at 440 nm in the CD spectrum of Np-bR_{442} (Figure 5c). After incubation of Np-bR_{503} with hydroxylamine, the positive CD band and the absorption band due to Np-bR_{503} disappeared, and a large positive CD band appeared at the wavelengths corresponding to the λ_{max} of hydroxylamine-treated Np-bR_{503} (Figure 5d; see curve 7 in Figure 2a). The CD band of Np-bR_{442} also disappeared with hydroxylamine treatment.

The difference between CD bands in Np-bR_{503} and Np-bR_{442} means that two pigments are different in the chromophoric arrangement. Exciton coupling of CD spectrum of Np-bR_{503} suggests that the chromophore alignment of Np-bR_{503} is similar to that of native bR.

Proton Pumping Activity. The one major purpose of using Np-retinal in the present investigation is to check the role of the β-ionone ring in the proton pumping activity of bR.

Np-bR_{503} incorporated in DMPC vesicles has its λ_{max} around 470 nm. Scattering was corrected for by subtracting the spectrum of vesicles treated with hydroxylamine. The shift of λ_{max} of Np-bR_{503} in vesicles is probably related to changes in protein conformation resulting from incorporation into the vesicles. In the case of bR, incorporation into the vesicles caused a shift of the λ_{max} to a shorter wavelength (our unpublished observation).

The vesicles with Np-bR_{503} or Np-bR_{442} showed proton pumping activity under illumination with white light (Figure 6b,c). Several kinds of measurements were performed in order to exclude a possibility that the observed proton pumping activity was derived from bR that remained in bPM. The vesicles with the same amount of bPM used for reconstitution of Np-bR's showed only less than 6% of the activity of Np-bR's. The vesicles formed with Np-bR's showed a larger activity by irradiation with 440-nm light than that observed with 560-nm light of the same quanta. After incubation with hydroxylamine in the dark, the vesicles formed with Np-bR_{442} did not pump protons at all (Figure 6d). Thus, Np-bR's can transport protons by light, indicating that the naphthalene ring that is larger than the β-ionone ring in retinal did not abolish the proton pumping activity.

Discussion

Several kinds of retinal analogues have so far been used for analysis of the retinal binding site in bR. We found in the present work that naphthylretinal binds with bacterioopsin at the correct "binding site", Lys-216. Akhtar et al. (1982) have reported the formation of a single species of the pigment with Np-retinal having its λ_{max} at 504 nm, which probably corresponds to our Np-bR_{503}. Contrary to their results, we found Np-bR_{503} only under specific experimental conditions where the amount of added retinal was quite small compared to the total retinal binding sites, and another form, Np-bR_{442}, was found to be produced with a stoichiometric amount of Np-retinal. A part of these results have already been presented at the annual meeting of the Japanese Biochemical Society (Tokunaga et al., 1981b).

The formation of different pigments dependent on the mixing ratio would be explained as follows: The large head of Np-retinal may perturb the smooth fitting of chromophore and bacterioopsin. The small amount of Np-retinal and a long enough time interval after addition allow the formation of a stable state (Np-bR_{503}) in which the chromophore–protein interaction may be similar to that of *trans*-bR as suggested by CD spectrum. The high amount of Np-retinal, however, may not allow such smooth fitting and result in another stable

state between chromophore and bacterioopsin.

It is interesting that both kinds of Np pigments contain almost exclusively the all-trans isomer of Np-retinal. The difference between Np-bR$_{503}$ and Np-bR$_{442}$ is attributable to the extent of the interactions between chromophore and the protein as shown by different sensitivity to hydroxylamine and by CD spectra: (1) The exciton CD band, which is characteristic of trimer structure, was not observed in Np-bR$_{442}$. (2) The negative CD band at 310 nm, which probably results from steric hindrance of the chromophore (Becher & Cassim, 1976), was larger in Np-bR$_{442}$ than in Np-bR$_{503}$. (3) Np-bR$_{442}$ was much more sensitive to hydroxylamine than Np-bR$_{503}$ (Figure 2c), though Np-retinal formed a Schiff-base linkage in both pigments (Figure 3).

The wavelength at maximum absorption of the free Np-retinal in ethanol was at 370 nm (2.70×10^4 cm^{-1}), not so different (800 cm^{-1}) from that of *all-trans*-retinal (381 nm; 2.62×10^4 cm^{-1}). This was rather interesting, because Np-retinal seems to have a longer conjugation of the double bonds as shown in the inset in Figure 1a. It may be that the π-electron system of Np-retinal is partially separated into two parts, the naphthalene ring and the polyene chain. The λ_{max} of 5,6-didehydroretinal, which has five double bonds, is at 368 nm in ethanol solution (Mao et al., 1981). Similarly, the main absorption band of Np-retinal at 370 nm may result from the polyene chain. When Np-retinal was incorporated in bacterioopsin, the products, Np-bR$_{442}$ and Np-bR$_{503}$, represented their λ_{max}'s at shorter wavelengths than that of *trans*-bR (λ_{max} = 570 nm). In the case of phenylretinal, the λ_{max} of which is at 391 nm in ethanol, the complex with bacterioopsin has its λ_{max} at 510 nm (Bayley et al., 1981b). In these pigments only the structure of the head region of the chromophore was different. These results suggest a possible importance of the torsion between the C_5–C_6 double bond and polyene side chain of retinal in a large bathochromic shift of the λ_{max}.

When incorporated into DMPC vesicles, Np-bR$_{442}$ or Np-bR$_{503}$ pump protons on absorption of light. This fact and additional evidence reported by Bayley et al. (1981b) and Towner et al. (1980) elucidated that the *retro*-retinyl type of intermediates is not responsible for proton pumping mechanism.

The molecular species with similar absorption maximum to Np-bR$_{442}$ was observed as an intermediate on the regeneration process of *trans*-bR by Schreckenbach et al. (1977). Its absorption spectrum has three peaks (400, 430, and 460 nm). In the case of Np-bR$_{442}$, the absorption spectrum does not show such a structure. Upon incubation at 4 °C, Np-bR$_{442}$ was slowly converted to Np-bR$_{503}$. This reaction was so slow that it took several days to complete the reaction. This observation does not necessarily indicate that the Np-bR$_{442}$ is an intermediate on the formation pathway of Np-bR$_{503}$, because a pigment having its absorbance around 400 nm was observed as the intermediate on the regeneration process of Np-bR$_{503}$ (data not shown). This observation can be explained by the presence of the thermal equilibrium between Np-bR$_{503}$ and Np-bR$_{442}$ (preliminary observation).

Acknowledgments

We thank Prof. M. Hatano for the kind permission to use the spectropolarimeter and Y. Ito for her technical assistance. Prof. A. Maeda is also thanked for helpful discussions and critical reading of the manuscript.

Registry No. Np-retinal, 81455-08-5; 13-*cis*-Np-retinal, 81455-09-6; *all-trans*-retinal, 116-31-4; (*E*)-triethyl phosphonosenecionate, 39760-56-0; (*E*)-3-(2-naphthyl)-2-butenal, 81826-92-8; *all-trans*-ethyl 3-methyl-7-(2-naphthyl)-2,4,6-octatrienoate, 81826-94-0; DMPC, 13699-48-4; hydroxylamine, 7803-49-8; hydrogen ion, 12408-02-5.

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