Photochemical Properties of Naphthylbacteriorhodopsins Differing in Their Protein–Chromophore Interactions†

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ABSTRACT: Artificial pigments of bacteriorhodopsin (bR) were synthesized with naphthylretinal [Np-retinal; (*all-E*)-3-methyl-7-(2-naphthyl)-2,4,6-octatrienal] and termed Np-bR_{442}, Np-bR_{503}, and Np-bR_{480} after their λ_{max}'s. Although both Np-bR_{442} and Np-bR_{503} have the same *all-trans*-naphthylretinal binding to the same position of apoprotein as the native one, they are different in color and CD spectra, probably due to different steric interactions at the ring portion [Iwasa, T., Takao, M., Yamada, M., Tsujimoto, T., & Tokunaga, F. (1984) *Biochemistry 23*, 838–843]. Upon irradiation at room temperature, Np-bR_{480} is produced from Np-bR_{442} but not from Np-bR_{503}. Np-bR_{480} has a larger amount of 13-*cis*-retinal as its chromophore (60%) and reverts to Np-bR_{442} in the dark at room temperature. Np-bR_{442} and Np-bR_{503} formed their own batho intermediates, which were photoreversibly converted to their mother pigments at −185 °C. Meta intermediates were formed from both pigments upon irradiation at −65 °C. The meta intermediate of Np-bR_{503} disappeared at lower temperatures than that of Np-bR_{442} and reverted to Np-bR_{503} upon warming, while that from Np-bR_{442} was converted to Np-bR_{480}. These results indicate that the main photochemical reaction pathway is not disturbed by the substitution of a naphthyl group in place of a β-ionone ring or by the difference in the chromophoric structures around the ring portion.

The light-dependent proton pump bacteriorhodopsin (bR)[1] is the chromoprotein containing retinal as the chromophore. The chromophoric retinal interacts with the apoprotein, and the interaction determines the color of the protein. On absorbing light, bR undergoes the photoreaction through several intermediates characterized by their color. The color change suggests that the interaction between the chromophore and apoprotein changes during the photoreaction. The changes may trigger physiological processes.

In order to investigate the interaction between the chromophore and the apoprotein, many different types of retinal analogues were used to form artificial pigments of bR [see the recent review by Crouch (1986)]. Previously we used a retinal analogue, (*all-E*)-3-methyl-7-(2-naphthyl)-2,4,6-octatrienal (we designated it *all-trans*-naphthylretinal; Np-retinal), having a large head group (Figure 1) to investigate the interaction around the ring portion and found that the large head group does not perturb the proton-pumping activity of bR (Iwasa et al., 1984). We also found that two different pigments of Np-bR, such as Np-bR_{442} and Np-bR_{503}, were formed depending on conditions of regeneration, though Akhtar et al. (1982) reported only one species (λ_{max} 502 nm). When the relatively small amount of the analogue retinal is added to the suspension of apoprotein of bR (bop) (molar ratio of retinal to bop is ca. 1/10), the 400-nm complex which shows λ_{max} around 400 nm appears first and then converts to Np-bR_{503}. The formation of Np-bR_{503} finishes within 2 h at room temperature, and the formed pigment is stable in the dark. If nearly equimolar amounts of retinal analogue and bop are mixed at once, Np-bR_{442} appears immediately (Figure 1). Np-bR_{442} slowly converts to Np-bR_{503} at room temperature during several days. *all-trans*-Naphthylretinal was extracted from both pigments, and by several experiments it was confirmed that the binding site is the same in both pigments as that in bR (Iwasa et al., 1984). We concluded that the apoprotein–chromophore interaction around the ring portion induces the difference in color between Np-bR_{503} and Np-bR_{442} and in their CD spectra. Recently, several authors also reported about the steric interaction at the ring portion of bR and the presence of two different conformational states of analogue pigments (Maeda et al., 1984; Sheves et al., 1984).

In the present paper we investigated the photochemical properties of naphthylbacteriorhodopsins paying attention to the following two points. If the β-ionone ring portion of the retinal interacts with the apoprotein and the interaction plays an important role in the photoreaction of bR, it can be expected that Np-bRs will show different photochemical properties from those of bR. Another purpose is to compare the photochemical properties of the Np-bRs with each other in order to know whether or not the different conformational states of the same chromophore alter their photochemical properties.

Materials and Methods

Halobacterium halobium R_1m_1 was cultured, and the purple membrane was prepared according to standard methods with slight modification (Iwasa et al., 1980). The purified purple membrane was suspended in 10 mM HEPES buffer (pH 8.0) and bleached in the presence of hydroxylamine. The retinal oxime was extracted according to Tokunaga and Ebrey (1978).

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[1] Abbreviations: bR, bacteriorhodopsin; *trans*-bR, bR having *all-trans*-retinal as its chromophore; λ_{max}, wavelength of maximum absorbance; Np-retinal, (*all-E*)-3-methyl-7-(2-naphthyl)-2,4,6-octatrienal; Np-bR_{503}, Np-bR_{442}, and Np-bR_{480}, pigments reconstituted with Np-retinal and bacterioopsin and named after their λ_{max}'s; bop, apoprotein of bR; PSS420, photo-steady-state mixture produced by irradiation of Np-bR_{442} at 420 nm at −185 °C; PSS600, photo-steady-state mixture produced by irradiation of PSS420 with red light (>600 nm) at −185 °C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

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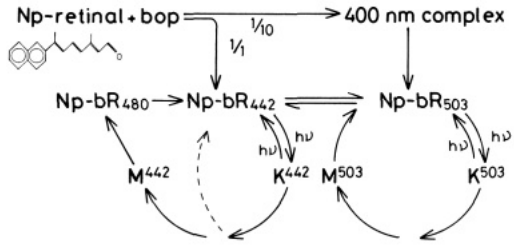

FIGURE 1: Summarized scheme of photoreaction cycles and dark reactions of Np-bRs. The supersuffix 503 or 442 means that the K (batho) or M (meta) intermediate is formed from Np-bR_{503} or Np-bR_{442}. The ratios beside the arrows indicate the molar ratios of Np-retinal to bacterioopsin. The arrows with *hν* indicate the photoreactions and the others, thermal reactions.

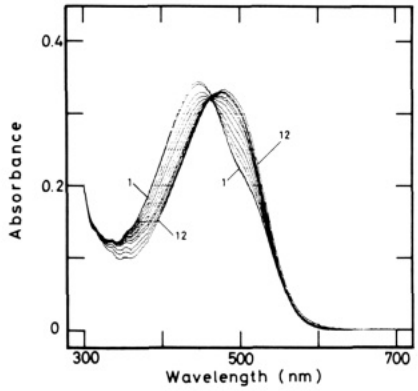

FIGURE 2: Formation of Np-bR_{480}. Np-bR_{442} (curve 1) was irradiated at 18 °C at 420 nm for 1, 5, 5, 10, 20, 40, 80, 160, 320, and 640 min (curves 2–11, respectively). Np-bR_{480} (curve 12) was obtained after 46.3-h irradiation.

Np-retinal was synthesized and purified on an HPLC system (JASCO Trirotar II) just before use as previously reported (Iwasa et al., 1984). Np-bR_{442} and Np-bR_{503} were prepared by the same method as reported previously (Iwasa et al., 1984). Np-bRs were mixed with glycerol (75% v/v), and the mixtures were used as samples for low-temperatures experiments. After extraction of the chromophoric retinal from Np-bRs according to Tsuda et al. (1980), the isomeric composition was analyzed by use of the same HPLC system as used for purification.

The absorption spectra were recorded on an Hitachi 320 spectrophotometer. For measurements at low temperatures, a Dewar especially equipped for the Hitachi 320 spectrophotometer was used. The temperature of the sample was monitored with a copper–constantan thermocouple attached to the sample holder. The wavelengths of irradiation light from a xenon lamp (500 W, Ushio) were selected with a long-pass filter [Toshiba O-50 (>480 nm), O-56 (>540 nm), or R-62 (>600 nm)] or a combination of an interference filter and a long-pass filter [Toshiba L-39 + KL-42 (420 nm) or L-43 + KL-45 (450 nm)].

RESULTS

Photochemical Reactions at Room Temperature. On irradiation with 420-nm light at 18 °C, Np-bR_{442} was converted to a new species having its λ_{max} in the longer wavelength region (Figure 2), and the subsequent dark incubation reversed reaction to the original pigment (data not shown), indicating that Np-bR_{442} undergoes a light and dark adaptation like native bR. The light adaptation of Np-bR_{442} took a longer irradiation time, and the dark process is also slower than that of native bR. The light-adapted state formed from Np-bR_{442}, designated as Np-bR_{480}, was different from Np-bR_{503}, because their λ_{max}'s are different and Np-bR_{480} reverts to Np-bR_{442}, but not to Np-bR_{503}, in the dark at room temperature.

Table I: Isomeric Composition of Np-bRs

	isomeric composition (%)[a]	
	13-cis[b]	all-trans
Np-bR_{503} (dark)	13 ± 3	87 ± 3
Np-bR_{503} (light)	17 ± 2	83 ± 2
Np-bR_{442}	12 ± 2	88 ± 2
Np-bR_{480}	63 ± 6	37 ± 6

[a] These values were averages of four to seven independent extractions. Three HPLC spectra were taken for each extraction. The isomeric composition was estimated by assuming that the molar extinction coefficients of both isomers at 350 nm are the same. In the case of retinal, the extinction coefficient of *all-trans*-retinal at 350 nm was almost similar to that of 13-*cis*-retinal (1.063-fold that of 13-*cis*-retinal; Hubbard, 1956). [b] 2-*cis*-3-Methyl-7-(2-naphthyl)-2,4,6-octatrienal. The position of the cis bond is the same as that of 13-*cis*-retinal.

Only a small absorbance change was observed upon irradiation of Np-bR_{503} with 450-nm light at room temperature. This change is due to the light adaptation of Np-bR_{442} contamination in the sample because the difference spectrum of this change was the same as that between Np-bR_{442} and Np-bR_{480}. Thus, we concluded that Np-bR_{503} does not show any light-induced change in its absorption spectrum at room temperatures.

The isomeric compositions of the chromophore were analyzed on the irradiated products of Np-bR_{442} and Np-bR_{503} (Table I). As previously reported (Iwasa et al., 1984), the isomeric compositons were almost equal in the chromophores extracted from Np-bR_{442} and Np-bR_{503}; *all-trans*-Np-retinal (nearly 90%). The 13-cis isomer of Np-retinal was in more than 60% of the chromophores extracted from Np-bR_{480}. Therefore, the light irradiation shifts the equilibrium of the chromophoric Np-retinal in Np-bR_{442} toward the 13-cis form. This phenomenon was different from the light adaptation in native bR, where light isomerizes chromophoric retinal from 13-cis to all-trans form. On the other hand, when Np-bR_{503} was irradiated with 450-nm light for 4 h at 0 °C, the isomeric composition of the extracted Np-retinal was slightly changed (4% increase of 13-*cis*-Np-retinal). This increase of 13-*cis*-Np-retinal is probably due to the light adaptation of the contaminating Np-bR_{442}, and the isomeric composition in Np-bR_{503} should not change on irradiation at room temperature.

Formation of Batho Intermediates. In order to elucidate whether or not Np-bRs form batho intermediates, they were irradiated at liquid nitrogen temperatures. It was so difficult to prepare pure Np-bR_{442} that the photoreaction of Np-bR_{442} was analyzed with the mixture of Np-bR_{442} and Np-bR_{503} as the sample. Its absorption spectrum represented a peak at 450 nm and a shoulder at 520 nm at −185 °C, corresponding to Np-bR_{442} and Np-bR_{503}, respectively (curve 1 in Figure 3). Upon irradiation with 420-nm light, the absorbance decreased at wavelengths shorter than 480 nm and increased at longer wavelengths. The final photoequilibrium state (curve 2 in Figure 3) was designated as PSS420 (*p*hoto *s*teady *s*tate formed by irradiation at 420 nm).

It was elucidated by following irradiation that PSS420 contained a mixture of two batho intermediates from Np-bR_{442} and Np-bR_{503}. Upon irradiation of PSS420 with red light (>600 nm), which is absorbed only by the photoproduct(s) having λ_{max} at the longer wavelength, the absorbance decreased at wavelengths longer than 540 nm and increased around 510 nm (curve 3 in Figure 3). This suggests that the batho intermediate produced from Np-bR_{503} reverted to the original pigment, Np-bR_{503}. The mixture in this state was designated as PSS600. Further irradiation of PSS600 with an orange

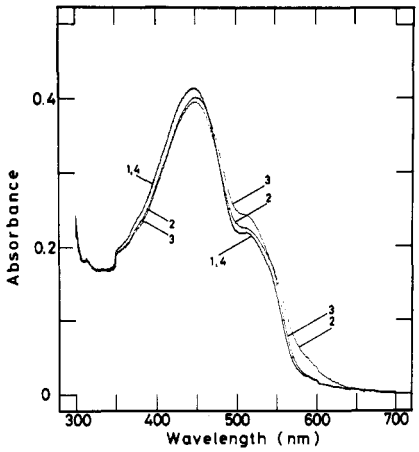

FIGURE 3: Formation of batho intermediates from Np-bR$_{442}$ and Np-bR$_{503}$ and their photoreversion at −185 °C. The mixture of Np-bR$_{442}$ and Np-bR$_{503}$ (curve 1) was irradiated at 420 nm. The photo-steady-state mixture (curve 2), designated as PSS420, was obtained by irradiation for 32 min, indicating the formation of batho intermediates. Further irradiation with light of wavelengths longer than 600 nm for 8 min produced the photo steady state, designated as PSS600 (curve 3). Subsequent irradiation with light of wavelengths longer than 540 nm for 256 min caused reversion of the absorption spectrum (curve 4).

light (>540 nm) caused an increase in absorbance around 430 nm. The absorption spectrum, finally, reverted to the original one (curve 4 in Figure 3). This spectral change should be the reversion of batho intermediate produced from Np-bR$_{442}$ to Np-bR$_{442}$. The difference spectrum between curves 3 and 2 and that between curves 4 and 3 in Figure 3 support the above explanation for the spectral changes, because the shapes of the difference spectra are similar to that between native bR and its batho product except wavelengths of the difference maximum and minimum. This will be discussed later. When the sample containing a large amount of Np-bR$_{503}$ (>90%) was irradiated at −180 °C with 420-nm light, the observed spectral change was similar to that between curves 4 and 3 in Figure 3. This is supporting evidence for the above explanation.

In order to clarify the final product from the batho intermediates of Np-bRs, two different photo steady states were warmed to 0 °C. When PSS420 (curve 2 in Figure 3) and PSS600 (curve 3 in Figure 3) were warmed to 0 °C, the final absorption spectra showed an absorbance increase around 510 nm and a decrease around 430 nm from the starting spectrum in both cases. The shapes of the difference spectra between those of warmed products and the starting one were similar to that between those of Np-bR$_{480}$ and Np-bR$_{442}$. These results suggest that the batho intermediate from Np-bR$_{442}$ converts to Np-bR$_{480}$ by being warmed and that from Np-bR$_{503}$ reverts to Np-bR$_{503}$. These results coincided with the observations from irradiation of both pigments at 0 °C (Figure 2).

Formation of Meta Intermediates. The formation of the meta intermediate (M) from Np-bR was investigated at −65 °C, where meta intermediates from Np-bRs were stable in the dark. The same sample was used as in the experiment shown in Figure 3. The sample (Np-bR$_{442}$ and Np-bR$_{503}$) was irradiated with light of wavelengths longer than 480 nm (Figure 4a). Two-minute irradiation caused the large decrease of the absorbances around 500 nm (curve 2 in Figure 4a). Successive irradiation caused a continuous spectral change with an isosbestic point at 420 nm (curves 3–9 in Figure 4a). The difference spectrum of the early stage (curve 2 − curve 1 in Figure 4a) has a minimum at 520 nm, and that of the later stage (curve 9–curve 2 in Figure 4a) has a minimum at 470

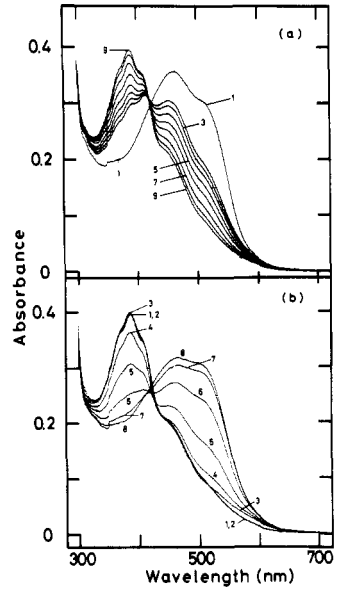

FIGURE 4: (a) Formation of meta intermediates from Np-bR$_{503}$ and Np-bR$_{442}$ at −65 °C. The mixture of Np-bR$_{442}$ and Np-bR$_{503}$ (curve 1) was irradiated with light of wavelengths longer than 480 nm. The absorption spectra of the products were measured after irradiation for 2, 4, 9, 19, 39, 79, 159, and 259 min (curves 2–9, respectively). (b) Thermal reaction of meta intermediates by warming. The mixture of meta intermediates from Np-bR$_{503}$ and Np-bR$_{442}$ [curve 1, same as curve 9 in (a)] was warmed gradually, and absorption spectra were measured at −55, −35, −25, −19, −13, −7, and 0 °C (curves 2–8, respectively).

nm. This indicates that Np-bR$_{503}$ was first changed into its meta intermediate followed by the conversion of Np-bR$_{442}$. The meta intermediates formed from Np-bR$_{442}$ and Np-bR$_{503}$ were not distinguishable in their absorption spectra (λ_{max} 390 nm). The mixture of meta intermediates (curve 9 in Figure 4a) was slowly warmed. Up to −55 °C the spectrum did not show any change. When it was warmed to −35 °C, absorbances increased at wavelengths longer than 500 nm (Figure 4b, curve 3). As the maximum increase was located at ca. 570 nm, this small increase may be due to reversion from the meta intermediate of the native bR contaminating the sample. The spectral change between curves 3 and 4 in Figure 4b showed the difference maximum at 530 nm and minimum at 380 nm, suggesting that this spectral change is due to reversion of the meta intermediate to Np-bR$_{503}$. Upon further warming to −13 °C (curve 6), the wavelength at the difference maximum shifted to shorter wavelength up to 500 nm, and absorbances largely increased at wavelengths between 440 and 500 nm.

When the meta intermediates were warmed, absorbances first increased in the longer wavelength region and then in the shorter wavelength region at higher temperatures. This phenomenon suggests that the meta intermediate from Np-bR$_{503}$ was less stable than that from Np-bR$_{442}$. When it was warmed up to 0 °C, the absorption spectrum of the product (curve 8) was not similar to that of the original pigment at 0 °C, indicating that the meta intermediate produced from Np-bR$_{442}$ changed to another species. The spectrum shows an absorbance increase around 520 nm and a decrease around 430 nm, probably due to the conversion of Np-bR$_{442}$ into Np-bR$_{480}$. The spectrum also shows a small increase in absorbance around 390 nm, probably due to the residual meta intermediate.

DISCUSSION

Previously we reported that Np-retinal and bacterioopsin form two different pigments, probably resulted from the

difference(s) in retinal–protein interaction caused by the larger ring structure of Np-retinal (Iwasa et al., 1984). Recently several authors found the simultaneous formation of two or more analogue pigments with a pure isomer of analogue retinal. Maeda et al. (1984) reported that two kinds of analogue pigments were formed from phenylretinal. They confirmed that both pigments have *all-trans*-phenylretinal as their chromophores (Maeda et al., 1984). Using retinal analogues having the C4 hydrogen atom replaced with methyl (CH_3) or butyl (C_4H_9), Sheves et al. (1984) found the formation of two different pigments based on the flash photolysis experiments. They concluded that the retinal moiety may adopt at least two conformations. These analogue pigments have the retinal analogues modified in the ring portion. These results support our previous explanation that there is a steric interaction between the β-ionone ring of retinal and protein, which results in the two different stable states of Np-bRs.

In the present work, we investigated the effect of these conformational differences in the ring portion on the photochemical properties of the pigments. The results mentioned above clearly showed that the change in the interaction around the ring portion of retinal does not disturb the main events in photochemical reactions. As summarized in Figure 1, both Np-bRs have their own batho and meta intermediates. We compare the shape of the difference spectra between Np-bRs and batho intermediates with that of the native *trans*-bR system (Iwasa et al., 1980). The shape of the difference spectrum between Np-bR_{503} and its batho intermediate was very close to that of *trans*-bR in the wavelength shift from the difference maximum to the minimum (*trans*-bR, 2.48×10^3 cm^{-1}; Np-bR_{503}, 2.37×10^3 cm^{-1}) and in the half band width of the difference absorbance increase (*trans*-bR, 1.63×10^3 cm^{-1}; Np-bR_{503}, 1.63×10^3 cm^{-1}). This indicates that the energy difference from the pigment to the batho intermediate is equal in both systems. Therefore, the photoevent in Np-bR_{503} should be cis–trans isomerization at the C=C double bond of the chromophoric retinal like in the *trans*-bR system. The difference spectrum in the Np-bR_{442} system, however, was different from that of native *trans*-bR and Np-bR_{503}. The half band width is wider in Np-bR_{442} (2.48×10^3 cm^{-1}), and the shift of the wavelength is larger (3.87×10^3 cm^{-1}). This difference would be predictable from the fact that Np-bR_{442} has a different retinal–protein interaction as shown in its CD spectrum (Iwasa et al., 1984).

In the case of Np-bRs, the change in the interaction around the ring portion caused a further red shift of the λ_{max} from 442 to 503 nm. Sheves et al. (1984) found that the increase in the bulkiness of the C4 substituent correlated with the formation of the pigment in the shorter wavelength region. These results indicate the necessity of an appropriate interaction at the ring portion for a larger red shift of λ_{max}.

Light adaptation of Np-bR_{442} is an isomerization from *trans*-Np-retinal to the 13-cis one. This phenomenon is quite different from the bR system; light adaptation of native bR is an isomerization from the cis form to the trans one. On an analogue pigment formed with α-retinal, the light isomerizes from the trans form to the cis one like in the Np-bR system (Towner et al., 1980). It is noteworthy that both pigments have a retinal modified in the ring portion as the chromophore. These observations suggest that the difference in the interaction around the ring portion as shown in Np-bR_{442} and that of α-retinal resulted in the formation of a stable 13-cis pigment on irradiation at room temperature. Since it is generally considered that the 13-cis pigment of bR does not pump protons, the formation of a stable 13-cis pigment on irradiation results in a decrease in the number of pigments which can pump protons and reduces the efficiency of proton pumping.

Here we reported the photochemical properties of these two conformers of Np-bRs. We also studied the physicochemical properties and the relationship of both pigments, which will be published elsewhere.

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