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Changes in structure of the chromophore in the photochemical process of bovine rhodopsin as revealed by FTIR spectroscopy for hydrogen out-of-plane vibrations

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Abstract

The hydrogen out-of-plane bending (HOOP) vibrations were studied in the difference Fourier transform infrared spectra of lumirhodopsin and metarhodopsin I by use of a series of specifically deuterated retinal derivatives of bovine rod outer segments. The 947 cm^{-1} band of lumirhodopsin and the 950 cm^{-1} band of metarhodopsin I were assigned to the mode composed of both 11-HOOP and 12-HOOP vibrations. This result suggests that the perturbation near C_{12}–H of the retinal in the earlier intermediate, bathorhodopsin (Palings, van den Berg, Lugtenburg and Mathies, Biochemistry, 28 (1989) 1498–1507), is extinguished in lumirhodopsin and metarhodopsin I. Unphotolyzed rhodopsin and metarhodopsin I exhibited the 14-HOOP bands in the 12-D derivatives at 901 and 886 cm^{-1}, respectively. Lumirhodopsin, however, did not show the 14-HOOP in the 12-D derivatives. The result suggests a change in geometrical alignment of the C_{14}–H bond in lumirhodopsin with respect to the N–H bond of the Schiff base.

Keywords: Rhodopsin; Hydrogen out-of-plane vibration; FT-IR spectroscopy; Lumirhodopsin; Metarhodopsin I; Deuterated retinals

1. Introduction

Rhodopsin converts the light stimulus into the electric signal at the plasma membrane of the rod outer segments [1–3] through the activation of transducin on its surface. The mechanism to attain the active state could be revealed by the analysis of the structural features of the intermediate states, which have been defined spectroscopically as discrete entities in a time-resolved manner or by stabilizing at low temperatures [4–6].

Fourier transform infrared (FTIR) spectroscopy is quite advantageous for detecting structural changes in the functional residues because it detects signals of particular chemical bonds of not only both the

Abbreviations: FTIR, Fourier transform infrared; Batho, bathorhodopsin; HOOP, hydrogen out-of-plane; Lumi, lumirhodopsin; MetaI, metarhodopsin I; MetaII, metarhodopsin II; Rho, unphotolyzed rhodopsin

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chromophore and protein parts [7] but the water molecules in the interior of the protein [8–10]. Infrared spectroscopy is also preferable for the photosensitive pigments because of its non-invasiveness.

A visual pigment, rhodopsin has 11-*cis* retinal linked to Lys-296 of the protein by a protonated Schiff base. Its positive electrical charge interacts with the negative charge of Glu-113 [11–13]. Upon light absorption, the chromophore isomerizes to the all-*trans* form, while keeping the Schiff base in a similar strong H-bonding state, as judged from the extent in the shift of the C=N stretching vibration band upon replacement in D_2O [14].

Both resonance Raman and FTIR spectra of the early intermediate, bathorhodopsin (Batho), measured at 77 K exhibit intense hydrogen out-of-plane vibration (HOOP) bands [15–20]. Since the HOOP modes of A_u [2] symmetry are inactive with conventional all-*trans* retinal in Raman spectra, the emergence of intense HOOP bands is regarded to reflect twisting around the single bonds in the chromophore. A similar distribution of HOOP bands in the infrared spectrum of Batho [20] suggests that their origin would be also twisting of the retinal. At more elevated temperatures, we established the conditions to record pure FTIR spectra of lumirhodopsin (Lumi) and metarhodopsin I (MetaI) [9]. These spectra clearly exhibit the HOOP bands around 950 cm^{-1}.

In the traditional scheme based on low temperature spectroscopy, Lumi is a precursor of MetaI, which is in equilibrium with metarhodopsin II (MetaII) whose Schiff base is in the unprotonated state. MetaII is known to be responsible for the GDP–GTP exchange reaction in transducin. An experiment on Schiff base-methylated rhodopsin [21] showed that the deprotonation of the Schiff base is a prerequisite for the activation of transducin. An opsin derivative in which either Lys-296 or Glu-113 is replaced by a neutral amino acid residue is constitutively active [22]. This is also compatible with the thesis that the proton transfer from the Schiff base to Glu-113 is necessary for creating active conformation in wild type rhodopsin [23,24]. Elucidation of the mechanism on the Schiff base deprotonation is thus extremely important.

In our previous studies on bacteriorhodopsin [10], we suggested that strong interaction of Asp-85 with an intervening water molecules in the L intermediate causes distortion in the retinal chromophore around the site close to the Schiff base. Such a structure of the L intermediate is supposed to be obligatory for the proton transfer from the Schiff base to Asp-85 in bacteriorhodopsin.

A structural analysis of MetaI in comparison with Lumi is one of the ways to reveal a mechanism for the deprotonation of the Schiff base in the photolyzed rhodopsin. Although the FTIR spectra of both Lumi and MetaI are identical in the O–H stretching vibrations [9], the spectral changes due to the chromophore differ from each other.

Analysis of HOOP bands does not only simply result in the discovery of twists as described above. An unusually low frequency of the 12-HOOP in Batho is a probe of a specific perturbation in a particular environment around the C_{12} position of the retinal chain [18]. In the systematic analysis of the HOOP bands of Lumi and MetaI by use of various isotope derivatives, the appearance of the steric perturbation between the C_{14}–H and N–H bonds was implied in Lumi.

2. Materials and methods

2.1. Preparation of opsin

The method for the preparation of opsin was described previously by Sasaki et al. [20]. Bovine rod outer segments were collected from the red layer in the interface between discontinuous 29 and 36% (w/v) sucrose solutions obtained after the centrifugation at $74\,000 \times g$ for 20 min. The outer segments were then suspended in 0.1 M hydroxylamine in 10 mM HEPES buffer (pH 7.0) and irradiated for 30 min with >540 nm light from a 1 kW halogen-tungsten lamp in a slide projector. After the centrifugation at $74\,000 \times g$ for 20 min, the precipitates

[2] For retinal double bonds with hydrogens substituted *trans* to each other, the A_u HOOP mode is defined as the normal vibration in which the two coupled hydrogens always move in the same direction perpendicular to the plane of the double bond; the B_g HOOP mode is defined as the normal vibration in which the two hydrogens always move in opposite directions perpendicular to the plane.

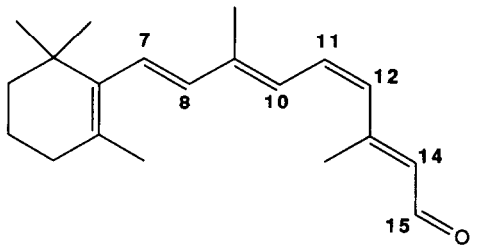

Fig. 1. Notations in 11-*cis* retinal.

were suspended in 0.01 M HEPES buffer. The centrifugation and the suspension were repeated 7 times for the complete removal of the hydroxylamine. The final pellet was lyophilized. The powders thus obtained were washed 5 times with hexane (centrifugation at $2000 \times g$ for 15 min in a swing bucket rotor) in order to remove the retinal oxime. The residual hexane was evaporated under a N_2 gas stream and the powder was suspended in 0.01 M HEPES buffer (pH 7.0) and used as opsin.

2.2. The synthesis of deuterated retinals

10,12-D_2 Retinal (see Fig. 1 for the notations of the numbers of carbon atoms) was synthesized and characterized by the procedure described in Groesbeek and Lugtenburg [25]. 10,11-D_2 Retinal synthesized by the same procedure showed the same characteristics as that previously synthesized by the procedure of Broek and Lugtenburg [26]. 11,14-D_2 and 12,14-D_2 Retinals were synthesized as described by Pardoen et al. [27]. The other deuterated retinals were same as used in the previous paper by Sasaki et al. [20].

2.3. Preparation of rhodopsin with deuterated retinals

The opsin suspension was mixed with a 1.5-fold molar excess of a 1/100 volume of the ethanol solution of each of the retinals. The mixture was incubated for 2 h under gentle shaking. The excess retinals were converted to the oxime by adding hydroxylamine to the final concentration of 0.01 M. After the hydroxylamine was removed by washing with 0.01 M HEPES buffer (pH 7.0), the pellet was lyophilized and then washed with hexane as described above. The powder thus obtained was suspended in water.

2.4. Sample manipulation and measurement of spectra

A 40-μl aliquot of the sample ($A_{500nm} = 2$) in water was dried on a BaF_2 window in a dark chamber with 30% relative humidity. The sample hydrated with 0.5 μl of water was mounted in an Oxford CF1204 cryostat. The temperature was maintained in a range of 0.1 K with an Oxford ITC-4 temperature controller. Liquid nitrogen was used as a coolant.

FTIR spectra were recorded by collecting 512 interferograms in a Nicolet SX60 spectrometer with 2 cm^{-1} resolution. Difference FTIR spectra were calculated as differences between the spectra taken after and before irradiation of rhodopsin (Rho). Infrared spectra were recorded 4–8 times for renewed samples. The Lumi/Rho and MetaI/Rho spectra were obtained by irradiating the sample at 200 K for 2 min and at 240 K for 1 min, respectively as described by Maeda et al. [9]. The light source for the irradiation was a 1-kW halogen-tungsten lamp in a slide projector. The wavelength was selected by passing through a cut-off filter of Toshiba VR58 (> 560 nm).

3. Results

The Lumi/Rho, MetaI/Rho and MetaII/Rho spectra in the 1800–800 cm^{-1} region, which were presented in the previous paper [9] in separate figures, were combined in Fig. 2 to compare these spectra on the basis of the same molar amount of the photoproducts. They were normalized by adjusting the amplitude of the negative HOOP band at 969 cm^{-1} of Rho [28], which is unaffected by different photoreactions, to the same height. The 947 cm^{-1} band of Lumi (Fig. 2a) and the 950 cm^{-1} band of MetaI (Fig. 2b) are attributable to the HOOP bands. In contrast, no bands due to the HOOP modes were observed for MetaII (Fig. 2c). This may be a result of low intensities generally observed for the unprotonated retinal species [29] and does not necessarily mean the absence of twists in the chromophore.

The HOOP modes of the ethylenic protons across the 11,12 double bond of all-*trans* retinal derivatives

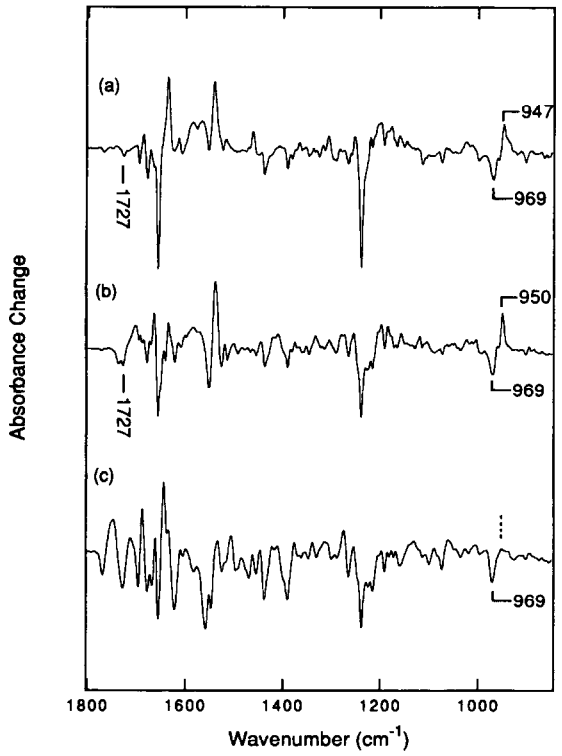

Fig. 2. Difference FTIR spectra of (a) Lumi/Rho, (b) MetaI/Rho and (c) MetaII/Rho in the 1800–850 cm−1 region.

form A_u and B_g^2 local symmetry combinations around 960 cm^{-1} and 830 cm^{-1}, respectively, while the HOOP modes adjacent to the methyl group across the double bond, 10-HOOP and 14-HOOP are located as isolated ones around 880 cm^{-1} [30,31]. The deuteration of these protons causes shifts of the HOOP modes to the region between 800 and 600 cm^{-1}. Our system for FTIR spectroscopy is unable to detect these bands below 800 cm^{-1}. The features between 850 and 800 cm^{-1} are difficult to identify as the bands exceeding the noise, as judged from the base line recorded as the difference between two spectra in the dark. Below we will analyze the constituents of the HOOP bands in the 1000–850 cm^{-1} region by use of specifically deuterated retinals.

Fig. 3I and 3II are the Lumi/Rho and MetaI/Rho spectra, respectively, for unlabeled (a), 7-D (b), 8-D (c), 10-D (d), 14-D (e) and 15-D (f) samples. For each intermediate, the amplitudes of the spectra with different deuterations were scaled by adjusting the protein band of the unphotolyzed rhodopsin at 1727 cm^{-1} [19] (see also Fig. 2) to the same height. A distinct feature of the 947 cm^{-1} band in the unlabeled sample of Lumi (Fig. 3Ia) is a 5 cm^{-1} up-shift in the 7-D derivative (Fig. 3Ib). However, a shift in the 8-D derivative was not detected (Fig. 3Ic) as opposed to the expectation for the presence of a combined mode of 7- and 8-HOOP modes around 966 cm^{-1} [30]. Shifts in the 10-D and 14-D derivatives of Lumi (Fig. 3Id) are small. The 950 cm^{-1} band of the unlabeled sample of MetaI (Fig. 3IIa) did not shift in the 7-D (Fig. 3IIb), 8-D (Fig. 3IIc) and 15-D (Fig. 3IIf) derivatives and exhibited a 5 cm^{-1}

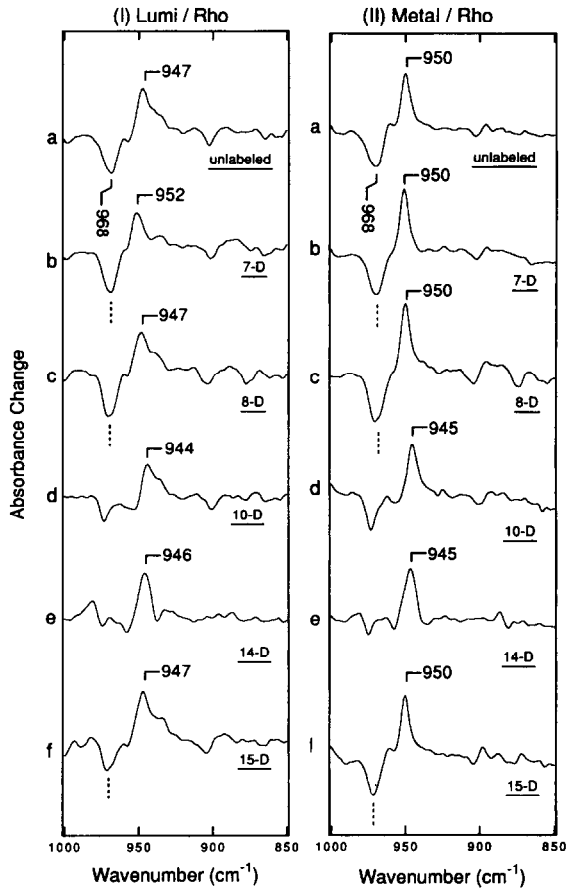

Fig. 3. Difference FTIR spectra of Lumi/Rho (I) and MetaI/Rho (II) in the 1000–850 cm−1 region. (a) unlabeled, (b) 7-D, (c) 8-D, (d) 10-D, (e) 14-D, and (f) 15-D samples. The full height of the vertical axis is 0.026 absorbance unit for (a).

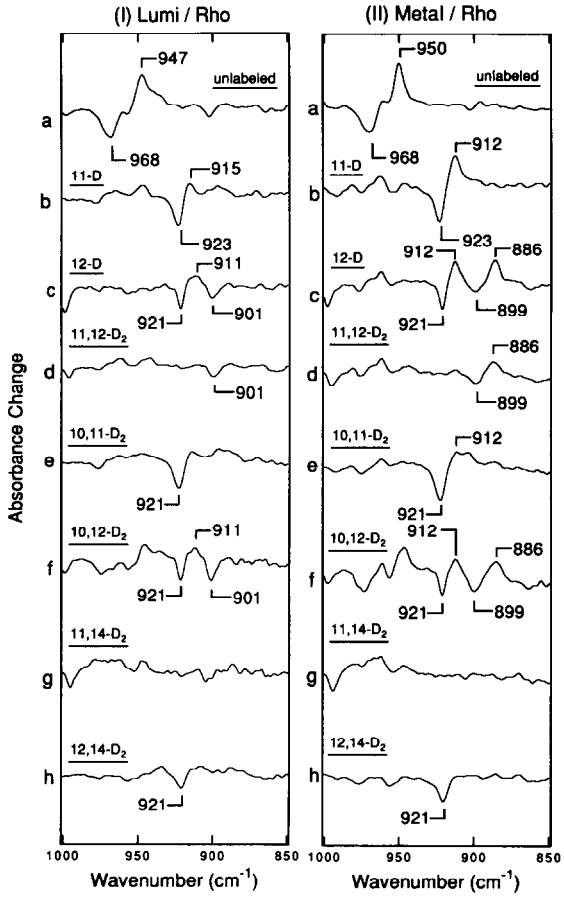

Fig. 4. Difference FTIR spectra of Lumi/Rho (I) and MetaI/Rho (II) in the 1000–850 cm − 1 region. (a) unlabeled, (b) 11-D, (c) 12-D, (d) 11,12-D2, (e) 10,11-D2, (f) 10,12-D2, (g) 11,14-D2, and (h) 12,14-D2 samples. The full height of the vertical axis is 0.031 absorbance unit for (a).

shift in the 10-D (Fig. 3IId) and 14-D (Fig. 3IIe) derivatives.

Fig. 4I and Fig. 4II show the Lumi/Rho and MetaI/Rho spectra of the unlabeled (a), 11-D (b), 12-D (c), 11,12-D_2 (d), 10,11-D_2 (e), 10,12-D_2 (f), 11,14-D_2 (g) and 12,14-D_2 (h) samples. The 947 cm^{-1} band of Lumi (Fig. 4Ia) considerably shifted to 915 cm^{-1} in the 11-D derivative (Fig. 4Ib) and 911 cm^{-1} in the 12-D derivative (Fig. 4Ic) with decrease in intensity. These bands then shifted to below 850 cm^{-1} in the 11,12-D_2 derivative (Fig. 4Id). Thus, the 11-HOOP mode of Lumi forms the coupled A_u mode with the 12-HOOP mode at 947 cm^{-1} in Lumi, in contrast to either bathorhodopsin or hypsorhodopsin in which the 11-HOOP is uncoupled from the 12-HOOP mode [18,20].

Likewise, the 950 cm^{-1} band of MetaI (Fig. 4IIa) gave rise to the 912 cm^{-1} band in the 11-D (Fig. 4IIb) and 12-D (Fig. 4IIc) derivatives. These bands shifted to below 850 cm^{-1} in the 11,12-D_2 derivative (Fig. 4IId). Thus, as the 947 cm^{-1} band of Lumi, MetaI also forms the coupled mode of the 11- and 12-HOOP modes at 950 cm^{-1}.

In distinction from the result upon deuteration at C_{11}, deuterium-substitution at C_{12} induced the appearance of a 886 cm^{-1} band in MetaI (Fig. 4IIc). This band was unobserved in the corresponding spectrum of Lumi (Fig. 4Ic). The additional deuteration of either C_{10} or C_{14} was performed to the mono-deuterated derivatives of C_{11} and C_{12} because similar bands in the 11-D and 12-D derivatives of all-*trans* retinal entail the 10-HOOP and 14-HOOP modes [30]. The 886 cm^{-1} band of MetaI persisted in the 11,12-D_2 (Fig. 4IId) and 10,12-D_2 (Fig. 4IIf) derivatives, while disappeared in the 12,14-D_2 (Fig. 4IIh) derivative. Thus, the 886 cm^{-1} band newly appeared in the 12-D derivative of MetaI (Fig. 4IIc) does not contain the 10-HOOP mode and is composed of only the 14-HOOP mode. The HOOP bands of Lumi at 947 cm^{-1} (Fig. 4Ia) and MetaI at 950 cm^{-1} (Fig. 4IIa) resemble each other in the unlabeled sample. Nevertheless, in the 12-D derivatives MetaI is distinct from Lumi in showing the 886 cm^{-1} band.

The other bands at 912 cm^{-1} in the 11-D (Fig. 4IIb) and 12-D (Fig. 4IIc) derivatives of MetaI, while persisting in the 10,11-D_2 (Fig. 4IIe) and 10,12-D_2 (Fig. 4IIf) derivatives, disappeared completely from the 1000–850 cm^{-1} region in the 11,14-D_2 (Fig. 4IIg) and 12,14-D_2 (Fig. 4IIh) derivatives, respectively, indicating that the 14-HOOP mode also mixes individually with the 11-HOOP and 12-HOOP modes at 912 cm^{-1}. For Lumi, the 915 cm^{-1} band in the 11-D derivative (Fig. 4Ib) and the 911 cm^{-1} band in the 12-D derivative (Fig. 4Ic) exhibited the same responses to the isotope substitutions as MetaI but with less intensities than MetaI.

Next, we discuss the bands of Rho appearing as the negative peaks in the difference spectra. Since these are common to the series in Fig. 4I and Fig. 4II, we will refer only to that of Fig. 4I. The

negative 968 cm^{-1} band of unlabeled Rho (Fig. 4Ia) shifted to 923 cm^{-1} in the 11-D derivative (Fig. 4Ib) and split into 921 and 901 cm^{-1} bands in the 12-D derivative (Fig. 4Ic). The 923 cm^{-1} band in the 11-D derivative (Fig. 4Ib) and the 921 cm^{-1} band in the 12-D derivative (Fig. 4Ic) shifted below 850 cm^{-1} in the 11,12-D_2 derivative (Fig. 4Id). Thus, both the 11- and 12-HOOP modes of Rho forms a coupled mode with each other at a higher frequency than Lumi and MetaI. The additional 901 cm^{-1} band of Rho in the 12-D derivative (Fig. 4Ic), while persisting in the 11,12-D_2 (Fig. 4Id) and 10,12-D_2 (Fig. 4If) derivatives, shifted below 850 cm^{-1} in the 12,14-D_2 derivative (Fig. 4Ih). Thus, this band is assigned to the 14-HOOP mode with a behavior similar to the 886 cm^{-1} band of the 12-D derivative of MetaI (Fig. 4IIc).

The 923 cm^{-1} band of Rho in the 11-D derivative (Fig. 4Ib), which persisted in the 10,11-D_2 derivative (Fig. 4Ie), disappeared completely in the 11,12-D_2 (Fig. 4Id) and 11,14-D_2 (Fig. 4Ig) derivatives. The other band of Rho at 921 cm^{-1} in the 12-D derivative (Fig. 4Ic) persisted in the 10,12-D_2 derivative (Fig. 4If) and disappeared in the 11,12-D_2 derivative (Fig. 4Id). These responses to the isotope substitutions are same as those of Lumi and MetaI. However, the 921 cm^{-1} band of the 12-D derivative (Fig. 4Ic) persisted even with additional deuteration at C_{14} (Fig. 4Ih) in distinction from Lumi and MetaI, the all-*trans* intermediates. Thus, the 14-HOOP mode of Rho does mix with the isolated 12-HOOP mode (Fig. 4Ib) but not with the 11-HOOP mode (Fig. 4Ic).

4. Discussion

The resonance Raman spectrum of Batho exhibits many intense HOOP bands as a reflection of the twisting in the retinal chromophore [15,16,18]. Although the A_u HOOP of retinal are allowed modes in IR, a considerable part of the intensities of the HOOP bands in the FTIR spectrum also may be derived from twisting in the chromophore; the HOOP bands of Batho in the FTIR spectrum exhibits a similar intensity distribution to that of the resonance Raman spectrum [18,20]. For exploring the structural differences of the retinal chromophore among Batho, Lumi and MetaI, we analyzed the HOOP bands in the FTIR spectra of Lumi and MetaI on the basis of the frequency shifts upon various deuterium substitutions along the polyene chain of the chromophore, and compared the HOOP bands also with those of Batho in the previous studies [20].

Both Lumi and MetaI show relatively intense HOOP bands at 947 and 950 cm^{-1}, respectively, in the difference FTIR spectra. These exhibited typical features of $HC_{11}{=}C_{12}H$ A_u HOOP as observed for the 959 cm^{-1} band of all-*trans* retinal [30] and all-*trans* bacteriorhodopsin [32]. These are distinct from earlier intermediate, Batho, whose 11-HOOP is decoupled from the 12-HOOP. A nearly isolated band at 921 cm^{-1} of Batho is a result of an unusually low force constant of the 12-HOOP and a low extent of the coupling with the 11-HOOP. These characteristics in Batho are supposed to be the result of the presence of a local perturbation near C_{12}–H [18]. An extensive coupling between the 11- and 12-HOOP modes in Lumi and MetaI indicates the disappearance of the perturbation in these late intermediates. However, persistent intense features of these bands may reflect the twisted structure in the C_{11} and C_{12} region in Lumi and metaI. The $HC_{11}{=}C_{12}H$ HOOP mode of the 11-*cis* chromophore is only infrared active due to twists of the chromophore caused by the steric interaction of the 13-methyl group with 10-H. However, the chromophore in 9-*cis* rhodopsin, which exhibits the same mode [20], may have a similar conformation to that of 11-*cis* rhodopsin. It could be enforced by the binding site in the protein.

An important difference between Lumi and MetaI is observed in the HOOP bands in the 12-D derivatives (Fig. 4Ic and 4IIc). The 886 cm^{-1} band which was assigned to the 14-HOOP mode appeared for MetaI but not for Lumi. It was reported for all-*trans* retinal [30,31,33] that the 14-HOOP band at 862 cm^{-1} in the IR spectrum is much more intense in the 12-D derivative than others. This has been interpreted as the disappearance of a destructive coupling of the 14-HOOP vibration with the 12-HOOP vibration which constructs the $HC_{11}{=}C_{12}H$ B_g mode. Similar intensification of the 14-HOOP band in the 12-D derivatives is valid for Rho in the present study and isorhodopsin appearing in the previous study on hypsorhodopsin [20]. Thus, an intense 14-HOOP band in the 12-D derivatives is noticeable not only

for MetaI but also for all-*trans* retinal, and 11-*cis* and 9-*cis* retinal pigments. An intense 14-HOOP band was observed at 850 cm^{-1} in Batho [18], which is in a similar state as the 12-D derivatives of other intermediates. In these respects *Lumi is unusual.*

Since the 11-HOOP vibration in the 12-D derivative (Fig. 4Ic and 4IIc) and 12-HOOP in the 11-D derivative (Fig. 4Ib and 4IIb) are located at similar frequencies for both Lumi and MetaI, the coupling of the 14-HOOP with the $HC_{11}{=}C_{12}H$ B_g mode might be common in these intermediates. The only reason for the absence of 14-HOOP in the 12-D derivatives of Lumi might be the result of a decrease in intrinsic intensity of the 14-HOOP in Lumi. Specific increase in intensity of 14-HOOP reflects the increase in magnitude of the dipole moment in the C_{14}–H bond. This could be brought about by the neighboring charge of the protonated Schiff base. Even aldehyde of the all-*trans* retinal has a polar character [30]. Although not identical in mechanism, the resonance Raman spectrum of all-*trans* bacteriorhodopsin exhibits a greater intensity for 14-HOOP [32]. The absence of such an inductive effect on 14-HOOP vibration in Lumi may indicate *a change in a geometrical alignment* of the C_{14}–H bond with respect to the N–H bond.

The 14-HOOP mode was detected in the two bands at 912 and 886 cm^{-1} in the 12-D derivative of MetaI (Fig. 4IIc vs. 4IIh). The corresponding 911 cm^{-1} band in the 12-D derivative of Lumi contains the 14-HOOP mode (Fig. 4Ic vs. 4Ig). On the other hand, this mode could not be detected in the 921 cm^{-1} band of the 11-HOOP mode in the 12-D derivative of Rho. The mixing of the 14-HOOP mode with the 11-HOOP mode commonly observed for the all-*trans* intermediates is thus devoid in Rho. Since the mixing of the 14-HOOP mode with the 12-HOOP mode at 923 cm^{-1} in the 11-D derivative was achieved even for Rho (Fig. 4Ib vs. 4Ih), the absence of the mixing between 11-HOOP and 14-HOOP would be due to the presence of a *cis* bond between C_{11} and C_{12} in Rho. This kind perturbation did not appear in Lumi and MetaI.

In the previous study by Ganter et al. [34], it was found that 13-desmethyl rhodopsin is capable of activating transducin. Apparently no corresponding HOOP bands were observed for Lumi and MetaI of this analog. Hence it was concluded that the HOOP bands are indifferent to the function of the protein. However, this is superficial. The removal of the 13-methyl group from all-*trans* retinal results in the formation of a coupled band at 984 cm^{-1} from both $HC_{11}{=}C_{12}H$ A_u and $HC_{13}{=}C_{14}H$ A_u HOOPs [30]. In the difference spectrum of Batho of the analog, a negative band due to its unphotolyzed state was seen at 990 cm^{-1} [34]. The absence of this band in the negative side in the corresponding difference spectrum of Lumi indicates that the same HOOP band also occurs for Lumi which simply canceled out in the difference spectrum. Thus, these facts indicate that the twisting in the chromophore is present at least in Lumi.

It is known that Lumi is very weak in H-bonding interaction of the Schiff base compared to MetaI and Batho, as judged from the extent of the deuteration shift of the C=N stretching vibration [19]. A possible interaction change between the 9-methyl group with the surrounding in the Lumi–MetaI conversion was proposed [4]. These changes in interaction with the protein moiety in MetaI may be brought about by the compensation of the local twist of the chromophore in Lumi.

A local twist close to the protonated Schiff base has been proposed for the L intermediate of bacteriorhodopsin [10,35,36]. This may be brought about by strong H-bonding of the protonated Schiff base with the negatively charged Asp-85 with an intervention of a few molecules of strong H-bonding water [10]. Lumi is similar to the L intermediate of bacteriorhodopsin with respect to the presence of the local twisting but distinct from it in weak H-bonding of the Schiff base. However, the water molecules with strong H-bonding are detected for both Lumi and MetaI [9].

Recently, it has been proposed that not only MetaI but also Lumi is at the stage before the deprotonation of the Schiff base [6]. This could be related to an assumed distortion in Lumi. Differences between Lumi and MetaI in relation to the function must be explored in further studies.

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