Orientation of retinal in purple membrane determined by polarized Raman spectroscopy

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ABSTRACT The orientation of the retinal molecule in the purple membrane was determined by polarized Raman spectroscopy for stacked purple membranes. The depolarization ratios of C=C stretching vibration mode were measured for three scattering geometries of purple membrane films. From the depolarization ratios we estimated the tilt angle of the transition dipole moment of retinal to the membrane normal and the rotational angle of the molecular plane along the transition dipole moment of retinal. The molecular plane of M intermediate was found to be almost perpendicular to the membrane plane. We confirmed that the tilt angle was 65 ± 2° for both bR and M intermediates.

INTRODUCTION

Bacteriorhodopsin (bR) in the purple membrane of *Halobacterium halobium* works as a light-driven proton pump. The determination of the site and orientation of retinal in the photochemical intermediates is important to clarify the mechanism of proton transportation through the membrane. The tilt angle of the long axis of retinal, which is considered to be parallel to the transition dipole moment, to the membrane normal, θ_0, was determined by linear dichroism (Heyn et al., 1977) and by electric dichroism measurement (for example, Kimura et al., 1981). Recently, Earnest et al. (1986) studied the orientation of retinal by a polarized FT-IR method. They reported that θ_0 was ~65–70° for bR_{570}, M_{412}, and K_{630} intermediates, and that the molecular plane of retinal was almost perpendicular to the membrane. In this paper we determined θ_0 and the rotational angle of the molecular plane with polarized Raman scattering of a well-oriented purple membrane stack. We estimated the above two parameters from the depolarization ratios of the C=C stretching vibration mode for three different scattering geometries. We concluded that the molecular plane of retinal in M intermediate is almost perpendicular to the membrane. This result complements the FT-IR study. Furthermore, the values θ_0 for bR and M well agreed with that obtained by the FT-IR and dichroism studies.

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EXPERIMENTAL

Purple membrane was purified from *Halobacterium halobium* strain Et1001 according to the well-established procedure (Oesterhelt et al., 1974). Purple membrane was stacked on a nesa glass by an electrophoresis method and was dried slowly (Varo and Keszthelyi, 1983). The thickness of the film was typically ~2–3 μm, calculated by the total content of PM fragments in a drop and the radius of the obtained purple membrane film (PM film). The PM film thus obtained was well oriented. The mosaic spread in dried film was estimated to be less than 5° by a small angle x-ray diffraction method. During the Raman measurements, the PM films were soaked in a buffer solution (10 mM Hepes, pH 7.0). Although the orientational deviation of the PM film must be worse than that of dried film in this condition, we used 5° in the calculation. The PM films were not dissolved into the buffer solution at least for 5 h and were not damaged by laser beam during Raman measurement for ~30–40 min from a Raman spectroscopic point of view, as mentioned below.

Raman spectra were obtained on a Jobin-Yvon double monochromator with a photon counting detection and a digital accumulating system. A quartz polarizer for incident beam, a polaroid film analyzer, and a quartz scrambler for scattered light were used. The depolarization ratio of a Raman band is defined as the intensity ratio I_{VH}/I_{VV}. I_{ij} is the Raman intensity, where i and j refer to the polarization direction of incident and scattered light relative to the scattering plane. The 488.0-nm line of an Ar ion laser was used as an excitation source to balance the Raman intensities between bR and M intermediates. Because high laser power degenerates the sample, the laser power was kept lower than 10 mW at sample position. Under this condition, the sample remained in a quasistable state, that is, the decrease in Raman intensity for successive laser irradiation was sufficiently small. To minimize this breaching effect, we obtained the depolarization ratio by averaging the intensities of the Raman peaks in VV and VH spectra for successive VH, VV, and VH measurements.

Among the Raman bands specific to retinal, the C=C stretching vibration band near 1,500 cm^{-1} was used in this depolarization measurement for the following reasons. The frequency of this band is known to be different for each photoreaction intermediate, that is, 1,530 cm^{-1} for bR_{570} and 1,570 cm^{-1} for M_{412} (Smith et al., 1985). These bands are well

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separated, so that the peak intensity ratio for each line, I_{VH}/I_{VV}, can be used as the depolarization ratios for each intermediate. This band is strong enough to estimate the depolarization ratios definitely with weak laser power.

The procedure to obtain the orientation of retinal from the depolarization ratio of C=C stretching band is outlined below. As shown in Fig. 1, axes fixed to a PM film are designated XYZ, and axes of Raman tensor of this band fixed to a retinal molecule are designated $\xi\,\eta\,\zeta$. Axes $\xi\,\eta\,\zeta$ are connected to axes XYZ with Euler's angles θ, ϕ, and ψ. θ is a tilt angle of ζ axis to Z axis. ϕ is an angle between XZ plane and $Z\zeta$ plane. ψ is an angle between $Z\zeta$ plane and $\xi\,\zeta$ plane, that is, an internal rotational angle of ξ axis around ζ axis. ζ axis is taken to be parallel to the transition dipole, that is, to the polyene chain direction. η axis is taken in the molecular plane. The Raman tensor fixed to a retinal molecule can be described in the following way. Because this C=C stretching band exhibits a strong resonant Raman effect for visible light, $(\zeta\zeta)$ component is expected to be dominant (Behringer, 1967). $(\xi\xi)$ component (out of molecular plane) and off diagonal elements can be neglected and $(\eta\eta)$ component is supposed to be smaller than $(\zeta\zeta)$ component. Thus we can write the Raman tensor of this band fixed to retinal as follows.

$$\sigma_R = \begin{pmatrix} 0 & 0 & 0 \\ 0 & b & 0 \\ 0 & 0 & 1 \end{pmatrix}, \qquad (1)$$

where $b < 1$. The value b can be derived by the depolarization ratio of isotropic PM suspension applying the following equation,

$$\rho_{iso} = (b^2 - b + 1)/(3b^2 + 2b + 3), \qquad (2)$$

which can be derived from the ordinary equation of isotropic suspension.

From this Raman tensor fixed to retinal we can calculate the Raman tensor components σ_{ij} (i, j = X, Y, Z) fixed to a PM film, using Euler's angles θ, ϕ, and ψ from σ_R. The representations of σ_{ij} are listed in Table 1. When incident light and scattered light are polarized in X and Y direction, respectively, the Raman intensity is proportional to $(\sigma_{XY})^2$. Depolarization ratios were obtained for three scattering geometries, cases A, B, and C shown in Fig. 2. The depolarization ratio for each scattering geometry is simply represented as follows,

$$\rho_A = I_{XY}/I_{XX} = (\sigma_{XY})^2/(\sigma_{XX})^2, \text{ etc.}, \qquad (3)$$

assuming that the incident laser power is kept constant and that the efficiency of the detection system does not depend on the polarization direction. In this equation we neglect the absorption effect of incident and scattered lights, which will be discussed in the next section. From the measured depolarization ratios ρ for PM film in these scattering geometries and for PM suspension (isotropic), we can obtain the values

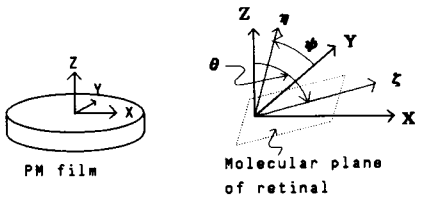

FIGURE 1 Relation between axes XYZ fixed to PM film and principal axes of Raman tensor fixed to retinal for 1,570 cm^{-1} C=C stretching mode.

TABLE 1 Raman tensor components fixed to a PM film represented by Raman tensor components fixed to retinal ($\sigma_{R\xi\xi}$ = 1 and $\sigma_{R\eta\eta}$ = b) and Euler's angles

σ_{XX}	$b(\cos\theta\cos\phi\cos\psi - \sin\phi\sin\psi)^2 + \sin^2\theta\cos^2\phi$
σ_{ZZ}	$b\sin^2\theta\sin^2\psi + \cos^2\theta$
σ_{XY}	$b(\cos\theta\cos\phi\cos\psi - \sin\phi\sin\psi)(\cos\theta\sin\phi\cos\psi + \cos\phi\sin\psi) + \sin^2\theta\cos\phi\sin\phi$
σ_{XZ}	$b(-\cos\theta\cos\phi\cos\psi + \sin\phi\sin\psi)\cdot\sin\theta\cos\psi + \sin\theta\cos\theta\cos\phi$

of ϕ_0, ψ_0, and b. Practically, we test the fitting of the measured value of ρ to the calculated one for given θ_0, ψ_0, and b values as mentioned in the following section.

Generally, it is difficult to perform a quantitative analysis on polarized Raman scattering of opaque samples. The most serious problem is the effect of multiple scattering. It causes the uncertainty in polarization direction of incident and scattered lights, and as a result the depolarization ratio approaches 1.0. We measured several depolarization ratios of natural emission lines of Ar ion laser to estimate the relative intensity of multiple scattering. For completely opaque samples of white powder of microcrystalline or white gel, the intensities of each Raman line and natural emission line were the same for VV and VH components, that is, the depolarization ratios ρ were 1.0. On the contrary, the depolarization ratios ρ of natural emission lines for PM film were $<10^{-2}$, which means that the relative intensity of multiple scattering is very weak. This must be partly because of the large absorption coefficient of PM film. So we adopt the ρ value farthest from 1.0 as the most probable ρ value among the values obtained from a series of experiments.

RESULTS AND DISCUSSION

In Fig. 3, polarized Raman spectra of PM film thus selected for three scattering geometries are shown. The ρ values for PM film and PM suspension are listed in Table 2 (each left-side column for bR and M). The values of Raman tensor component b (= $\sigma_{R\eta\eta}$) estimated from ρ_{iso} for bR and M are also shown.

The observed ρ values were corrected by the following points. (a) The transmittance of polarized Raman scattering light at membrane surface depends on the polarization direction. In cases B and C in Fig. 2, the observed values were corrected by a factor of ~1.2. (Appendix 1). (b) The absorption coefficient of incident and scattered lights depend on the polarization direction. The correc-

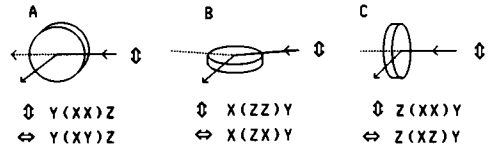

FIGURE 2 Three scattering geometries studied are drawn by the laboratory frame. Polarization directions of incident and scattered light are shown. (↕) Vertical. (⟷) Horizontal. (A) $\rho_A = I_{XY}/I_{XX} = \sigma^2_{XY}/\sigma^2_{XX}$. ($B$) $\rho_B = I_{ZX}/I_{ZZ} = \sigma^2_{ZX}/\sigma^2_{ZZ}$. ($C$) $\rho_C = I_{XZ}/I_{XX} = \sigma^2_{XZ}/\sigma^2_{XX}$.

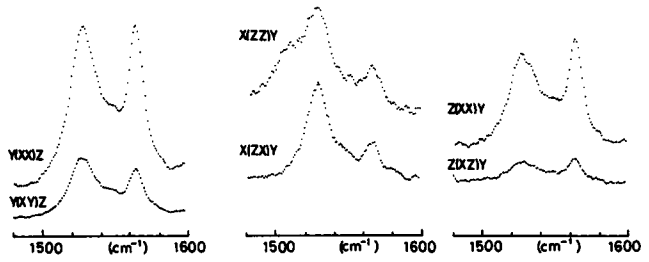

FIGURE 3 Polarized Raman spectra for three scattering geometries in Fig. 2 excited by 488-nm line.

tion factor is ~1.1 for the cases *B* and *C* in Fig. 2. (Appendix 2).

In Table 2 the corrected depolarization ratios are listed for bR and M intermediates (each middle column).

Furthermore, we took into account the following effects on the calculation of the depolarization ratios for given θ_0 and ψ_0 values. (*a*) Mean orientational deviation of PM in the membrane stack was estimated to be ~5° by small angle x-ray diffraction study. The distribution around θ_0 value was represented by the Gaussian distribution function $g(\theta)$ shown in Eq. 4, and the Raman scattering activities α_{ij}^2 was averaged by this distribution function.

$$g(\theta) = 1/(\pi\,\delta) \cdot \exp\left(-(\theta - \theta_0)^2/\delta^2\right), \qquad (4)$$

where $\delta = 5°$. (*b*) Polarized laser light induces the uneven distribution of bR or M intermediate concerning with ϕ value in the membrane plane, because the fraction of M intermediate parallel to the polarization direction of incident light is larger than the one perpendicular to that direction. This inhomogeneity factor strongly appears in case *A*. We assume that this effect is represented by the distribution function $f(\phi) = 1 - k \cdot \cos^2\phi$ for bR and $f(\phi) = 1 + k \cdot \cos^2\phi$ for M, where k is a fitting parameter in case *A*. In cases *B* and *C*, we can put $f(\phi) = 1$ for both bR and M.

TABLE 2 Depolarization ratios of C=C stretching Raman line for bR and M

	bR (1,530 cm^{-1})			M (1,570 cm^{-1})		
	Meas*	Corr‡	Calc§	Meas*	Corr‡	Calc§
ρ_A	0.36	0.36	0.36	0.30	0.30	0.30
ρ_B	1.1	1.4	1.6	0.8	1.0	1.0
ρ_C	0.36	0.28	0.30	0.31	0.23	0.22
ρ_{iso}	0.33	—	—	0.29	—	—
b	—	0.01	—	—	0.2	—

*Measured values at 488 nm excitation. ‡Corrected experimental values according to Appendices. §Calculated depolarization ratio assuming $\theta_0 = 64°$ for bR, $\theta_0 = 66°$ for M, $\psi_0 > 60°$ for M, and $k = 0.2$ for ρ_A.

Finally, the equation of Raman scattering activities are modified as follows.

$$I_{ij}(\theta_0, \psi_0) \propto \int_0^{2\pi}\int_0^{\pi} \sigma_{ij}^2 \cdot g(\theta) \cdot f(\phi) \cdot d\theta \cdot d\phi. \qquad (5)$$

The procedure to determine the fitting parameters such as θ_0 is as follows. The parameter b was first determined by Eq. 2 for bR and M. θ_0 was roughly estimated by ρ_C and then ψ_0 was chosen to satisfy the value ρ_B. Finally, k was estimated by ρ_A. When we chose 64° and 66° as θ_0 values for bR and M respectively, and 60–90° as ψ_0 value for M, the calculated depolarization ratios were well fitted to the experimental values as shown in Table 2. The θ_0 value that satisfies the experimental values of both ρ_B and ρ_C can be almost uniquely chosen within the error ±2°. ψ_0 value for bR could not be determined because the value of b was too small to exhibit a planar character of the Raman tensor. The value k was estimated as 0.2, which seems to be a reasonable value for the distribution of bR and M.

The values θ_0 thus obtained for bR and M intermediates correspond to the tilt angle of the transition dipole moment, and well agree with the values (~65–70°) obtained by FT-IR or other absorption studies. Furthermore, the result that ψ_0 is >60° for M intermediate indicates that the molecular plane of retinal is almost perpendicular to the membrane plane. Almost the same result was reported by FT-IR method for bR, K, and M intermediates (Earnest et al., 1986).

As shown in Fig. 3*b*, there is an extra response near 1,510 cm^{-1} only in (*ZZ*) component, which indicates that ζ axis defined as the most dominant axis of the Raman tensor fixed to retinal molecule is almost parallel to the membrane normal of the PM film (*Z*), that is, $\theta_0 \sim 0°$. This Raman band is usually assigned to belong to O_{640} intermediate (Smith et al., 1983). Considering that θ_0 value is so different from bR or M intermediates, however, there may be another possible explanation that this band belongs to another photoproduct induced by rather intense laser light.

In conclusion, the molecular plane of retinal in M intermediate is almost perpendicular to the membrane plane, and the tilt angle of the long axis of retinal in PM film is concluded to be 65 ± 2°.

APPENDIX

Correction factor for transmittance coefficient at membrane surface

The transmittances for parallel and perpendicular polarization to the membrane plane are

$$T\| = (\sin 2\theta_i \cdot \sin 2\theta_t)/[\sin^2(\theta_i + \theta_t)] \cdot [\cos^2(\theta_i - \theta_t)]$$

$$T\perp = \sin 2\theta_i \cdot \sin 2\theta_t / \sin^2(\theta_i + \theta_t)$$

$$n_{12} = \sin \theta_i / \sin \theta_t,$$

where θ_i and θ_t are the light propagation angles to the membrane plane for incident and transmittant light. In cases *B* and *C*, n_{12} (refractive index) and θ_i are estimated as 1.1 and 89°, respectively. The correction factor is then derived as 1.2.

Correction factor for absorption by bR

For example, we explain this correction for case *B*. For simplicity, we assume that the light propagation direction is parallel to *Y*-axis and the tilt angle is 70°. The average transition dipole moments for *X* and *Z* direction are

$$P_X = \left|1/\pi \int_0^{\pi} \sin\psi \cdot P \cdot \sin 70° \cdot d\psi\right| = 0.6P$$

$$P_Z = \left|1/\pi \int_0^{\pi} P \cdot \cos 70° \cdot d\psi\right| = 0.17P,$$

where *P* is a molecular dipole moment. When we denote the molar absorption coefficients for *X* and *Z* polarization direction as ϵ_X and ϵ_Z, ϵ_X and ϵ_Z are proportional to P_X and P_Z, respectively. The intensity of scattered light which propagates through the pass length *l* before and after scattering can be written as follows.

$$I_{ZX}(l) \propto \exp(-\epsilon_Z \cdot c \cdot l) \cdot \exp(-\epsilon_X \cdot c \cdot l)$$

$$I_{ZZ}(l) \propto \exp(-\epsilon_Z \cdot c \cdot l) \cdot \exp(-\epsilon_Z \cdot c \cdot l),$$

where *c* is the molar density of the PM film. When the pass length which contributes to the Raman intensity is restricted to l_0, the depolarization ratio can be described as

$$\rho_{ZX/ZZ} = (\alpha^2_{ZX}/\alpha^2_{ZZ}) \cdot f(l_0),$$

where

$$f(l_0) = \int_0^{l_0} I_{ZX}(l) \cdot dl \Big/ \int_0^{l_0} I_{ZZ}(l) \cdot dl.$$

If l_0 can be assumed as $2 \cdot \epsilon_Z \cdot c \cdot l_0 = 1$, the intensity ratio $f(l_0)$ is

$$f(l_0) = \frac{2\epsilon_Z}{\epsilon_X + \epsilon_Z} \cdot \frac{\exp(-(\epsilon_X + \epsilon_Z) \cdot c \cdot l_0) - 1}{\exp(-2 \cdot \epsilon_Z \cdot c \cdot l_0) - 1}.$$

The value $f(l_0)$ can be estimated as 0.9.

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