

Optical Rotation of the Second Harmonic Radiation from Retinal in Bacteriorhodopsin Monomers in Langmuir-Blodgett Film: Evidence for Nonplanar Retinal Structure

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ABSTRACT We observed optical rotation of the plane of polarization of the second harmonic (SH) radiation at 532 nm (in resonance with the retinal absorption) generated in reflection geometry in Langmuir-Blodgett film of bacteriorhodopsin (bR). The analysis of the experimental data showed that this effect arises from the nonvanishing contribution of the antisymmetrical part of the hyperpolarizability tensor. This requires that the dipole moment of the resonant electronic transition, the change of the dipole moment upon electronic excitation, and the long axis of the retinal not be coplanar. Such conditions are satisfied only if the retinal has a nonplanar geometry, a conclusion that could lend support to the heterogeneity model of the origin of the biphasic band shape of the linear CD spectrum of the retinal in bR. On the basis of our theoretical analysis, we were able to estimate the angle between the induced dipole moment and the plan that contains the long axis of the chromophore and the transition dipole moment of the retinal absorption.

INTRODUCTION

Bacteriorhodopsin (bR) is the light-energy transducing retinal protein in the purple membrane of *Halobacterium salinarium*. bR produces a transmembrane electrochemical gradient by pumping the proton across the membrane upon the absorption of light (Stoeckenius et al., 1982). Light-adapted bR contains an all-*trans*-retinal, which is covalently bound to the protein and which is the only chromophore in bR absorbing light at 568 nm. The process of the photoinduced proton pumping is initiated by the absorption of a photon and the photoisomerization of retinal.

Because of the key role of the retinal chromophore in the bR, its structural behavior and interaction with the protein environment were extensively studied by CD spectroscopy. The origin of chirality of the retinal visible absorption spectrum in bR has been a subject of numerous studies since the 1970s and is still an open question. Two models are presented in the literature to account for the biphasic line-shape of the observed visible CD spectrum of the retinal in bR. In the first one (Cassim, 1992; Becher and Ebrey, 1976; Ebrey et al., 1977; Kriebel and Albrecht, 1977), the exciton coupling between the retinal molecules in the hexagonal bR lattice, is considered to be responsible for the observed biphasic nature of the spectrum. In the exciton model, appropriate calculations, assuming a strong exciton coupling case, were performed to support this model quantitatively and to explain the biphasic nature of the CD spectrum. Although the exciton model explained a number of

observations, the assumption of strong coupling case, however, is questionable in light of the observed relatively slow energy transfer rate between the coupled retinal moieties (El-Sayed et al., 1981).

In the second model, the heterogeneity model (Wu and El-Sayed, 1991), the biphasic nature of the CD spectrum was proposed to result from the presence of two (or more) distributions of twisted (nonplanar) retinals with different signs and magnitudes for their twist angle. The sign of the twist angle of one part of the retinal molecule around the long axis relative to the other part determines the sign of the optical rotation. Assuming that two distributions of oppositely twisted retinals have absorption maxima that differ by as little as 400 cm^{-1} from one another, Wu et al. were able to reproduce the biphasic CD spectrum of retinal in bR. The heterogeneity model thus requires a nonplanar structure for the retinal in bR.

An attractive opportunity to examine the retinal structure in bR is to employ the recently developed nonlinear optical approach to the study of chiral molecules (Petralli-Mallo et al., 1993). This approach makes use of the polarization characteristics of the second harmonic generation (SHG) in the orientational ordered molecular layers (Corn and Higgins, 1994). Because SHG is governed on the microscopic level by the third-rank tensor of the first hyperpolarizability, β_{ijk} , this process is more sensitive to the molecular structure than conventional light scattering. The latter is governed by the second-order polarizability tensor, which does not depend on molecular handedness. Moreover, the symmetry of the first hyperpolarizability tensor is different from the symmetry of the molecular tensor; these are responsible for the CD and circular birefringence (Barron, 1982). This means that the SHG technique should give a new information on the retinal structure in comparison with conventional CD spectroscopy.

Received for publication 9 September 1996 and in final form 22 September 1997.

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0006-3495/97/12/3164/07 \$2.00

The first observation of the SHG in bR LB films (Aksipetrov et al., 1987) was followed by investigations of polyvinyl alcohol films (Huang et al., 1989; Bouevich et al., 1995). Results of these experiments were successfully interpreted in terms of the simplest model of the retinal nonlinear optical response. Specifically, it was presumed that the β_{333} (index 3 labels the long axis of the retinal) is the only nonzero component of the first hyperpolarizability tensor. These studies, therefore, ignored the possible twisted architecture of the retinal chromophore in bR (Siebert, 1984; Humphrey et al., 1994; Seibert et al., 1995), which may result in nonparallel orientations of the dipole moments of the different electronic transitions. Correspondingly, additional components of the first hyperpolarizability tensor may contribute to the nonlinear optical response of the film. It has been suggested (Chen et al., 1994) that the mirror symmetry is slightly relaxed for the retinal chromophore in bR upon light excitation.

In this work we studied the polarization state of the wave of the second harmonic generated light in LB films of monomerized bR. The films are rotationally invariant around the surface normal. The second harmonic frequency was chosen to be within the retinal absorption band. Our experimental approach followed the methodology developed to measure the second-order susceptibility for ordered molecular layers of chiral molecules (Petralli-Mallo et al., 1993; Byers et al., 1994). By comparing the experimental results with the theory developed, we found that the retinal chromophore must have a nonplanar architecture. This is dictated by our conclusion that the transition dipole moment, the induced change in the permanent dipole moment vector upon excitation, and the long axis of the retinal chromophore cannot lie in the same plane.

THEORY

SHG on reflection from LB film

We use the axis system shown in Fig. 1 in our present analysis of the SHG. The LB film of bR, which is placed on

the surface of the fused silica support, is illuminated by a light wave of frequency ω at an angle α with respect to the surface normal. The wave at the frequency 2ω is emitted at the specular reflection angle. The magnitude of its electric field is given by (Mizrahi and Sipe, 1988; Hecht and Barron, 1996)

$$\vec{E}(2\omega) = \frac{4\pi i\omega}{c \cos \alpha} \vec{P}(2\omega) \quad (1)$$

Here $P(2\omega)$ is the magnitude of the electric dipole moment per unit area at the frequency 2ω , which is induced on the surface by the incident light wave. $P(2\omega)$ may be calculated by the summation of dipole moments of the individual molecules over the surface:

$$\vec{P}_i(2\omega) = \frac{1}{S} \sum_{nes} \beta_{ijk}^{(n)} \vec{E}_j \vec{E}_k \quad (2)$$

S is the total surface area, $\beta_{ijk}^{(n)}$ is the tensor of the first hyperpolarizability of the n th molecule, and E is the electric field magnitude of the incident wave. Subscripts in Eq. 2 label Cartesian coordinates in the laboratory frame. The tensor of the first hyperpolarizability β_{ijk} is symmetrical with respect to the permutation of the last two indices, i.e., $\beta_{ijk} = \beta_{ikj}$ (Shen, 1984).

When molecules are homogeneously distributed on the surface, Eq. 2 is reduced to $\vec{P}_i = N \langle \beta_{ijk} \rangle \vec{E}_j \vec{E}_k$, where N is the molecular surface density. Angle brackets denote an average over the orientation distribution of the molecules. That is, the magnitude of the i th Cartesian component of the electric field at the frequency 2ω (Eq. 1) is determined by the tensor of nonlinear susceptibility, $\chi_{ijk} = N \langle \beta_{ijk} \rangle$.

To calculate χ_{ijk} , we assume that the long axis of the chromophore has a preferred tilt, θ , to the outward normal to the surface, but an arbitrary azimuthal angle. This means that LB film is supposed to be invariant with respect to rotations about the surface normal. The orientational averaging reduces the number of nonzero elements of the third-rank tensor χ_{ijk} down to 11 (Shen, 1984). Its four independent components may be expressed as follows:

$$\begin{aligned} \chi_{xxz} &= \chi_{xzx} = \chi_{yyz} = \chi_{yzy} \\ &= \frac{N}{2} (\beta_{113} + \beta_{223}) \cos^3 \theta + \frac{N}{4} (2\beta_{333} - \beta_{311} - \beta_{322}) \cos \theta \sin^2 \theta \end{aligned} \quad (3)$$

$$\begin{aligned} \chi_{zxx} &= \chi_{zyy} \\ &= \frac{N}{2} (\beta_{311} + \beta_{322}) \cos^3 \theta + \frac{N}{4} (2\beta_{333} - \beta_{113} - \beta_{223}) \cos \theta \sin^2 \theta \end{aligned} \quad (4)$$

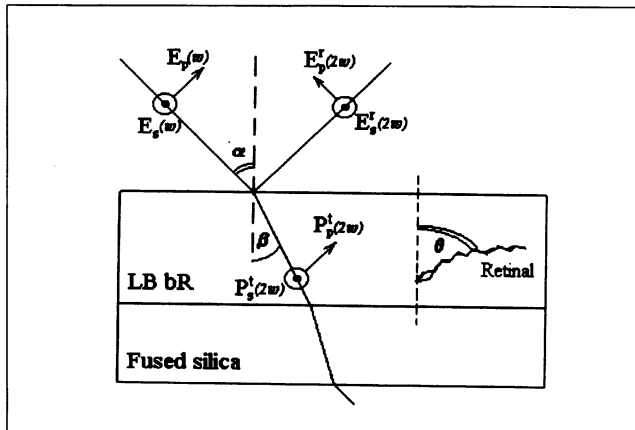


FIGURE 1 Geometry of the SHG experiment.

χ_{zzz}

$$= N\beta_{333}\cos^3\theta + \frac{N}{2}(2\beta_{113} + 2\beta_{223} + \beta_{311} + \beta_{322})\cos\theta\sin^2\theta \quad (5)$$

$$\begin{aligned} \chi_{xyz} = \chi_{xzy} = -\chi_{yxz} = -\chi_{yzx} \\ = \frac{1}{2}N(\beta_{123} - \beta_{213})\left(\frac{3}{2}\cos^2\theta - \frac{1}{2}\right) \end{aligned} \quad (6)$$

Here β_{333} , β_{311} , β_{322} , β_{113} , β_{223} , β_{123} , and β_{213} are components of the first hyperpolarizability tensor in the molecular frame (its axis 3 coincides with the long axis of retinal).

The four components of the nonlinear susceptibility tensor listed in Eq. 8 determine the so-called chiral contribution to the nonlinear optical response. They survive only if molecules on the surface have no planes of symmetry, i.e., they are chiral (Petralli-Mallo et al., 1993; Hecht and Barron, 1996). However, β_{ijk} becomes symmetrical with respect to the permutation of all indices when the frequency dispersion is negligible (Shen, 1984). Therefore, if frequencies ω and 2ω are far from resonance, the antisymmetrical part of the first hyperpolarizability tensor, $\beta_a = \beta_{123} - \beta_{213}$, is equal to zero. Correspondingly, the components of the second-order susceptibility tensor listed in Eq. 8 vanish. This means that the magnitude and polarization state of the wave of the second harmonics generated in the absorption-free film do not depend on the handedness of the molecule.

Let us consider the p -polarized incident wave, i.e., $\vec{E} = \vec{e}_p(\omega)A_p(\omega)\exp\{-i\omega t + ik_1\vec{r}\} + \text{c.c.}$, where $\vec{e}_p(\omega) = \{-\cos\alpha, 0, \sin\alpha\}$, $V_1 \Rightarrow$ and $\vec{K}_1 = (\omega/c)\{-\sin\alpha, 0, -\cos\alpha\}$. The magnitudes of the orthogonal p - and s -polarization components of the reflected wave at frequency 2ω , $A_{p,s}(2\omega) = (\vec{e}_{p,s}(2\omega) \cdot \vec{E}(2\omega))$, where $\vec{e}_s(2\omega) = \{0, 1, 0\}$, $\vec{e}_p(2\omega) = \{\cos\alpha, 0, \sin\alpha\}$, $V_2 \Rightarrow \vec{K}_2 = 2\omega/c\{\sin\alpha, 0, \cos\alpha\}$ may then be obtained from Eq. 1:

$$A_p(2\omega) \propto \chi(p\text{-}pp)A_p^2(\omega) \quad (7)$$

$$A_s(2\omega) \propto \chi(s\text{-}pp)A_p^2(\omega) \quad (8)$$

Here the parameters

$$\chi(p\text{-}pp) = \vec{e}_p(2\omega) \cdot \chi: \vec{e}_p(\omega)\vec{e}_p(\omega) \quad (9)$$

$$= \chi_{zzz}\sin^3\alpha + (\chi_{zzx} - 2\chi_{xxz})\cos^2\alpha\sin\alpha$$

$$\chi(s\text{-}pp) = \vec{e}_s(2\omega) \cdot \chi: \vec{e}_p(\omega)\vec{e}_p(\omega) = 2\chi_{xyz}\sin\alpha\cos\alpha \quad (10)$$

account for the geometry of the nonlinear reflection. These parameters determine the azimuth of the polarization plane ϕ of the wave at the frequency 2ω (Hecht and Barron, 1996):

$$\tan 2\phi = \frac{2\text{Re}\{\chi(p\text{-}pp)\chi^*(s\text{-}pp)\}}{|\chi(p\text{-}pp)|^2 - |\chi(s\text{-}pp)|^2} \quad (11)$$

Correspondingly, the SHG intensity obtained with a linear

polarization analyzer in the path of the reflected wave is (Byers et al., 1994)

$$I(2\omega) \propto 1 + B\cos^2(\Phi - \phi) \quad (12)$$

Here Φ is the angle between the analyzer plane and $e_p(2\omega)$, and the parameter B depends on the phase difference between the s - and p -polarizations of the wave. It follows from Eq. 12 that in the experiment ϕ may be obtained from the offset with respect to the $\cos^2\Phi$ function in the dependence of the SHG intensity on the analyzer orientation Φ .

Molecular properties

Let us consider what information on the molecular architecture may be obtained from the polarization state of the reflected wave at the frequency 2ω . We will begin from the equation for the first hyperpolarizability of a two-level molecule (Zyss and Oudar, 1982; Corn and Higgins, 1994):

$$\begin{aligned} \beta_{ijk} = \frac{1}{2\hbar^2(\omega_0^2 - \omega^2)} \\ \cdot \left\{ \delta\vec{\mu}_i\vec{\mu}_j\vec{\mu}_k + \vec{\mu}(\delta\vec{\mu}_j\vec{\mu}_k + \vec{\mu}_j\delta\vec{\mu}_k) \frac{\omega_0^2 + 2\omega^2}{\omega_0^2 - 4\omega^2} \right\} \end{aligned} \quad (13)$$

Here $\vec{\mu}$ and ω_0 are the dipole moment and frequency of the electronic transition, respectively, and $\delta\vec{\mu}$ is the difference between the dipole moments of the molecule in excited and ground states. From Eq. 13 we can readily find the antisymmetrical part of the first hyperpolarizability, $\beta_a = \beta_{123} - \beta_{213}$, which determines $\chi(s\text{-}pp)$:

$$\beta_a = \beta_{123} - \beta_{213} = \frac{3\omega^2(\vec{\mu} \cdot \vec{\zeta})([\delta\vec{\mu} \times \vec{\mu}] \cdot \vec{\zeta})}{\hbar^2(\omega_0^2 - 4\omega^2)(\omega_0^2 - \omega^2)} \quad (14)$$

Here $\vec{\zeta}$ is a unit vector along the long axis of the molecule.

We can see from Eq. 14 that if the vectors $\vec{\mu}$, $\delta\vec{\mu}$, and $\vec{\zeta}$ lie in the same plane, $\beta_a = 0$. Therefore, the departure of a molecule from the rodlike or planar architecture manifests itself in the nonlinear optical experiment as a nonzero value of $\chi(s\text{-}pp)$.

Equation 13 does not account for the resonant absorption that is important when ω or 2ω is within the bandwidth Γ of the electronic transition. To take into account the resonance phenomena, we can replace ω_0 with $\omega_0 - \frac{1}{2}i\Gamma$ (Barron, 1982) in Eq. 13. If $|2\omega - \omega_0| \leq \Gamma$, the antisymmetrical part of the first hyperpolarizability (Eq. 14) may be rewritten as

$$\beta_a = \frac{(\omega_0^2 - 4\omega^2 + 2i\omega\Gamma)(\vec{\mu} \cdot \vec{\zeta})([\delta\vec{\mu} \times \vec{\mu}] \cdot \vec{\zeta})}{\hbar^2((\omega_0^2 - 4\omega^2)^2 + 4\omega^2\Gamma^2)} \quad (15)$$

Similarly, one may obtain from Eq. 13 that

$$\beta_{333} = \frac{(\omega_0^2 - 4\omega^2 + 2i\omega\Gamma)(\vec{\mu} \cdot \vec{\zeta})(\delta\vec{\mu} \cdot \vec{\zeta})}{\hbar^2((\omega_0^2 - 4\omega^2)^2 + 4\omega^2\Gamma^2)} \quad (16)$$

If β_{113} , β_{223} , β_{311} , and β_{322} are small in comparison with

β_{333} , we arrive at

$$\chi(p\text{-}pp) = \frac{N}{2} \beta_{333} (2 \cos^3 \theta \sin^3 \alpha - \cos \theta \sin^2 \theta \cos^2 \alpha \sin \alpha) \quad (17)$$

$$\chi(s\text{-}pp) = \frac{N}{2} \beta_{333} \left(\frac{3}{2} \cos^2 \theta - \frac{1}{2} \right) \sin 2\alpha \quad (18)$$

Substitution of Eqs. 17 and 18 into Eq. 11 gives the azimuth of the polarization plane of the wave at the frequency 2ω . In particular, when $|\chi(s\text{-}pp)| \ll |\chi(p\text{-}pp)|$, Eq. 11 may be rewritten as

$\tan 2\phi$

$$= \frac{([\vec{\mu} \times \delta\vec{\mu}] \cdot \vec{\zeta})}{(\vec{\mu} \cdot \vec{\zeta})(\delta\vec{\mu} \cdot \vec{\zeta})} \times \frac{(3 \cos^2 \theta - 1) \cos \alpha}{(2 \cos^2 \theta \sin^2 \alpha - \sin^2 \theta \cos^2 \alpha) \cos \theta} \quad (19)$$

The ratio

$$\Delta = \frac{([\vec{\mu} \times \delta\vec{\mu}] \cdot \vec{\zeta})}{(\vec{\mu} \cdot \vec{\zeta})(\delta\vec{\mu} \cdot \vec{\zeta})} \quad (20)$$

depends on the orientation of the $\delta\vec{\mu}$ with respect to the $\mu\zeta$ plane. This ratio may be obtained from the analysis of the polarization state of the wave of the second harmonics. The value of Δ allows us to estimate the angle between the induced dipole moment and the plane containing the long axis of the chromophore and dipole moment of the electronic transition.

This may be clearly seen when the chromophore departs slightly from the rodlike architecture, i.e., when $\vec{\mu}$ and $\delta\vec{\mu}$ are mainly along $\vec{\zeta}$. In such a case, in the denominator of formula 20, we can replace $(\vec{\mu} \cdot \vec{\zeta})$ and $(\delta\vec{\mu} \cdot \vec{\zeta})$ with $|\mu|$ and $|\delta\mu|$, respectively. One may readily obtain from (20) that $\Delta \cong \delta\psi \times \delta\varphi$, where $\delta\psi$ is the angle between the retinal axis $\vec{\zeta}$ and the dipole moment of the optical transition $\vec{\mu}$, and $\delta\varphi$ is the angle between the induced dipole moment $\delta\vec{\mu}$ and the $\mu\zeta$ plane.

MATERIALS AND METHODS

The purple membrane was prepared according to a standard procedure (Oesterhelt and Stoekenius, 1974). The LB films were made of light-adapted bR membrane fragments, following the method of Furuno et al. (1988), with some modifications. The solvent used is a mixture of hexane, water, and dimethylformamide (DMF) in volume proportions of 1.0:0.5:0.2, respectively. The concentration of the purple membrane fragments suspended in the solvent was 0.1 mM. Compression started 1 h after spreading. The compressed film was deposited on a fused quartz plate at 32 dyn/cm by a horizontal transfer. The transfer ratio was between 0.8 and 1.3. After every transfer, the film was dried under a stream of nitrogen gas.

Absorption and one-photon CD spectra of all of the samples were taken with a Bechman DU-650 spectrophotometer and a Jasco J-720 spectropolarimeter, respectively. It was found that the optical density was increasing with the number of depositions while the absorption maximum for all samples was ~ 568 nm. The observed CD spectra was monophasic, which showed that the LB films contained bR in the form of monomers.

The experimental setup for nonlinear optical measurements is shown in Fig. 2. A Q-switched Nd:YAG laser with a 10-Hz repetition rate and a 8-ns pulsewidth was used. The laser gave an infrared beam at 1064 nm with a spot diameter of 3 mm and energy density of 0.25 J/cm². To create orthogonal linear polarizations, the laser radiation passed through a Glan-Thompson prism and a half-wavelength plate.

The SH intensity was measured in both reflective and transmission geometries (see Fig. 2). The transmitted SHG radiation was focused onto the monochromator slit, detected by photomultiplier tube, and averaged with a boxcar integrator (Princeton Applied Research). The reflected SHG radiation was focused onto a polychromator slit (Acton Research Corporation, SpectraPro-275) and then detected by a CCD camera (model LN/CCD-1024 EVU; Princeton Research).

RESULTS AND DISCUSSION

The absorption spectra represented in Fig. 3 show the growth of the optical density with increasing numbers of depositions. The absorption maximum for all of the samples was ~ 568 nm.

The one-photon CD spectra (Fig. 4) are monophasic for the sample with bR fragments in hexane, and DMF (*dashed line*) and biphasic for bR membrane fragments in water solution (solid line). This suggests that the hexagonal trimetric structure of bR in purple membrane is dissolved in the organic solvent we use for the deposition. Thus, in the LB films, which were made from hexane and DMF solution, bR was in the monomeric form.

The SH signal is mainly attributed to the retinal in the LB film, because its frequency is in resonance with the electronic transition of retinal in bR. We measured the electric-quadrupole contribution from our substrate (clean fused silica plate) according to the method of Shen (Guyot-Sionnest and Shen, 1987), and found that the intensity of its SH was 10–20 times smaller than that from the bR LB film.

The measurements in the transmission geometry were performed to obtain the orientation of the chromophore. For the *p*- and *s*-polarization of the fundamental beam, we measured intensities of the *p*-polarized component of the

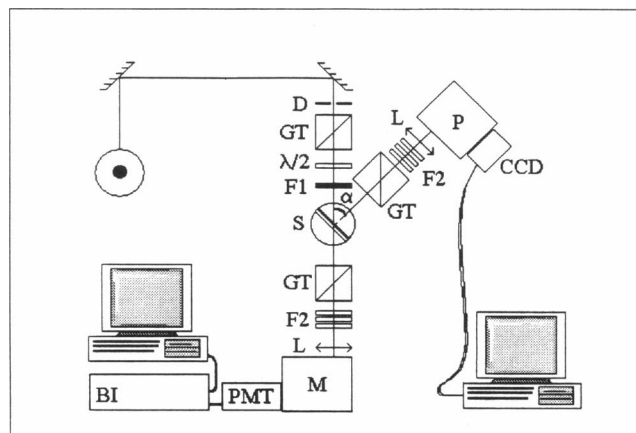


FIGURE 2 Experimental set-up for SHG measurement. D, diaphragm; GT, Glan-Thompson prism; F1, filter to cut 532 nm; F2, filter to cut 1064 nm; S, sample; L, lens ($F = 9$ cm); M, monochromator; P, polychromator; BI, boxcar integrator; α , angle of incidence.

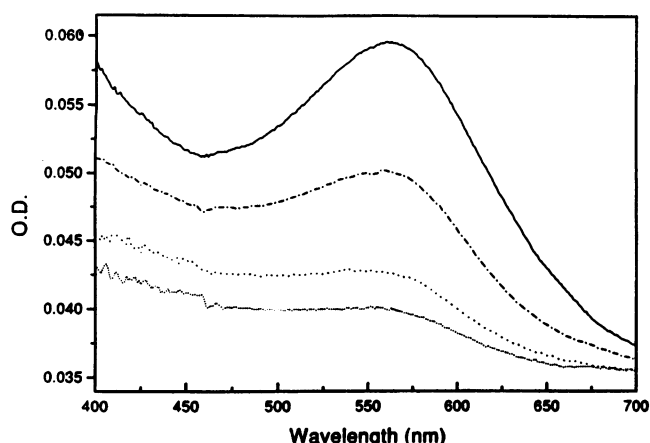


FIGURE 3 The dependence of the optical density on the number of depositions: 9, 4, 3, and 1.

second harmonic wave. Under the assumption that β_{333} is the major component of the first hyperpolarizability tensor, the ratio of these intensities may be written as (Mizrahi and Sipe, 1988)

$$\frac{I_{p-ss}(2\omega)}{I_{p-pp}(2\omega)} = \frac{\sin^2\theta \sin^2\alpha}{2 \cos^2\theta \sin^2\alpha + 3 \sin^2\theta \cos^2\alpha} \left[\frac{I_s^2(\omega)}{I_p^2(\omega)} \right] \quad (21)$$

where $I_{p,s}(\omega)$ are the intensities of the transmitted waves at frequency ω .

The measurements were done at an angle of incidence of $\alpha = 45^\circ$ and $I_p(\omega)/I_s(\omega) \cong 1$. From the experimental data on the ratio $I_{p-ss}(2\omega)/I_{p-pp}(2\omega)$ and Eq. 21, we calculated the chromophore tilt angle. The results of this calculation are presented in Table 1. It was found that θ in one- or two-layer film was close to that found by neutron scattering (King and Schoenborn, 1982), electrochromism (Clark et

TABLE 1 Average angles of the retinal orientation in bR in the LB films having different numbers of depositions (layers)

No. of depositions	$I_{p-ss}(2\omega)/I_{p-pp}(2\omega)$	θ , grad
1	0.312	72.1
3	0.276	60.9
4	0.226	49.9
9	0.073	23.4

al., 1980), and polarized Fourier transform infrared spectroscopy (Earnst et al., 1986) measurements. The decreasing of the average chromophore tilt angle with the number of layers deposited may be caused by nonuniform deposition of the additional membrane fragments.

In reflection geometry, we measured the dependence of the SHG intensity on the analyzer orientation at various angles of incidence, α . The fundamental beam was p -polarized. To set up the experimental configuration, the test experiment was performed for each angle of the incidence. Specifically, we measured the dependence of the SHG radiation, reflected from the Rhodamine 6G (R6G) film on the orientation of the analyzer. The R6G molecule is achiral. The wave of the second harmonics reflected from the R6G film is p -polarized for either p - or s -polarization of the fundamental (Heintz et al., 1982).

Fig. 5 shows the dependence of the SHG intensity on Φ at $\alpha = 25^\circ$. The open circles give the results for the bR LB film with four depositions, and the solid squares represent the results for the R6G film. The wave at frequency 2ω reflected from the R6G film was linearly polarized, $I(2\omega)\alpha \cos^2(\Phi + 9.3^\circ)$. That is, the analyzer angle $\Phi_s = -9.3^\circ$ corresponded to the s -polarization of the second harmonic at that angle of incidence. This angle is determined by the optical and instrumental conditions for the R6G sample and is used as a reference in the measurement of the bR LB film. The wave of the second harmonic reflected from bR LB

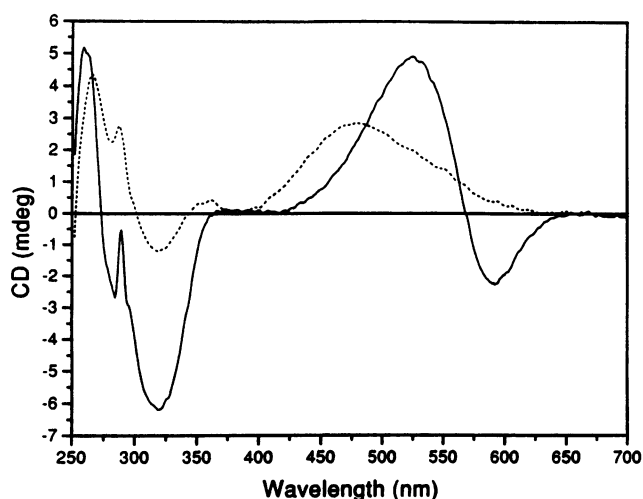


FIGURE 4 A comparison of the monophasic CD spectra of bR fragments in hexane solution and DMF (---) and the biphasic spectrum of bR membrane in water solution (—).

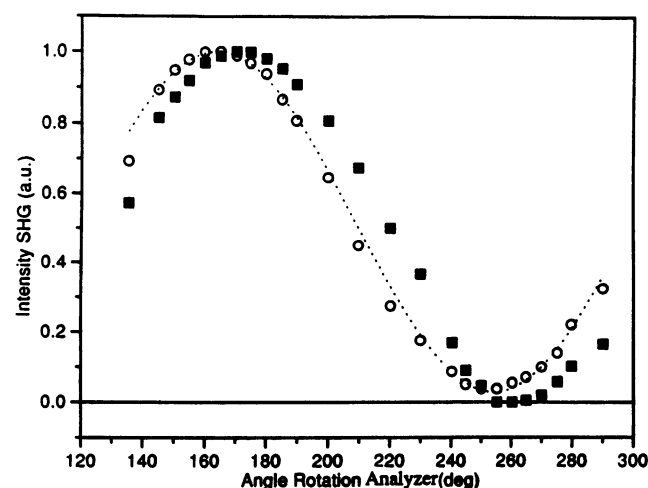


FIGURE 5 The dependence of the second harmonics intensity, reflected from the bR LB film with 4 depositions (○) and R6G film (■), on the analyzer orientation. The dotted line is the theoretical fit.

film was elliptically polarized, $I(2\omega) \propto 1 + A \cos^2(\Phi + 16.1^\circ)$. That is, at $\alpha = 25^\circ$, the azimuth of the polarization plane of the wave of the second harmonic wave is at $\phi = -16.1^\circ - \Phi_s = -6.8^\circ$.

The measurements of ϕ were performed for different angles of incidence for the bR LB film with four depositions. The data on the azimuth of the rotation of the plane of polarization are given in Table 2. By substituting into Eq. 19 the measured value of $\phi = -6.9^\circ$ and $\theta = 49.9^\circ$, it was found that $\Delta \cong 0.22$. This shows a significant change in the structure of the chromophore under electronic excitation. In particular, if the dipole moment of the retinal is in the $\mu\zeta$ plane in the ground electronic state, it departs from this plane considerably.

Note that Eq. 19 was derived under the assumption that $|\chi(s-pp)| \ll |\chi(p-pp)|$. Therefore, this equation is valid when $\tan(2\phi) \ll 1$. One may see from Eq. 19 that for the bR LB film with four depositions ($\theta = 49.9^\circ$), the denominator in formula 19 is equal to zero at $\alpha \cong 40^\circ$ when $\chi(p-pp) = 0$. Therefore, when α approaches 40° , the accuracy of our estimate decreases.

The measurements of ϕ were performed for different angles of the incidence for the bR LB film with four depositions. The data on the azimuth of the polarization plane rotation are given in Table 2. By substituting into Eq. 19 the measured value of ϕ and $\theta = 49.9^\circ$, it was found that $\Delta \cong 0.22$. This shows a significant modification of the chromophore under electronic excitation. In particular, if the dipole moment of the retinal lies within the $\mu\zeta$ plane in the ground electronic state, it departs from this plane considerably in the excited electronic state.

Finally, we would like to note that $\delta\vec{\mu}$ of the retinal chromophore in bR is found to be larger than that of the free chromophore (Huang et al., 1989) by a factor of 1.7. However, in free all-*trans* retinal, $\vec{\mu}$ and $\delta\vec{\mu}$ are along the charge transfer direction, which coincides with the molecular long axis (Ponder and Mathies, 1983). Our experiment showed that the protein environment crucially affects the electronic structure and geometry of retinal upon excitation. Specifically, in bR the electronic excitation of retinal is found to be accompanied by a departure from the planar or rodlike architecture. The breaking of the molecular symmetry could probably be attributed to the twisting around the C-C bonds (Humphrey et al., 1994), which was previously suggested to be the origin of the biphasic line shape of the CD spectrum

of the bR (El-Sayed et al., 1981). Studies of polarized infrared spectroscopy suggest the existence of distortions around different carbon atoms along the retinal chain (Seibert, 1984; Seibert et al., 1995). Recent solid-state NMR demonstrated that the retinal polyene backbone is slightly curved, with its methyl groups tilting away from the membrane normal (Ulrich et al., 1994). In addition, very recently, theoretical consideration of the retinal structure in bR suggested that possible distortions exist around certain carbon atoms along the retinal chain in bR (Xu et al., 1996).

The authors thank the Department of Energy, Basic Energy Sciences, for its support of this work (grant DE-FG05-95ER-14537). YPS thanks the Liverhulme Trust for a Fellowship.

REFERENCES

- Aksipetrov, O. A., N. Akhmediev, N. N. Vsevolodov, D. A. Esikov, and D. A. Shutov. 1987. Photochromism in nonlinear optics: photocontrolled second harmonic generation by bacteriorhodopsin molecules. *Sov. Phys. Dokl.* 31:219–223.
- Barron, L. D. 1982. *Molecular Light Scattering and Natural Optical Activity*. Cambridge University Press, Cambridge.
- Becher, B., and T. G. Ebrey. 1976. Evidence for chromophore-chromophore (exciton) interaction in the purple membrane of *Halobacterium halobium*. *Biochem. Biophys. Res. Commun.* 69:1–6.
- Boueviyeh, O., A. Lewis, and M. Sheves. 1995. Probing bacteriorhodopsin photochemistry with nonlinear optics: comparing the second harmonic generation of bR and the photochemically induced intermediate K. *J. Phys. Chem.* 99:10648–10657.
- Byers, J. D., H. I. Yee, and J. M. Hicks. 1994. A second harmonic generation analog of optical rotatory dispersion for the study of chiral monolayers. *J. Chem. Phys.* 101:6233–6241.
- Cassim, J. Y. 1992. Unique biphasic band shape of the visible circular dichroism of bacteriorhodopsin in purple membrane. *Biophys. J.* 63:1432–1442.
- Chen, Z., M. Sheves, A. Lewis, and O. Bouevitch. 1994. A comparison of the second harmonic generation from light-adapted, dark-adapted, blue and acid purple membrane. *Biophys. J.* 67:1155–1160.
- Clark, N. A., K. J. Rothschild, D. A. Luippold, and B. A. Simon. 1980. Surface-induced lamellar orientation of multilayer membrane array. *Biophys. J.* 31:65–96.
- Corn, R., and D. A. Higgins. 1994. Optical second harmonic generation as a probe of surface chemistry. *Chem. Rev.* 94:107–125.
- Earnst, T. N., P. Roepe, M. S. Braiman, J. Gillespie, K. J. Rothschild. 1986. Orientation of the bacteriorhodopsin chromophore probed by polarized Fourier transform infrared different spectroscopy. *Biochemistry*. 25:7793–7798.
- Ebrey, T. G., B. Becher, M. P. Kilbride, and B. Honig. 1977. Exciton interaction and chromophore orientation in the purple membrane. *Mol. Biol.* 112:377–397.
- El-Sayed, M. A., B. Karvaly, and J. M. Fukumoto. 1981. Primary step in the bacteriorhodopsin photocycle: photochemistry or exciton transfer? *Proc. Natl. Acad. Sci. USA*. 78:7512–7516.
- Furuno, T., T. Kouyama, A. Ikegami, and H. Sasabe. 1988. Photovoltaic properties of purple membrane Langmuir-Blodgett films. *Thin Solid Films*. 160:145–152.
- Guyot-Sionnest, P., and Y. R. Shen. 1987. Local and nonlocal surface nonlinearities for surface optical second-harmonic generation. *Phys. Rev. B*. 35:4420–4426.
- Hecht, L., and L. D. Barron. 1996. New aspects of second-harmonic optical activity from chiral surfaces and interfaces. *Mol. Phys.* 89:61–80.
- Heintz, T. F., C. K. Chen, D. Ricard, and Y. R. Shen. 1982. Spectroscopy of molecular monolayers by resonant second-harmonic generation. *Phys. Rev. Lett.* 48:478–481.

TABLE 2 The azimuth of the polarization plane of the wave of the second harmonics light, reflected from four depositions of bR LB film for different angles of incidence

α , grad	ϕ , grad	Δ
13	−4.5	0.22
25	−6.8	0.23
28	−8.7	0.26

The parameter Δ (see text) has been calculated from Eq. 19 at a retinal angle tilt to the outward perpendicular axis to the membrane plane of $\theta = 49.9^\circ$.

- Humphrey, W., I. Logunov, K. Schulten, and M. Sheves. 1994. Molecular dynamics study of bacteriorhodopsin and artificial pigments. *Biochemistry*. 33:3668–3678.
- Liang, J. Y., Z. Chen, and A. Lewis. 1989. Second harmonic generation in purple membrane-poly(vinyl alcohol) films: probing the dipolar characteristics of the bacteriorhodopsin chromophore in bR₅₇₀ and M₄₁₂. *J. Phys. Chem.* 93:3314–3320.
- King, G. I., and B. P. Schoenborn. 1982. Neutron scattering of bacteriorhodopsin. *Methods Enzymol.* 88:241–248.
- Kriebel, A., and A. Albrecht. 1977. Exciton interaction among three chromophores: an application to the purple membrane of *Halobacterium halobium*. *J. Chem. Phys.* 65:4575–4583.
- Mizrahi, V., and J. E. Sipe. 1988. Phenomenological treatment of surface second-harmonic generation. *J. Opt. Soc. Am. B.* 5:660–667.
- Oesterhelt, D., and W. Stoekenius. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* 31:667–678.
- Petralli-Mallo, T., T. M. Wong, J. D. Byers, H. I. Yee, and J. M. Hicks. 1993. Circular dichroism spectroscopy at interfaces: a surface second harmonic generation study. *J. Phys. Chem.* 97:13833–13838.
- Ponder, M., and R. Mathies. 1983. Excited state polarizabilities and dipole moment of diphenylpolyenes and retinal. *J. Phys. Chem.* 87:5090–5098.
- Seibert, F. 1984. Static and a time-resolved infrared difference spectroscopy applied to rhodopsin and bacteriorhodopsin. *NATO ASI Ser. C. Spectrosc. Biol. Mol.* 139:347–372.
- Seibert, F., F. Jaeger, O. Weidlich, and M. Sheves. 1995. The role of steric interaction between the chromophore and protein: FTIR and functional studies of rhodopsin and bacteriorhodopsin regenerated with modified chromophore. Conference Proceedings: Spectroscopy of Biological Molecules, 6th European Conference. 175–176.
- Shen, Y. R. 1984. The Principles of Nonlinear Optics. Wiley, New York.
- Stoeckenius, W., R. H. Lozier, and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 51: 587–616.
- Wu, Sh., and M. A. El-Sayed. 1991. CD Spectrum of bacteriorhodopsin. *Biophys. J.* 60:190:197.
- Zyss, J., and J. L. Oudar. 1982. Relations between microscopic and macroscopic lowest-order optical nonlinearities of molecular crystals with one-dimensional or two-dimensional units. *Phys. Rev. A.* 26: 2028–2048.