Determination of the absolute orientation of the retinylidene chromophore in purple membrane by a second-harmonic interference technique

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ABSTRACT The absolute direction of the retinal chromophore of bacteriorhodopsin relative to the membrane plane is investigated by using an optical second-harmonic interference technique. Compared with the known adsorbed geometry of free retinylidene Schiff base on a glass substrate, our data indicate the β-ionone ring of the chromophore of bacteriorhodopsin points away from the cytoplasmic surface of the purple membrane. The implication of this finding is discussed in light of other chemical and structural results on bacteriorhodopsin.

INTRODUCTION

Bacteriorhodopsin (bR) is a unique energy-transducing molecule that is the only protein found in the purple membrane which grows in the bacterium *Halobacterium halobium*. The physiological role of this molecule is to convert light energy into a proton gradient across the bacterial cell membrane (1–4). The initial step in this process of photoactivated proton pumping is the absorption of light by the retinylidene chromophore which is embedded and covalently attached to a protein matrix to form the bR molecule. One of the important questions is how the bR molecule stores the absorbed photon energy in the initial photochemical step. To account for this energy storage, a mechanism based on a generally accepted mechanism of color regulation in visual pigments (5) was proposed. In this model (6), excitation causes a large change in the dipole moment of the chromophore, which is then stabilized or destabilized by charges on the protein. The changes in retinal dipole moment could also separate charged groups on the protein, and thereby store large amounts of energy. The protein structural alterations are then stabilized by chromophore configurational changes. Thus, the crucial step in this mechanism is a large alteration in the electron distribution of retinal upon excitation.

Recently we applied optical second-harmonic generation (SHG) to investigate the dipole moment changes in free and bound retinal chromophores (7). In this study, we found both the free and the bound retinal chromophores have similar and significant dipole moment changes upon electronic excitation. In the present paper, we extend our study to probe the absolute orientation of the bound retinal chromophore in the bR molecule. The absolute direction of the bound retinal chromophore has important implications in understanding the dipolar interaction between the chromophore and surrounding amino residues, and the excitonic interaction within the bR trimer in purple membrane. In addition, the determination of the absolute chromophore orientation could have a significant impact on modeling the three-dimensional structure of bR.

The orientation angle of the retinal chromophore of bR has been measured by using neutron scattering (8), polarized Fourier transform infrared difference spectroscopy (9), and electrodichroism (10, 11). However, these physical techniques are insensitive to the absolute direction. On the other hand, Huang et al. (12) used a chemical approach to indirectly probe the absolute direction of the bound retinal chromophore. These workers used a photosensitive analogue of retinal which cross-linked to amino acids of the protein (12). This was followed by enzymatic and chemical degradations of the cross-linked protein and amino acid sequencing procedures. The conclusions of their study require knowledge of the detailed arrangement of the distribution of the polypeptide chain of bR in the membrane.

To appreciate the conclusions of Huang et al. (12), it is important to realize that bR is composed of a single polypeptide chain which traverses the membrane seven times. The seven transmembrane excursions are seen in low-resolution electron density maps of the naturally crystalline bR purple membrane (13). The membrane spanning amino acid sequences have been labeled with the numbers 1–7 in the electron density map and with letters A–G in the amino acid sequence. The letters A–G refer to regions of the amino acid sequence that obviously contain seven groups of membrane spanning amino acids with a strong tendency to form α-helical secondary structures. Extensive modeling has been performed to assign

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the letters from the sequence data to the numbers in the electron density map. The retinylidene chromophore of bR is known to be covalently linked to lysine 216 on helix G (14, 15) through a protonated Schiff base linkage (16). The photochemical labeling results of Huang et al. (12) suggest that the β-ionone ring, which is at the other end from the nitrogen in this linear polyene chain, is in close proximity to helix F. Assuming positions for lysine 216 and the serine 193 and glutamate 194 that the photochemical cross-linking agent of Huang et al. (12) complexes with, the absolute direction of the chromophore would be towards the extracellular side of the membrane.

In this paper we show how the absolute orientation of the bound retinal chromophore can be resolved by using a very simple method based on optical SHG. We demonstrate that by interfering the second-harmonic electric field, which is generated from oriented films of free and membrane bound retinal chromophores with that from a quartz crystal, the abolute direction of the chromophore with respect to the film plane can be determined. Our data indicate that this method, which was originally developed and applied to a monolayer of the simple molecule phenol by Kemnitz et al. (17), can also be readily extended to other relevant biological and chemical applications which require the investigation of absolute chromophore orientations.

MATERIALS AND METHODS

Experimental arrangement

A Q-switched frequency doubled Nd:YAG laser with a 10-Hz repetition rate and 10-ns pulse width was used. The Nd:YAG laser was adjusted to give an infrared 1064-nm beam of less than 60 mW which was focused onto a 3.5-mm-diam spot on the sample surface. The infrared nature of the fundamental beam is specifically important in preventing damage and bleaching of the bR molecules. The transmitted second-harmonic (SH) photons at 532 nm were detected by a cooled RCA C31034 photomultiplier tube (RCA New Products Div., Lancaster, PA) and averaged by a boxcar integrator.

The detailed experimental arrangement for obtaining the absolute chromophore orientation involves generating interference fringes of SH fields as shown in Fig. 1 *a*. Two SH sources are seen in this figure, one an x-cut quartz crystal plate and the other the retinylidene thin film sample. The SH fields generated by these two sources will have a definite phase relationship with respect to each other, as each has a specific relationship to the phase of the fundamental field squared. Between these two SH sources, a 1.61-mm-thick fused quartz glass plate was inserted to modulate their relative phase. In our experiment, the distance between the x-cut quartz crystal plate and the thin sample was fixed to 19 cm.

Three different orientations for each thin film sample were used. The first one is described in Fig. 1 *a* where the film surface faces the incident fundamental laser beam. In this case the glass substrate of the sample does not affect the relative phase between the SH electric fields generated by the sample and the x-cut quartz. Fig. 1 *b* describes the second arrangement where the exciting laser beam is incident on the substrate before it excites the molecules in the sample. An additional phase delay in the SH electric field from the x-cut quartz plate is generated by the glass substrate in this arrangement. In order to directly compare the result obtained from the setup of Fig. 1 *b* with that from Fig. 1 *a*, the phase delay induced by the glass substrate has to be compensated. This was done by inserting an identical quartz glass plate directly before the thin film sample as illustrated in Fig. 1 *c*. For measurement of simply the chromophore angle relative to the membrane plane an experimental arrangement similar to Fig. 1 *a* was used with the exception of the x-cut quartz crystal and the fused quartz plate.

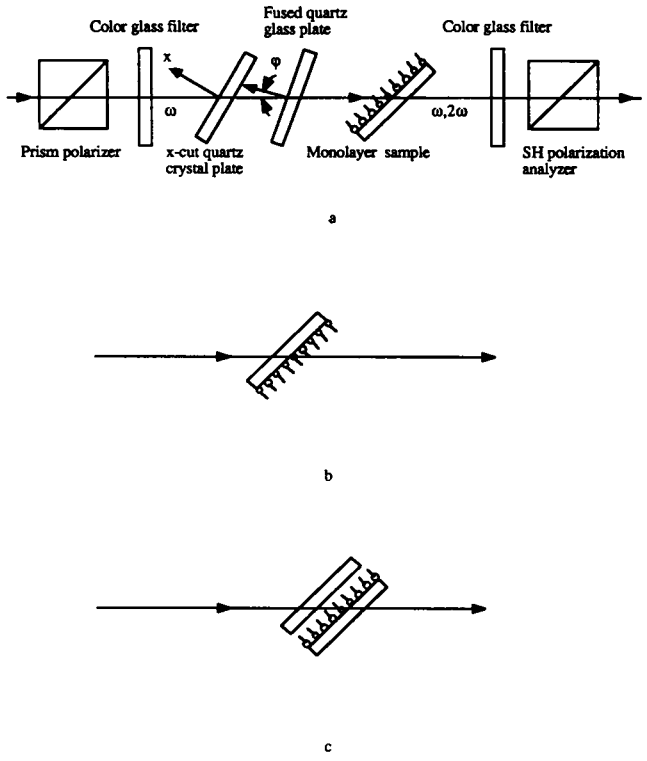

FIGURE 1 Experimental arrangement for the measurement of the SH interference pattern. (*a*) The film surface of the monolayer sample is oriented to face the incident fundamental laser beam; (*b*) the laser beam is incident on the substrate; and (*c*) a sample arrangement is shown, which is the complement to (*b*) and compensates for the phase delay in passing through the glass substrate.

Sample preparation

The purple membrane (PM) was harvested and purified from a fresh batch of *Halobacterium halobium* (S9) according to established procedures (18). The detailed method to make purple membrane–poly (vinyl alcohol) films (PM-PVA) has been reported previously (7). The typical thickness and optical density at 566 nm for a PM-PVA film used in our experiment were 168 μm and 0.1615. This corresponds to a number density of 9.1×10^{16} cm^{-3} for the retinal chromophores. The absorption spectrum of a PM-PVA film is shown in Fig. 2 by the dashed and dotted lines.

The dried, oriented PM sample on a transparent conducting SnO_2 layer was prepared by electrophoresis according to Váró (19). In order

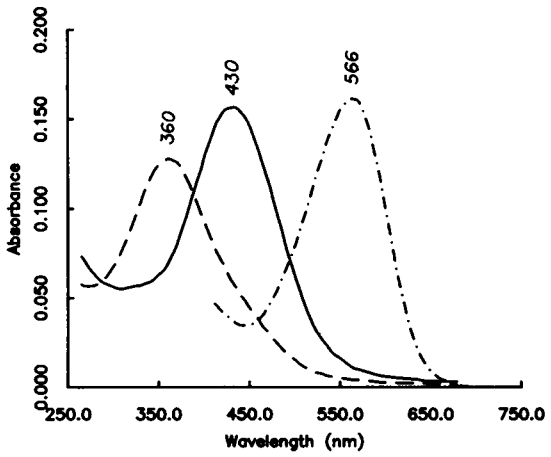

FIGURE 2 Absorption spectra of all-*trans* retinylidene *n*-butylamine Schiff base (NRB) monolayer on a glass surface (*dashed line*), a monolayer of the protonated Schiff base (N^+RB-HCl, *solid line*), and a purple membrane-poly (vinyl alcohol) film (PM-PVA, *dashed-dotted line*) are shown.

to avoid denaturing the protein, a 30-V/cm electric field was applied across the PM suspension for only 1 min. This keeps PM fragments in the purple state while still giving a satisfactory electrophoretic movement of the fragments. Typically a dried PM film with an ~0.2 optical density at 566 nm was used in order to minimize the alterations in the Fresnel coefficients for an air–glass interface that could be induced by a thick dried film on a glass substrate.

Monolayers of retinylidene *n*-butylamine Schiff base (NRB) and its protonated species (N^+RB-HCl) were spin-coated onto quartz glass substrates as detailed by Heinz et al. (20). The absorption spectra of these monolayers on glass surfaces are shown in Fig. 2. The *y*-scale of this figure shows the total absorbance of eight monolayers which were stacked together to increase the quality of the spectra. It was observed that the absorption peak of NRB (360 nm, *dashed line*) is blue shifted by about 5 nm from its peak position in a methanol solution. However, for the protonated species (*solid line*), N^+RB-HCl, a much larger blue shift (15 nm) from the 445-nm absorption in methanol was observed for the monolayer. This indicates that the Schiff base nitrogen which is positively charged in the protonated species not only lies close to the glass surface but also interacts with the hydroxyl groups on the surface.

RESULTS AND DISCUSSION

Determination of the orientational angle of the retinylidene chromophore

It has been recognized that the polarization dependence of the surface SHG can yield information about the average orientation of molecular absorbates (21). The general principle behind this determination by SHG can be easily understood by considering a thin layer of partially aligned molecules with a finite second-order nonlinear polarizability $\alpha(2\omega)$. Without local-field corrections, the surface nonlinear susceptibility $\chi_s^{(2)}$ can be written as $\chi_s^{(2)} = N_s\langle\alpha\rangle$, where N_s is the surface density of molecules and the angular brackets signify an average over all molecule orientations. With a knowledge of $\chi_s^{((2)}$ and $\alpha^{(2)}$, we can infer values for the components of the third-rank tensor relating the molecular axes to the fixed laboratory axes and, hence, find moments of the molecular orientation distribution.

Retinylidene chromophores are linear molecules where the polarizability α is dominated by an intramolecular charge transfer process which is along the molecular long axis ξ. Thus, for these molecules $\alpha^{(2)}$ is dominated by a single axial component $\alpha^{(2)}_{\xi\xi\xi}$ and the orientation determination can be further simplified. If the molecules on the substrate have no preferred direction in the plane (*xy*-plane), the film can be characterized by the substrate normal (*z*-axis) around which the film is rotationally invariant. The only independent nonvanishing components of $\chi_s^{(2)}$ in this case are (21–23):

$$(\chi_s^{(2)})_{zxx} = (\chi_s^{(2)})_{xzx} = (\chi_s^{(2)})_{xxz} = \tfrac{1}{2}\,N_s\langle\cos\zeta\sin^2\zeta\rangle\,\alpha^{(2)}_{\xi\xi\xi}$$
$$(\chi_s^{(2)})_{zzz} = N_s\langle\cos^3\zeta\rangle\,\alpha^{(2)}_{\xi\xi\xi} \qquad (1)$$

where ζ is the angle between the molecular ζ-axis and the film normal.

The formulas which were used for the calculations of the transmitted SH electric fields were derived previously by Dick et al. (22). With these formulas and Eq. (1), we can calculate the SH intensities for differently polarized fundamental and SH beams. Note that the Fresnel coefficients have been corrected according to the discussion of Guyot-Sionnest et al. (24). Our results with an incident angle of 45° for the fundamental laser beam are shown in Table 1, where the experimental data and the corresponding calculated values (enclosed in parentheses) for the relative polarization dependence for the samples we studied are compared. In these calculations, the indexes of refraction of NRB and N^+RB-HCl monolayers were taken to be equal to that of the ambient medium (i.e., air) (23). The inclination angle, ζ, of the free retinylidene chromophore in NRB and N^+RB-HCl monolayers was taken to be 30° away from the film normal. We also assumed the molecules are uniformly distributed around this angle with a half-width of ±5°, which was estimated by the fluctuations in the measured values of the polarized SH intensity. This gives good agreement between the experimental data and the calculated values for these two molecules. In the case of the purple membrane dried film, the Fresnel coefficients for an air-glass interface may be changed by the absorption of the dried film (24). The variations in the Fresnel coefficients were taken into account in our calculation by letting the indexes of refraction of the purple membrane dried film be 1.1 and 1.105 at the fundamental and the SH frequencies, respectively. To fit the experimental data of the PM dried film

TABLE 1 **Polarization dependences of the SH signals from monolayers of unprotonated Schiff base (NRB), protonated Schiff base (N^+RB-HCl), and an electric field-oriented PM-dried film**

Molecule	$I_{p\to p}$	$I_{s\to p}$	$I_{45°\to p}$	$I_{p\to s}$	$I_{s\to s}$
NRB monolayer	1.0	0.07	0.36	0.0	0.0
	(1.0)*	(0.06)*	(0.38)*	(0.0)*	(0.0)*
N^+RB-HCl monolayer	1.0	0.06 ± 0.01	0.38 ± 0.04	0.0	0.0
	(1.0)*	(0.06)*	(0.38)*	(0.0)*	(0.0)*
Electric field-oriented	1.0	0.21 ± 0.04	0.59 ± 0.05	0.0	0.0
PM-dried film	(1.0)‡	(0.22)‡	(0.54)‡	(0.0)‡	(0.0)‡

*The numbers enclosed in the parentheses were calculated for a monolayer with a molecular inclination angle of 30° relative to the substrate normal. The index of refraction of the monolayer was taken to be equal to that of the ambient medium (i.e., air).
‡The values were calculated for an electric field-oriented PM-dried film with the retinal chromophore inclining 65° relative to the substrate normal and with the indexes of refraction of the film being 1.1 and 1.105 at the fundamental and SH frequencies.

satisfactorily, the inclination angle of the bound retinylidene chromophore has to be increased to 65° away from the film normal. By choosing another angle for example 55°, the calculated normalized transmitted SH intensities, $I_{s\to p}/I_{p\to p}$ and $I_{45°\to p}/I_{p\to p}$, become 0.18 and 0.50, respectively, which gives values that are altered more than the error bars in our experimental measurement. Thus, the orientational parameter is estimated to be 65° with an accuracy of ±7°.

In the above calculation of the chromophore angle relative to the substrate normal we have assumed values for the index of refraction at the fundamental and SH frequencies. The assumed values of the index of refraction can be tested by repeating the measurements of the SH signal as a function of the rotation angle of the SH polarization analyzer described above. Thus, the signal was monitored at many different angles in order to get a complete picture of the alteration in the SH signal as the polarization was scanned and these results are shown as the filled circles in Fig. 3. The equations to deal with the curves generated by the polarization scanning have been previously described and we have applied these equations to calculate our experimental results. The theoretical curve generated is shown as the solid line in Fig. 3 and as can be seen there is excellent agreement between the theoretical and experimental results using the same parameters as were employed to get the calculated results in Table 1.

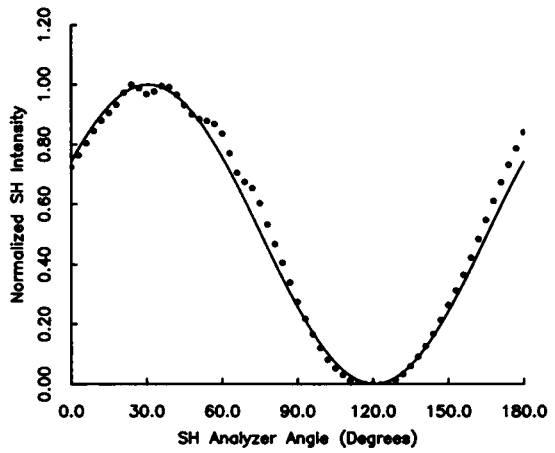

FIGURE 3 The SH signal from an electric field-oriented PM-dried film is plotted with the rotation angle of the SH polarization analyzer. (*Filled circles*) Experimental data. (*Solid line*) Calculated curve with the retinal chromophore of bR inclining 65° from the substrate normal and the indexes of refraction of the film being 1.1 and 1.105 at the fundamental and the SH frequencies, respectively. The incident fundamental laser beam is polarized 45° away from the *xz* incident plane which is defined by the substrate normal and the axis of propagation of the laser. Also, the fundamental beam is focused in this experiment on the film plane at a 45° angle of incidence. The s- and the p-polarized directions correspond to the positions of 0° and 90° in the *x*-axis of the figure.

Váró's method (19) of electric field deposition of PM results in the PM lying flat on a surface with no angle between the membrane plane and the substrate. Thus, the above SH data from these films indicate that the orientational angle between the polyene chain of the retinal chromophore and the purple membrane plane is about 25°. This agrees well with the results obtained from neutron scattering (8), polarized Fourier transform infrared difference spectroscopy (9), and electrodichroism (10).

Determination of the absolute direction of the retinylidene chromophores

The method described in the previous subsection to determine the average orientational parameter of molecule adsorbates on their substrates cannot distinguish whether the transition moment of the excited adsorbates is inclined towards or points away from the substrate. In addition, the answer to this question cannot be obtained from conventional linear optical spectroscopy. Recently Kemnitz and co-workers (17) developed a scheme for

inferring the absolute orientation of molecules at an interface with respect to the directed surface normal by measuring the absolute phase of the SH electric fields. The principle of the method can be understood simply by performing an inversion operation on Eq. (1), which leads to a change in sign of the nonlinear surface susceptibility, $\chi_s^{(2)}$. Therefore, by comparing the signs of the elements of $\alpha^{(2)}$ with the signs of $\chi_s^{(2)}$ unequivocal information can be obtained on the absolute sense of the molecular orientation.

We have slightly modified the method of Kemnitz et al. (17) such that it more closely meets our needs for investigating real biological membranes. In the original scheme, a quartz crystal plate is translated more than 22 mm to obtain one period of the interference pattern between the SH electric field from the thin film sample and that from the quartz crystal plate. Except for a well-collimated exciting laser beam, this procedure will induce significant signal variation and therefore obscure the interference pattern that we want to observe. We altered the above procedure to keep the distance between the quartz crystal plate and the thin film sample constant. To accomplish this, a fused quartz glass plate is inserted between the sample and the quartz crystal in order to modulate the relative phase of the two signals by rotation of the fused quartz plate (see Fig. 1 *a*). This method works well for both focused and collimated exciting beams.

The interference pattern obtained from the experimental arrangement which is shown in Fig. 1 *a*) can be expressed (25) as:

$$I = I_Q + I_s + 2\sqrt{I_Q} \cdot \sqrt{I_s} \cos(\Delta) \tag{2}$$

where Δ is the relative phase delay between the two SH electric fields and is given by:

$$\Delta = (\gamma_s - \gamma_Q) + D_A + D_G(\phi) + D_Q(\psi) + D_s \tag{3}$$

with

$$(\chi_s^{(2)}) = |\chi_s^{(2)}| \cdot e^{i\gamma_s}$$
$$(\chi_Q^{(2)}) = |\chi_Q^{(2)}| \cdot e^{i\gamma_Q}$$
$$D_A = 2\omega[n_a(\omega) - n_a(2\omega)]L_a/c,$$
$$D_G(\phi) = 2\omega[n_g(\omega)/\cos\phi_\omega - n_g(2\omega)]/\cos\phi_{2\omega}]L_g/c, \text{ and}$$
$$D_Q(\psi) = 0.5\pi[1 - L_Q/l_{coh}^Q(\psi)]. \tag{4}$$

Here γ_Q and γ_s are the phase factors of the nonlinear susceptibilities of the x-cut quartz crystal plate and the thin film sample; $D_A D_G$ (ϕ) are the dispersions between the fundamental and the SH frequencies induced by air, and the quartz glass plate. D_Q (or D_s) is the phase difference between the SH electric field generated from the quartz crystal (or the thin film sample) and the incident fundamental field squared. This phase delay factor depends on the thickness, L_Q, and the coherence length, l_{coh}^Q, of the plate. L_a and L_g in Eq. (4) are the path length in air and the thickness of the quartz glass plate. The propagation angles of the fundamental and the SH beams in the quartz glass plate are determined by Snell's law and are indicated by ϕ_ω and $\phi_{2\omega}$, respectively. With the known indexes of refraction of air, fused quartz glass, and quartz crystal, the values of these terms can be easily calculated.

We first apply this technique to a monolayer of unprotonated retinylidene butylamine Schiff base deposited on a native glass surface to test the feasibility of the method. The Schiff base nitrogen is the most hydrophilic part of the NRB and N^+RB-HCl molecules and therefore contacts with the native hydrophilic glass surface as the molecules are deposited. We measured the interference pattern of the SH electric field generated by the NRB monolayer with that from the quartz crystal plate. The results are shown in Fig. 4, where the solid and the dashed lines represent the interference fringes obtained from the monolayer being oriented according to Fig. 1, *c* and *b*, respectively. A phase shift of 180° between these two curves is clearly observed. This indicates that the molecules in the monolayer are indeed oriented with respect to the air–glass interface. The same measurements were also made on a monolayer of protonated retinylidene Schiff base (N^+RB-HCl). Fig. 5 shows the results. However in this figure, a phase shift which deviates from 180° is found. We believe this deviation is due to the thickness of the fused quartz glass plate used for the phase compensation not being exactly equal to the monolayer substrate. Since the coherence length of quartz glass is only 24 μm, a

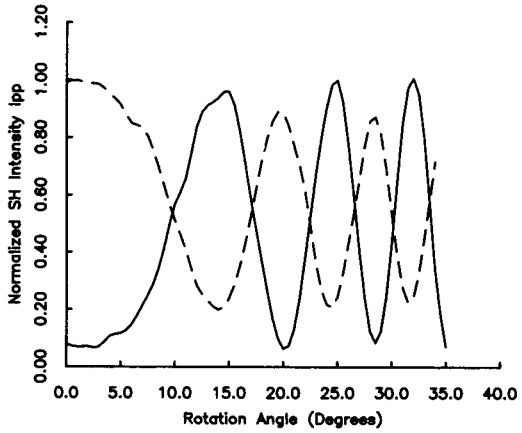

FIGURE 4 The interference pattern of the SH electric field generated from a monolayer of NRB with that from an x-cut quartz crystal is plotted with the rotation angle of the fused quartz, glass plate (see Fig. 1 *a*). (*Solid* and *dashed lines*) Results obtained from the NRB monolayer which was oriented according to Fig. 1 *c* and *b*, respectively.

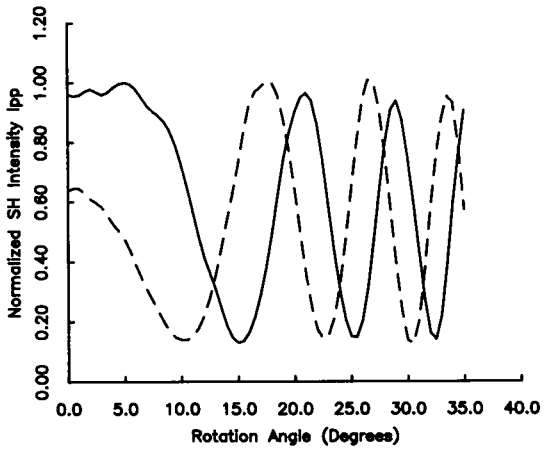

FIGURE 5 The same measurements as Fig. 4 except that the monolayer sample is N^+RB-HCl.

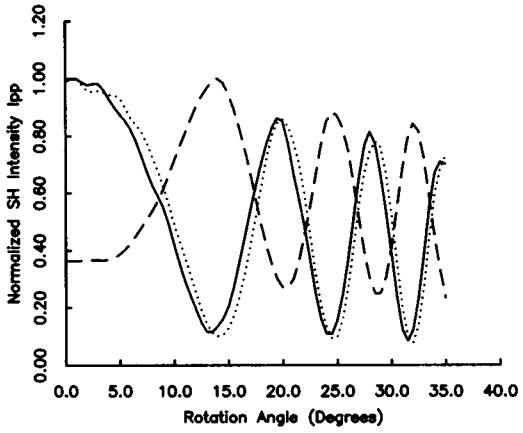

FIGURE 6 SH interference patterns of a free-standing PM-PVA film. The solid line is obtained from the arrangement in which the upper surface of the PM-PVA film faces the incident laser beam. The result with the PM-PVA film inverted is shown by the dashed line. For comparison, the SH interference pattern of a monolayer of N^+RB-HCl deposited on a quartz glass surface is also included (*dotted line*). The N^+RB-HCl monolayer was oriented to face the incident beam during the measurement.

slight difference in thickness can easily produce the observed deviation shown in Fig. 5. In support of this suggestion is the result on free standing PVA films with oriented PM to be discussed in the next section where a clear 180° phase shift is detected and where there is no need for a glass substrate or a compensating glass plate when the sample is inverted.

In our previous study of SHG in PM-PVA films, significant SH intensity was observed (7). Since the SHG process is forbidden in an isotropic medium in an electric dipole approximation, this result indicated that the purple membrane fragments must be oriented in the polymer matrix. In order to directly study this orientation, the SH interference technique was applied to these films. For this experiment, the PM-PVA films were first prepared on quartz glass substrates and then peeled from the substrates and attached to a metal frame. With this method, the phase compensation for the substrate is no longer needed. The results are shown in Fig. 6, where the solid line is the interference pattern obtained with the upper surface of the PM-PVA film facing the incident laser beam. We adopt the definition that the upper surface of the PM-PVA film is the one farthest from the glass on which it was formed. The dashed line in this figure is obtained by flipping over the film. The exact 180° phase shift shown in Fig. 6 provides clear-cut evidence that the purple membrane fragments are indeed oriented in the PVA matrix and supports our previous conclusions which was reached by the measurement of the SH intensity from a PM-PVA film (7). The interference pattern of a N^+RB-HCl monolayer on a glass substrate is also included in Fig. 6 (*dotted line*) for comparison. To obtain this interference pattern, the N^+RB-HCl monolayer is oriented to face the incident laser beam. The close resemblance of the solid and the dotted lines in Fig. 6 supports the notion that the substrate has no effect in the generation of the interference pattern in the case of the monolayer with an orientation such that the sample faces the incident beam. The lack of any phase shift between the PM-PVA and N^+RB-HCl results shown in Fig. 6 also indicate that the β-ionone ring of the bound retinylidene chromophore in a PM-PVA film points away from the substrate as it does in the N^+RB-HCl monolayer.

In order to unambiguously determine the absolute

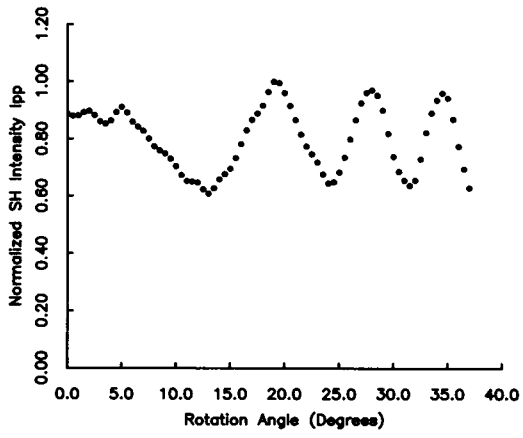

FIGURE 7 SH interference pattern of an electric field-oriented PM-dried film. The data were obtained with the PM film surface facing the incident laser beam.

orientation of the bound retinylidene chromophores in PM fragments, a PM film in which the orientations of the fragments are known is required. The electric field-oriented PM-dried film on a SnO_2 layer prepared according to Váró (19) is an ideal choice for this purpose since the electophoretic mobility of the fragments indicate that the cytoplasmic surface lies flat on the SnO_2 substrate. The SH interference pattern for an electric field-oriented PM-dried film is shown in Fig. 7. This result was obtained by letting the PM-dried film face the incident fundamental laser beam. The similarity of the SH interference pattern of the PM-dried film shown in Fig. 7 to that of N^+RB-HCl monolayer (see the dotted line in Fig. 6) clearly indicates that the β-ionone ring of the bound retinylidene chromophore of purple membrane fragments is inclined towards the extracellular surface of the fragments. The results obtained from this study are summarized in Fig. 8.

Implications of the absolute orientation of the bound retinylidene chromophore

A significant implication of our unequivocal determination of the absolute orientation of the bR chromophore is the fact that our result is in agreement with the suggestion obtained by Huang et al. (12) using chemical methods. In addition, it is interesting to try and correlate our data with recent energy transfer measurements suggesting that the chromohore is 10 ± 3 Å from the cytoplasmic side of the membrane (26). If the assumptions necessary for the energy transfer experiments are indeed correct for the donor/acceptor pairs used in this study, then either lysine 216 in the G helix has to be positioned close to the cytoplasmic side of the membrane or the lysine side chain assumes a structure that brings the chromophore close to the cytoplasmic surface even though the β-ionone ring is pointing to the extracellular surface with an angle of 25° relative to the membrane plane. Thus, if the energy transfer suggestion is correct, then our data and the information from the labeling experiments of Huang et al. (12) become somewhat difficult to reconcile with the energy transfer result.

It has been suggested that there are possibly three charges which are interacting with the polyene chain of the bound retinal chromophore (27). First, there is thought to be a negative charge near the β-ionone ring; next, there is the counter-ion of the Schiff base; and finally, there may be a third charge which is the counter-ion of the negative charge near the β-ionone ring. This positive charge appears to be located near the C_7-C_8 double bond. Our result on the absolute orientation of the retinal chromophore, therefore places additional criteria

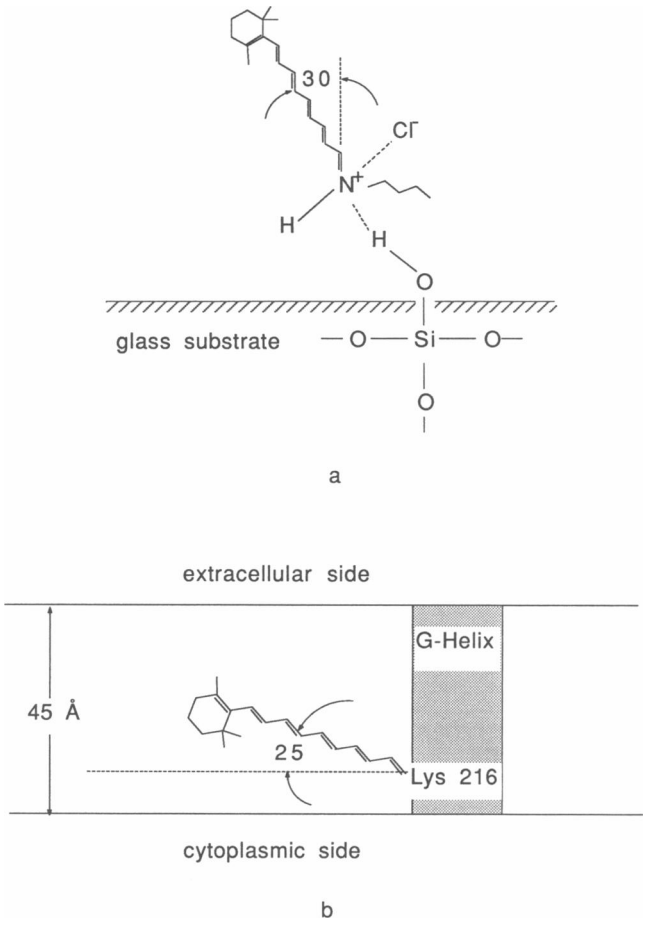

FIGURE 8 Schematic drawings that show (*a*) the adsorbed geometry of a N^+RB-HCl molecule on a glass surface, and (*b*) the *trans*-membrane orientation of the bound retinal chromophore of bR. Note our data give no information on how deep the chromophore is imbedded in the membrane. In this diagram the chromophore is placed close to the cytoplasmic surface because of the results of Otmoto et al. (26). However, as noted in the text, this result is difficult to reconcile with other data on the purple membrane.

for the three-dimensional distribution of these charged amino acid residues around the chromophore of bR.

Finally, it should be noted that the conclusion of this study is independent of the detailed *trans*-membrane structure of the α-helices of bR. Therefore this finding can be used as one of the objective criteria in order to determine the assignments of segments of the bR sequence to positions in the structural map (28).

Conclusion

In conclusion, we have used a simple method, based on the polarization and interfering properties of SHG, to determine the absolute orientation of the bound retinal chromophore of bR relative to the membrane plane. Our data

indicate that the β-ionone of the bound retinal chromophore of bR is inclined towards the extracellular surface of purple membrane with an angle of about 25° relative to the membrane plane. This information regarding the absolute orientation of the bound chromophore cannot be obtained by conventional linear optical spectrocopy. Our method, based on a SH interference technique of Kemnitz et al. (17), does not require knowledge of the detailed protein structure. Therefore the result of this study can be used as an objective criterion to help in the assignment of the helical segments in the bR sequence to positions in the structural map. This method can also be readily extended to other relevant biological and chemical applications which require the investigation of chromophore orientation between related systems.

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