A Comparison of the Second Harmonic Generation from Light-Adapted, Dark-Adapted, Blue, and Acid Purple Membrane

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ABSTRACT The second order nonlinear polarizability and dipole moment changes upon light excitation of light-adapted bacteriorhodopsin (BR), dark-adapted BR, blue membrane, and acid purple membrane have been measured by second harmonic generation. Our results indicate that the dipole moment changes of the retinal chromophore, $\Delta\mu$, are very sensitive to both the chromophore structure and protein/chromophore interactions. $\Delta\mu$ of light-adapted BR is larger than that of dark-adapted BR. The acid-induced formation of the blue membrane results in an increase in the $\Delta\mu$ value, and formation of acid purple membrane, resulting from further reduction of pH to 0, returns the $\Delta\mu$ to that of light-adapted BR. The implications of these findings are discussed.

INTRODUCTION

Bacteriorhodopsin (BR) is a unique energy-transducing protein in the purple membrane of *Halobacterium halobium*. This membrane protein has retinal as its chromophore and acts as a light-driven proton pump (Stoeckenius and Bogomolni, 1982). The photocycle and absorption spectrum of BR are sensitive to changes in both pH and ionic strength. At pH 2 or in deionized solutions, the absorption band of BR shifts from purple ($\lambda_{max} = 568$ nm) to blue ($\lambda_{max} = 605$ nm), forming what is referred to as the blue membrane (BR_{605}). Further reduction of the pH to 0 causes a blue-shift of the absorption, forming what is referred to as the acid purple membrane (BR_{565}) (Oesterhelt and Stoeckenius, 1971; Fischer and Oesterhelt, 1979). The resonance Raman spectrum of the blue membrane is very similar to that of dark-adapted BR, and the spectrum of the acid purple membrane is very similar to that of the light-adapted BR (Pande et al., 1984; Smith and Mathies, 1985). However, neither blue nor acid purple membranes produce the M state upon photoexcitation, and they do not transport protons (Chronister and El-Sayed, 1987; Mowery et al., 1979; Kobayashi et al., 1983). A molecular model that has been proposed to account for such acid-induced changes suggests that the formation of the blue membrane results from the changes in the interaction of the Schiff base and its counterion, either as a result of direct protonation of the counterion, or from a protein conformational change that increases the Schiff base counterion separation (Kriebel et al., 1979; Fischer and Oesterhelt, 1979; Mowery et al., 1979; Szundi and Stoeckenius, 1987, 1988). More recently, it has been shown that Asp-85 is protonated at low pH. This process induces the color change to give the blue form (Subramaniam et al., 1990; Otto et al., 1990; Metz

et al., 1992). Alternately, it has been suggested that the generation of BR_{565} is caused by either a protonation of a negative protein group near the β-ionone ring, or the association of a soluble anion near the Schiff base (Fischer and Oesterhelt, 1979; Szundi and Stoeckenius, 1987, 1988; Warshel and Ottolenghi, 1979). Despite extensive studies, the acid-induced structural changes in the protein and in the protein/retinal interactions that are responsible for the formation of BR_{605} and BR_{565} are still not entirely clear.

Second harmonic (SH) generation is the lowest order nonlinear optical process in which the second-order polarizability of a material is responsible for the generation of light at the SH frequency. For a conjugated chromophore like retinal, the charge transfer process provides a dominant contribution to the nonlinear polarizability, and the SH signal is a direct probe of the light-induced changes in the molecular electronic structure between the ground and the excited states (Oudar and Chemla, 1977). We have previously applied the SH generation technique to study the nonlinear optical properties of light-adapted bacteriorhodopsin, BR_{568}, and its intermediate state, M_{412} (Huang et al., 1989). Our results indicate that the dipole moment change between the ground and the excited states of BR is very sensitive to both the chromophore structure and the chromophore/protein interaction. To understand the effect of the protein environment on the excited state structure, we have also studied the nonlinear optical properties of the free retinal chromophore by SH generation (Huang et al., 1988). The results indicate that the light-induced dipole moment change of the retinal chromophore in BR_{568} is larger than that of the free chromophore. This indicates that the protein environment has a strong effect on the ground and excited states electronic structure. This conclusion is confirmed by the two-photon absorption experiment of Birge and Zhang (1990). In this paper, we extend our studies on the nonlinear optical properties of BR by investigating the SH generation of dark-adapted purple membrane (BR_{558}), blue membrane (BR_{605}), and acid purple membrane (BR_{565}). The aim of these studies is to develop a better understanding of the protein effects on the SH generation of

the retinylidene chromophore within the context of our present understandings of the molecular alterations in these species.

MATERIALS AND METHODS

Preparation of oriented purple membrane film

Purple membrane was harvested and purified from a fresh batch of *Halobacterium halobium* (S9) according to the established methods (Oesterhelt and Stoeckenius, 1974). The oriented purple membrane films were prepared by an electrophoretical method (Varo, 1981). Briefly, purple membranes were washed with deionized water several times. Approximately 300 μl of purple membrane solution with concentration of about 0.05 mM was placed between two glass plates coated with indium tin oxide (ITO) film. The glass plates were used as electrodes with the lower plate as an anode. A voltage of 4.5 V was applied to the electrodes, which are separated with a gap of 1.4 mm by a spacer. After about 1 min, purple membrane was deposited and oriented on the anode because of the negative charge of the membrane and large dipole moment associated with the membrane. The remaining solution was carefully removed with a pipette. The film was then dried in a high humidity environment to prevent cracking. The oriented film has an optical density of about 0.3 and a thickness of about 1 μm. The oriented BR film deposited on the conducting glass was covered with a micro slide to form a chamber with a spacer. The chamber was then filled with an appropriate buffer solution. The SH signal is very sensitive to the characteristics of the film such as uniformity and orientation. To minimize the effect of the film quality on the measurement, we carried out the measurement for light-adapted BR, dark-adapted BR, blue membrane, and acid purple membrane from the same film. Blue membranes were generated by changing the buffer of the soaking solution to pH 2. Acid purple membranes were generated by changing the soaking solution to pH 0 with 1 N of HCl. The films were not degraded in the buffer solution for at least 4 h.

Second harmonic generation measurement

A Q-switched Nd:YAG laser with a 10-Hz repetition rate and 10-ns pulse-width was used. The infrared beam at 1.064 μm with an average power of less than 8 mJ was used as the fundamental beam. A Glan laser polarizer and a half-wave plate were used to change the polarization state of the fundamental beam. The transmitted SH signal was measured by a cooled RCA C31034 photomultiplier tube, and the signal was averaged with a boxcar integrator. The fundamental beam was blocked by a BG5 filter and a monochromator. To minimize the error caused by possible nonuniformity of the sample, all of the measurements for the light-adapted, dark-adapted, blue, and acid purple membranes were carried out without any change of the sample and laser beam position. The detection sensitivity was calibrated against the SH intensity from a y-cut quartz plate, observed under the same experimental conditions. The details of the experimental set up have been described previously (Huang et al., 1989).

Theoretical considerations

For the retinal chromophore, the second-order molecular polarizability, β, is dominated by a single axial component $\beta_{\xi\xi\xi}$, where ξ is the long axis of the retinal. Because purple membranes are uniaxially oriented on the substrate, as a result of rotational invariance, the only two independent components of $\chi^{(2)}$ are $\chi^{(2)}_{zzz}$ and $\chi^{(2)}_{zxx}$ (Dick, 1985). The nonvanishing components of $\chi^{(2)}$ are given by (Mizrahi and Sipe, 1988; Mazely and Hetherington III, 1986)

$$\chi^{(2)}_{zxx} = \chi^{(2)}_{xzx} = \chi^{(2)}_{xxz} = \tfrac{1}{2}Nf_\omega f_\omega f_{2\omega} < \cos\Theta \sin^2\Theta > \beta_{\xi\xi\xi} \qquad (1)$$

$$\chi^{(2)}_{zzz} = Nf_\omega f_\omega f_{2\omega} < \cos^3\Theta > \beta_{\xi\xi\xi}, \qquad (2)$$

where f_ω and $f_{2\omega}$ are the local field factors at the appropriate frequencies and Θ is the angle between the molecular axis and the film normal.

The measured p-polarized SH signal as a function of the incident angle of the p-polarized fundamental beam for an oriented film of BR exhibits no observable Marker fringes and, thus, the oriented BR film behaves as if its coherence length is larger than that of the film thickness. In addition, the second harmonic generation of the film fits the thick film approximation because the magnitude of the p-polarized SH signal for an s-polarized fundamental beam, I_{sp}, for the case of the film facing the beam is similar to that of the film pointing away from the beam. In other words, the film behaves as if it has a thickness that is much larger than the wavelength, even though in our case the film thickness falls between the thick and thin film approximations. For these experimental conditions, the equation that relates the nonlinear susceptibilities defined by Eqs. 1 and 2 to the SH observed in transmission is given by

$$\frac{I(2\omega)}{I^2(\omega)} = \frac{32\pi^3}{c}|\chi^{(2)}_{zxx}|^2 p^2(\theta)(t_\omega)^4 T_{2\omega}\frac{\sin^2\Psi(\theta)}{(n^2_{2\omega} - n^2_\omega)^2}\exp[-(\alpha_\omega + \alpha_{2\omega}/2)L], \qquad (3)$$

where θ is the incident angle of the laser beam, t_ω and $T_{2\omega}$ are the Fresnel-like transmission factors, α_ω and $\alpha_{2\omega}$ are the absorption cross sections of the BR film at 1.064 and 0.532 μm, L is the thickness of the film, the *n*s are the refractive indices at the indicated frequencies of the BR film, $p(\theta)$ is a projection factor that projects the nonlinear susceptibility tensor onto a coordinate frame defined by the propagating electric fields, and $\Psi(\theta)$ is the phase difference between the bound and free wave (Jerphagnon and Kurtz, 1979).

Quantum chemical calculations have indicated that upon electronic excitation there is a dramatic charge redistribution from the β-ionone ring to the end group of the retinylidene chromophore. Charge transfer of this type provides a substantial contribution to the second-order molecular polarizability $\beta_{\xi\xi\xi}$ component along the charge-transfer direction ξ. Thus, such a system can be described by a two-level system that connects the molecular hyperpolarizability appearing in Eqs. 1 and 2 and the dipole moment change between the ground and excited states. The relation between this molecular hyperpolarizability and dipole moment change in the molecular species undergoing the charge transfer is given by (Oudar and Chemla, 1977)

$$\beta_{\xi\xi\xi} = \frac{3e^2}{2m}\frac{\omega_{ng}}{(\omega^2_{ng} - \omega^2)(\omega^2_{ng} - 4\omega^2)}f\Delta\mu, \qquad (4)$$

where ω_{ng} is the excitation energy, f is the oscillator strength, $\Delta\mu$ is the difference between the ground- and excited-state dipole moments, and ω is the incident laser frequency.

This equation, however, is valid only for the nonresonant case where both the fundamental and SH frequencies are far from ω_{ng} and the damping constant of the electronic state can be neglected. Because we used 1.06-μm laser as the fundamental beam, the wavelength of the SH photon is very close to the absorption peak of the BR molecule and the damping constant has to be included. The relation between the dipole moment change and molecular hyperpolarizability including the damping for a two-level system can be calculated by perturbation theory and is given by (Shen, 1984; Chemla and Zyss, 1987)

$$\beta_{\xi\xi\xi} = \frac{e^2}{2m\omega_{ng}}f\Delta\mu\left\{\frac{1}{(\omega_{ng} - \omega + i\Gamma)(\omega_{ng} - 2\omega + i\Gamma)}\right.$$
$$+ \frac{1}{(\omega_{ng} + \omega - i\Gamma)(\omega_{ng} + 2\omega - i\Gamma)} \qquad (5)$$
$$\left. + \frac{1}{(\omega_{ng} + \omega - i\Gamma)(\omega_{ng} - \omega + i\Gamma)}\right\},$$

where Γ is the damping constant.

The second-order susceptibility can be calculated using the above equations by measuring the polarization dependence of the SH signal I_{pp} and I_{sp}. Although the absolute value is sensitive to the approximation used, we are most interested in the relative value of molecular hyperpolarizability and the dipole moment change of BR_{568}, BR_{548}, BR_{605}, and BR_{565}. These relative values are insensitive to the approximation employed because all the measurements are taken from the same sample.

RESULTS AND ANALYSIS

The SH signal can be used to investigate the light and dark adaption processes of BR. Fig. 1 shows the light adaption process of purple membrane as monitored by the SH signal. Light was turned on at $t = 6$ min. The cross-symbols are the experimental data. The solid curve is drawn simply as a guide. Fig. 2 shows the effect of the reverse process of dark adaption on the SH signal. The open circles are the experimental data, and the solid curve is a fit of an exponential decay function with a decay constant of 30 min. To make sure that the decay in the signal is not caused by sample damage by the laser or dissolution of the film, we checked the SH signal from light-adapted BR again after the decay measurement by shining light on the film. The increase in the SH signal to the initial level that we observed clearly indicates that the decay is caused by the dark adaption process. Our results demonstrate that light-adapted BR generates a larger SH signal than that of the dark-adapted BR. The decay constant of the SH signal of ~30 min agrees well with the kinetics of the dark adaption process (Ohno et al., 1977).

The measured p-polarized SH signal as a function of the polarization of incident beam for light-adapted and dark-adapted BR is shown in Fig. 3. The plus symbols are experimental data from light-adapted BR, the open circles are from dark-adapted BR. The same measurements for blue and acid purple membranes are shown in Fig. 4, where the cross-symbols are the experimental results from blue membrane, and the open triangles are from acid purple membrane. Each data point is an average over 50 laser pulses. The results indicate that the SH signal from the acid purple membrane is larger than that of the light-adapted BR, and that the blue membrane and the dark-adapted BR have similar SH signals that are smaller than that of light-adapted BR.

Table 1 summarizes the experimental results of the polarization dependence of the SH signal of BR_{568}, BR_{558},

FIGURE 2   Dark adaption process monitored by the SH signal. Open circles are the experimental data points, and solid line is a fitting of the data with an exponential function. The decay constant from the fit is 30 min. Each data point is an average of 500 laser pulses.

FIGURE 3   P-polarized SH signal as a function of incident beam polarization for light-adapted and dark-adapted BR. Symbols: +, experimental data for BR_{568}; O, BR_{558}. Solid lines are drawn by the spline fit to the data.
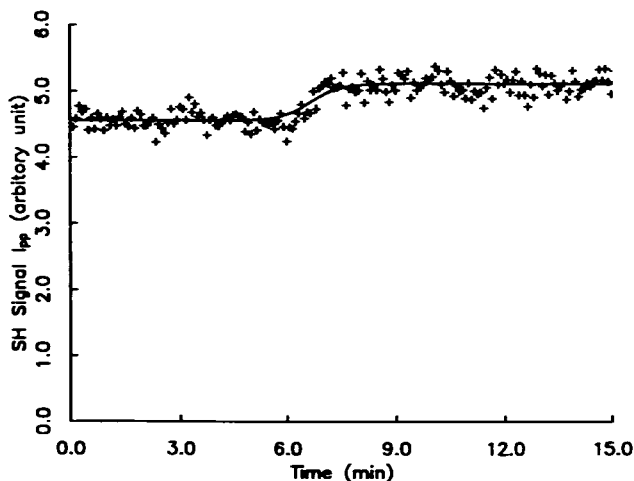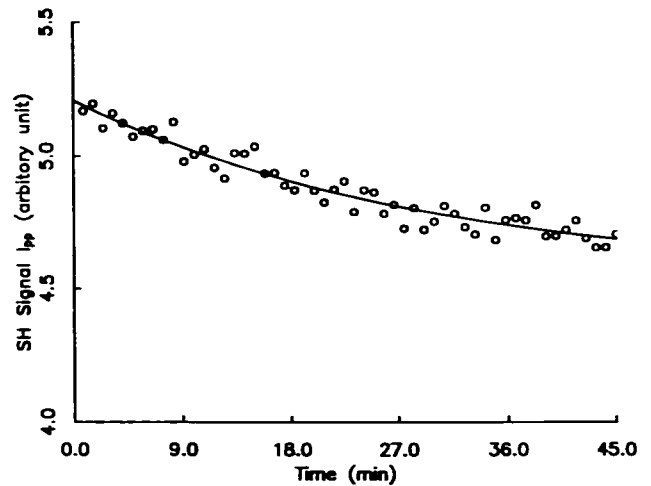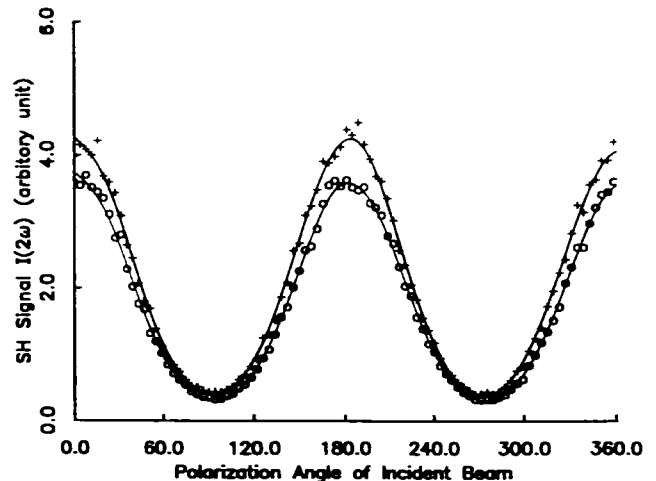

FIGURE 1   Light adaption process monitored by SH signal. Beam from a microscope lamp with an IR filter is turned on at about $t = 6$ min. Cross-symbols are the experimental data points, and the solid line is drawn as a guide. Each data point is an average of 50 laser pulses.

BR_{605}, and BR_{565} from a BR film with $OD_{568} = 0.26$. The zero signal of I_{ss} indicates that the purple membrane film is uniaxially deposited. The small but nonzero signal of I_{ps} is proportional to the square of the xyz components of the second-order susceptibility, $\chi^{(2)}_{xyz}$. This component vanishes if the film possesses a mirror plane containing the axis parallel to the film plane (Dick, 1985). Because molecules without mirror plane cannot be arranged in such a way as to give the whole ensemble mirror symmetry, the small but finite value of the I_{ps} observed indicates that the rigorous mirror symmetry is slightly relaxed for the retinal chromophore in BR. However, the signal from I_{ps} is about a factor of 100 smaller than that of I_{pp}, which supports our assumption that the dominant component of the molecular polarizability is $\beta_{\xi\xi\xi}$.

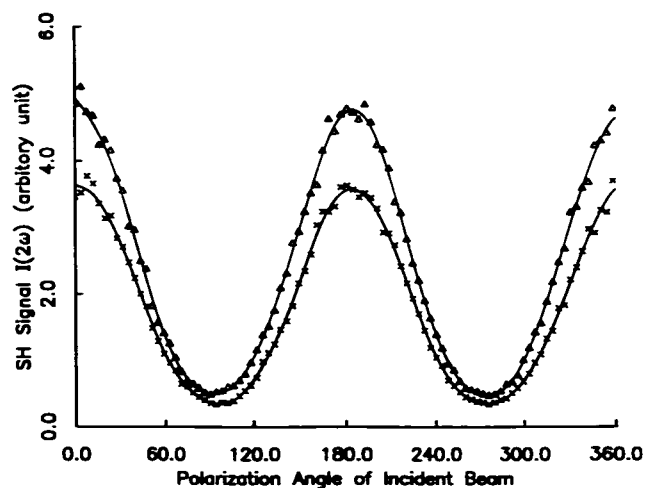From the measured value of the SH signal, the second-order susceptibility of the BR film can be calculated. To

FIGURE 4   P-polarized SH signal as a function of incident beam polarization for blue and acid purple membrane. Symbols: x, experimental data for BR$_{605}$; △, BR$_{565}$. Solid lines are drawn by the spline fit to the data.

TABLE 1   Polarization dependence of the transmitted second harmonic intensity from a BR film

| Molecule | SH signal I(2ω)/I²(ω) (10⁻²⁷ esu) | | | |
	$\dfrac{I_p(2\omega)}{I_p^2(\omega)}$	$\dfrac{I_p(2\omega)}{I_s^2(\omega)}$	$\dfrac{I_s(2\omega)}{I_p^2(\omega)}$	$\dfrac{I_s(2\omega)}{I_s^2(\omega)}$
BR$_{568}$	4.98 ± 0.13	0.52 ± 0.02	0.04	0.00
BR$_{558}$	4.22 ± 0.13	0.43 ± 0.02	0.04	0.00
BR$_{605}$	4.18 ± 0.10	0.43 ± 0.02	0.06	0.00
BR$_{565}$	5.58 ± 0.20	0.58 ± 0.03	0.05	0.00

deduce the absolute value of the molecular hyperpolarizability of the BR from the second-order susceptibility, we have to know the mosaic spread of the membrane. However, the relative value of the hyperpolarizability is not sensitive to the mosaic spread. Because the relative value is the most interesting parameter, we calculate only the relative value of the molecular hyperpolarizability. Table 2 summarizes the calculated nonlinear optical properties from the measured SH signal. The molecular polarizability is normalized to the value of the light-adapted BR. The dipole moment change is calculated from the two-level model of Eq. 5, which includes effects caused by resonance enhancement. This value is normalized to that of light-adapted BR. The damping constant Γ used is 1200 cm⁻¹, which is similar to the value obtained from the femtosecond hole-burning experiment (Mathies et al., 1988). Oscillator strengths are calculated from the respective absorption spectra.

TABLE 2   Nonlinear optical properties of light-adapted BR, dark-adapted BR, blue membrane, and acid purple membrane

Molecule	Oscillator strength	$\chi^{(2)}_{zzz}$(esu)	$\dfrac{\beta_{\xi\xi\xi}}{\beta_{\xi\xi\xi}(BR_{568})}$	$\dfrac{\Delta\mu}{\Delta\mu(BR_{568})}$
BR$_{568}$	1.02	1.16 × 10⁻⁹	1	1
BR$_{558}$	1.00	1.06 × 10⁻⁹	0.91	0.85
BR$_{605}$	1.03	1.03 × 10⁻⁹	0.89	1.12
BR$_{565}$	1.04	1.23 × 10⁻⁹	1.06	1.01

DISCUSSION

First, we will consider the SH generation from light- and dark-adapted BR. Both the nonlinear susceptibility and the dipole moment changes are larger for light-adapted BR than that for dark-adapted BR. It has been known that light-adapted BR and dark-adapted BR are different in their retinal isomer composition (Pettei et al., 1977; Sperling et al., 1977; Aton et al., 1977). The chromophore in light-adapted BR contains an all-*trans* protonated Schiff base of retinal, whereas dark-adapted BR contains a 2:1 mixture of the 13-*cis* and all-*trans* isomers (Scherrer et al., 1989). Our results indicate that the nonlinear optical polarizability and the dipole moment change of the 13-*cis* pigment are smaller than that of the all-*trans* pigment. The results can be explained by a slight difference in the extension of conjugation between the 13-*cis* and all-*trans* form. It has been known that for conjugated molecules, the dipole moment change and nonlinear polarizability increase when conjugation length increases (Oudar, 1977). Because the conjugation length is larger for the all-*trans* form than that of the 13-*cis* form, the SH signal generated from the all-*trans* isomer is larger than that of the 13-*cis* isomer. This is also consistent with the studies on polyenes (Heflin et al., 1991), where it was found that the all-*trans* isomer has a slightly larger nonlinear polarizability than the *cis* isomer.

Another possibility to explain the difference in the second harmonic signal in these two species involves different protein/chromophore interactions in the 13-*cis* and all-*trans*. It was suggested that the retinal 13-*cis* form in BR experiences a twist around the C14-C15 single bond and that this might induce a blue-shift in the spectrum of the 13-*cis* -form (Smith et al., 1987). Recently, it was suggested by molecular dynamics simulations (I. Lugonov, B. Humphrey, K. Schulten and M. Sheves, unpublished data) that the 13-*cis* form is characterized by a planar 14–15 single bond and by a smaller distance between the Schiff base positive charge and its counter ion. Such changes could also induce the alterations in the SHG that we have observed.

We now consider results from the BR$_{605}$, which is generally referred to as the blue membrane. Several lines of evidence indicate that the blue membrane has a percentage of its chromophores in the 13-*cis* configuration (Smith and Mathies, 1985; Fischer and Oesterhelt, 1979; Pande et al., 1984; Mowery et al., 1979; Gerwert et al., 1987). Despite this fact, the measured β yields a Δμ that is a factor of 1.12 larger than that of light-adapted BR and a factor of 1.3 larger than that of dark-adapted BR. Our previous studies indicated that the dipole moment is very sensitive to the protein/chromophore interactions (Huang et al., 1989).

One plausible mechanism for the change in Δμ is the change in the Schiff base and counterion interactions in these species. This is supported by the results of Albeck et al. (1989), which strongly suggest that the formation of the blue membrane is caused by an environmental change in which the counterion effect on the protonated Schiff base is reduced. Consistent with these studies are recent results

indicating that Asp-85 is protonated in the blue form (Subramaniam et al., 1990; Otto et al., 1990; Metz et al., 1992). Thus, it is now fairly well established that the blue membrane has a diminished Schiff base/counterion electrostatic interaction.

It is interesting to note that our previous SH studies indicate that dipole moment changes of the protonated Schiff base of the retinal chromophore in BR are larger than those of the free chromophore (Huang et al., 1989). The above results suggest that one possible contribution to the observed increase in $\Delta\mu$ value is the weakening of the Schiff base/counterion interaction of the chromophore complexed in the protein as compared with that of a retinal protonated Schiff base in solution (Legtenburg et al., 1986; Albeck, 1992).

We now turn to the results of the acid purple membrane species that absorbs at 565 nm. Acid purple membrane is generated by lowering the pH to 0. Previous investigators have shown that the acid purple membrane has a chromophore with an all-*trans* configuration (Smith and Mathies, 1985; Mowery et al., 1979), and the results presented in this paper indicate that the induced dipole in the acid purple form is similar to BR_{568}. One interpretation that could unify all of the above results is that the extent of the red-shift is correlated with the magnitude of the induced dipole.

These observations of changes in the induced dipole moment as a function of red- and blue-shifts in the chromophore can be explained by larger changes in the excited versus the ground state dipoles of the retinylidene moiety. This suggestion is based on the following considerations. The data on Stark effect spectroscopy of protonated retinal Schiff bases in solution (Mathies and Stryer, 1976) and the phase measurement from second harmonic generation of monolayer film of protonated retinal Schiff bases (Chen et al., unpublished results) support the view that the direction of positive charge movement upon optical excitation of the protonated retinal Schiff base is from the Schiff base nitrogen toward the β-ionone ring. On the other hand, it is known from vibrational spectroscopy (Smith and Mathies, 1985) that these red-shifts are accompanied by positive charge movement in the ground state, and the direction of this charge movement is also considered to be toward the β-ionone ring. Such changes lower the vibrational frequency of the C=C stretching mode. Therefore, if we assume that the excited state dipole does not change as a result of these spectral transitions, we would get a lowering in the induced dipole as a function of the red-shift in the acid blue species and in the initial pigment state BR_{568} versus the free retinylidene chromophore. Thus, the only remaining possibility is to assume that these red-shifts that result in an increase in the induced dipole moment of the chromophore are a result of an increase in the excited state dipole moment that is larger than any alteration in the dipole of the ground state.

CONCLUSION

We have applied the SH generation to measure the nonlinear optical properties of BR_{568}, BR_{558}, BR_{605}, and BR_{565}. Our

results indicate that the dipole moment change of the retinal chromophore in BR is very sensitive to both the structure and protein environmental change. Generation of blue membrane is accompanied by an increase in the $\Delta\mu$ value, and formation of acid purple membrane results in a return of the $\Delta\mu$ to the value of light-adapted BR. The results from the blue membrane indicate a weakening of the Schiff base/counterion interaction that results in an increases in $\Delta\mu$. Based on previous results, we argue that at the increase in $\Delta\mu$ as a function of increasing red-shift of the chromophore in the protein there is a larger increase in the excited state chromophore dipole moment as compared with changes that may be occurring in the chromophore ground state dipole.

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