Orientation of the protonated retinal Schiff base group in bacteriorhodopsin from absorption linear dichroism

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ABSTRACT Linear dichroism experiments are performed on light-adapted bacteriorhodopsin (BR_{568}) films containing native retinal (A_1) and its 3,4-dehydroretinal (A_2) analogue to measure the angle between the chromophore transition dipole moment and the membrane normal. QCFF/pi calculations show that the angle between the transition moment and the long axis of the polyene is changed by 3.4° when the C_3—C_4 bond is unsaturated. The difference vector between the two transition moments points in the same direction as the Schiff base ($N \rightarrow H$) bond for the all-*trans* BR_{568} chromophore. Because the plane of the chromophore is perpendicular to the membrane plane, a comparison of the transition moment orientations in the A_1- and A_2-pigments enables us to determine the orientation of the $N \rightarrow H$ bond with respect to the absolute chromophore ($N \rightarrow C_5$ vector) orientation. The angles of the transition moments are 70.3° ± 0.4° and 67.8° ± 0.4° for the A_1- and A_2-pigments, respectively. The fact that the change in the transition moment angle (2.5°) is close to the predicted 3.4° supports the idea that the chromophore plane is nearly perpendicular to the membrane plane. The decreased transition moment angle in the A_2-analogue requires that the $N \rightarrow H$ bond and the $N \rightarrow C_5$ vector point toward the same membrane surface. Available results indicate that the $N \rightarrow C_5$ vector points toward the exterior in BR_{568}. With this assignment, we conclude that the $N \rightarrow H$ bond points toward the exterior surface and its most likely counterion Asp-212. This information makes possible the construction of a computer graphics model for the active site in BR_{568}.

INTRODUCTION

Bacteriorhodopsin (BR) is an integral membrane protein in the purple membrane of *Halobacterium halobium* that functions as a light-driven proton pump. It contains a retinal chromophore that is attached by a Schiff base linkage to Lys-216 on helix *G*, one of seven α-helices that span the membrane (Stoeckenius and Bogomolni, 1982). Light absorption by the all-*trans* retinal chromophore initiates a femtosecond C_{13}=C_{14} double bond isomerization (Mathies et al., 1988) that is followed by a cyclic series of thermal reactions ($BR_{568} \rightarrow J \rightarrow K \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{550} \rightarrow O_{640} \rightarrow BR_{568}$). The initial double bond isomerization carries the protonated Schiff base into a new environment (Hsieh et al., 1983; Rothschild et al., 1984; Stern and Mathies, 1985). Subsequent protein relaxation and the deprotonation of the Schiff base lead to the formation of M_{412} and the release of protons to the external medium. Fourier transform infrared (FTIR) studies have assigned changes in the protonation states of nearby tyrosine (Lin et al., 1987; Roepe et al., 1987; Braiman et al., 1988*b*) and aspartate residues (Engelhard et al., 1985; Eisenstein et al., 1987; Braiman et al., 1988*a*) in the photocycle. These observations provide evidence for interactions between the Schiff base and protein residues, which play a critical role in the mechanism of the proton pump. However, information regarding the orientation of the Schiff base group in the protein is essential to determine the Schiff base-protein interactions in the various stages of the proton-pumping mechanism.

A number of experiments have been performed to get information about the orientation of the retinal chromophore in BR_{568}. The long axis of the chromophore is tilted ~20° from the membrane plane (Heyn et al., 1977; Bogomolni et al., 1977; Korenstein and Hess, 1978; Clark et al., 1980) and the plane of the chromophore is almost perpendicular to the membrane plane (Earnest et al., 1986; Ikegami et al., 1987). Energy transfer experiments by Otomo and coworkers (1988) suggest that the chromophore is closer to and tilted toward the cytoplasmic membrane surface. However, results from photoaffinity labeling experiments (Huang et al., 1982) indicate that the chromophore is tilted toward the exterior. None of these studies provide information on the absolute orientation of the $N \rightarrow H$ bond in the protein.

The orientation of the Schiff base group in BR_{568} can be determined from linear dichroism studies. Because the chromophore plane is perpendicular to the membrane plane, the $N \rightarrow H$ bond may either point toward the cytoplasm or the exterior. To determine the orientation of the Schiff base group, we have performed absorption linear dichroism experiments on BR_{568} containing native retinal (A_1) and its 3,4-dehydroretinal (A_2) analogue.

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Fig. 1 illustrates that in the all-*trans* form, the transition moment of the A_2-retinal is tilted more towards the cyclohexene ring compared with the A_1-transition moment as a result of the additional double bond between C_3 and C_4. Because the chromophore in BR_{568} has a 6-s-*trans* conformation (Harbison et al., 1985; van der Steen et al., 1986) and a $C{=}N$ anti configuration (Smith et al., 1984; Harbison et al., 1984), the $N \rightarrow H$ bond and the $C_4 \rightarrow C_3$ bond of the ionone ring point in the same direction. Thus, the angle of the A_2-transition moment relative to that of the A_1-transition moment can be used to specify the orientation of the $N \rightarrow H$ bond relative to that of the $N \rightarrow C_5$ vector.

The linear dichroism measurements presented here demonstrate that the $N \rightarrow H$ bond and the $N \rightarrow C_5$ vector point toward the same membrane surface. The available data on the orientation of the $N \rightarrow C_5$ vector are summarized and the evidence indicates that the $N \rightarrow C_5$ vector is tilted toward the exterior. Based on this assignment, we conclude that the $N \rightarrow H$ bond points toward the exterior and is directly hydrogen-bonded to its most likely counterion, Asp-212. A molecular graphics model of the Schiff base environment in BR_{568} is presented and the possible roles of other amino acids in the photocycle are discussed.

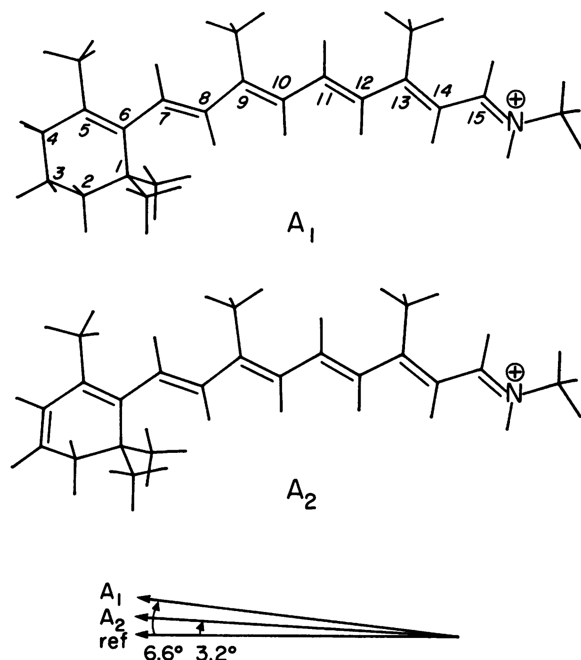

FIGURE 1 Projected structures of the 6-s-*trans* A_1- and A_2-retinal protonated Schiff bases from QCFF/pi calculations. The transition moment directions for the A_1- and A_2-chromophores are also indicated. The molecules are oriented such that the common reference axis and the transition moment vectors lie in the plane of the figure.

MATERIALS AND METHODS

Preparation of membrane films

Purple membrane was isolated from *Halobacterium halobium* (ET1001) following Braiman and Mathies (1980). Native purple membrane fragments suspended in distilled water were deposited onto a circular glass coverslip (~0.2 mm thickness) by centrifugation in a SW-28 swinging bucket rotor (Beckman Instruments Inc., Palo Alto, CA) at 15,000 rpm for 30 min (Clark et al., 1980). The coverslip rested on a cylindrically surfaced epoxy plug at the bottom of the centrifuge tube. Excess water was removed and the sample was dried in a dessicator for ~2 h so that the stacked layers of membrane fragments formed a dry film which adhered to the coverslip. To prepare the A_2-bacteriorhodopsin film, a suspension of purple membrane was bleached in 1 M NH_2OH at pH 7.8 in the dark for 12 to 20 h. The apomembrane suspension was then regenerated with 3,4-dehydroretinal and the A_2-membrane suspension was centrifuged down as described above. The absorption maximum (~595 nm) and the band shape of the light-adapted A_2-bacteriorhodopsin in distilled water was consistent with previous measurements (Marcus et al., 1977; Tokunaga and Ebrey, 1978). A regenerated A_1-bacteriorhodopsin film was also prepared as a control. The optical densities of light-adapted A_1- and A_2-films were ~0.40 o.d. corresponding to a thickness of ~1.3 μm for all of the samples (Clark et al., 1980). Finally, an apomembrane film of comparable thickness was prepared from a suspension of bleached purple membrane.

Absorbance measurements

Absorbance measurements were performed on a DW-2C spectrophotometer (SLM-Aminco, Urbana, IL). The sample compartment was fitted with a polarizer and a rotary stage on which the film samples were mounted (Fig. 2). The uncertainty in the value of the tilt angle α measured from the incident beam direction to the coverslip plane was at most $\pm 0.2°$. The compartment was humidified with air saturated at 28°C ± 1°. The high-humidity environment is essential for proper light/dark adaptation (Kouyama et al., 1985).

Absorption spectra of each sample in the 500 to 780 nm region were

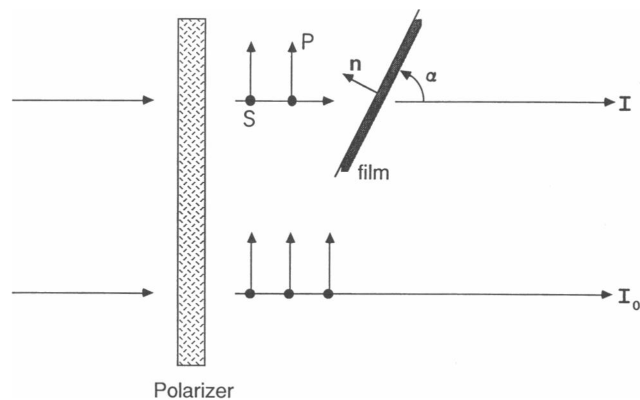

FIGURE 2 Experimental set-up for linear dichroism measurements. Arrows and dots represent the polarization of the incident beam in the plane (P) and perpendicular (S) to the plane of incidence which is also the plane of the figure. I_0 and I denote the intensities of the reference and sample beams, respectively. The film sample is rotated by angle α about an axis that is parallel to the S-polarization vector.

recorded with the incident beam polarized perpendicular (S) and parallel (P) to the plane of incidence. Measurements were made at α = 50°, 53°, 57°, 60°, 64°, 70°, 75°, and 90°. The dichroic ratio D, defined as the ratio of the P-polarized absorbance (A_P) over the S-polarized absorbance (A_S), was computed for each angle. The absorption spectra were corrected for scattering and reflection losses by subtracting the apomembrane spectra for that particular angle and polarization. This correction yielded A_P and A_S spectra which overlapped outside the absorption region (>650 nm for A_1 and >680 nm for A_2) as expected. The anomalous dispersion which contributes to a reflectivity variation and birefringence across the chromophore absorption band is unimportant. The maximum change in the refractive index across the absorption band was estimated to be ~0.025 and ~0.022 for A_1- and A_2-films, respectively, from Kramers-Kronig relations and will not lead to large intensity variation of the reflected beam. This was verified by measuring the reflectivity of each sample at selected wavelengths (488, 514, 532, 568, 647, and 676 nm) for three different angles of α. Furthermore, differential reflectivity of the two polarized beams arising from birefringence is also negligible since the maximum birefringence is calculated to be only ~0.005. Values estimated for the refractive index (n) of purple membrane range from 1.4 to 1.5 (Ebrey et al., 1977; Heyn et al., 1977; Clark et al., 1980). We used an average value of 1.45 in our calculations for the A_1- and A_2-transition moment angles. The sensitivity of the absolute angles to ±0.05 change in refractive index is ~±1.7°. However, the difference between the A_1- and A_2-transition moment angles is less sensitive to n, changing by +0.6° for n = 1.5.

The mosaic spread of the membrane planes in the sample was measured by small angle X-ray diffraction carried out with a X-ray generator (Rigaku USA Inc., Danvers, MA) and an area detector (Nicolet Instrument Corp., Madison, WI). The film samples were mounted inside a mylar chamber and hydrated at room temperature with helium at 100% relative humidity. The mosaic spread was estimated from Ω, the angular half maximal/half width of the intensity of the (001) meridional or lamellar diffraction maxima relative to the direct beam. The angular widths were found to be 4.2° ± 0.2° (1 = 3), 5.4° ± 0.3° (1 = 3), and 8.6° ± 0.4° (1 = 2) for the A_1-, regenerated A_1-, and A_2-films, respectively.

Linear dichroism analysis

The equation relating the transition moment angle Φ and the dichroic ratio D for rotational symmetry of the transition moments about the sample normal vector **n** is given by (Heyn et al., 1977; Clark et al., 1980)

$$D = 1 + \frac{3P_2(\Phi)}{n^2(1 - P_2(\Phi))}\cos^2\alpha \ , \qquad (1)$$

where $P_2(\Phi) = (3\cos^2\Phi - 1)/2$ is the second order Legendre polynomial for angle Φ measured from **n**. The magnitude of Φ is determined by plotting $D - 1$ v.s. $\cos^2\alpha$.

Mosaic spread in the stacking of the membrane fragments reduces the experimental value of $P_2(\Phi)$ and leads to an underestimation of the transition moment angle. Correction for the mosaic spread is included by assuming a nested cone arrangement of the transition moment vector and **n** (Rothschild and Clark, 1979). The correction to the measured value $P_2(\Phi)$ is given by

$$P_2(\Phi) = P_2(\Omega)\cdot P_2(\theta), \qquad (2)$$

where $P_2(\Omega)$ and $P_2(\theta)$ are the Legendre polynomials for the rms angle Ω of the mosaic spread and the corrected transition moment angle θ measured from the membrane normal, respectively.

Transition dipole moment calculations

Calculations of the π-electron structure of the 6-s-*trans* A_1- and A_2-retinal protonated Schiff bases were performed using a modified version of Warshel's QCFF/pi program (Warshel and Karplus, 1974). The direction of the electronic transition dipole moment was computed in the principal-axis system of the molecule. Because the polyene chain geometries of the two molecules are essentially identical, a best fit line through carbon atoms C_8 to C_{13} was used as the reference axis for the transition moment angle (Fig. 1).

RESULTS

The results of the QCFF/pi calculations for the electronic transition dipole moments are summarized in Fig. 1. The transition moment of A_1-retinal subtends an angle of 6.63° from the reference axis in the figure. The A_2-retinal transition moment makes a smaller 3.24° angle and is tilted more towards the ring because of the extension of the π-electron system into the β-ionone ring. The difference vector between the A_1- and A_2-transition moments points along the $C_4 \rightarrow C_3$ bond and the predicted angle difference is 3.4°.

The evidence indicates that the A_1- and A_2-retinals have very similar orientations and structures in the pigments. The computed minimized structures of the polyene chain are nearly identical in the A_1- and A_2-retinals (Fig. 1). The extra double bond in A_2-retinal slightly alters its ring conformation resulting in a ~10° larger torsion about the C_6—C_7 bond compared with that found in A_1-retinal. The net effect is to displace the positions of the ring methyls of A_2-retinal by ~0.5 Å from the positions in A_1-retinal. This is a minor change and the A_2-pigment manifests properties nearly identical to the native pigment. The 4,560 cm^{-1} opsin shift for the A_2-pigment is very close to the 4,830 cm^{-1} value for the A_1-pigment (Spudich et al., 1986). This indicates that the environment around the chromophores is very similar. Resonance Raman spectra of the light-adapted A_2-pigment indicate that ~100% of the chromophores are in the all-*trans* state (Massig et al., 1982). The A_2-pigment photocycles efficiently and forms an analogous blue-shifted M-intermediate (Tokunaga and Ebrey, 1978). The magnitude and the kinetics of proton pumping by the A_2-pigment is comparable with that of the native pigment (Tokunaga et al., 1977). These observations argue that the A_2-chromophore is bound to the protein with the same orientation as the A_1-chromophore.

Absorption spectra of A_1- and A_2-bacteriorhodopsin films for P and S polarizations are presented in Figs. 3 and 4. The absorption spectra at various tilt angles display the expected qualitative behavior based on the knowledge that the molecular transition moments lie

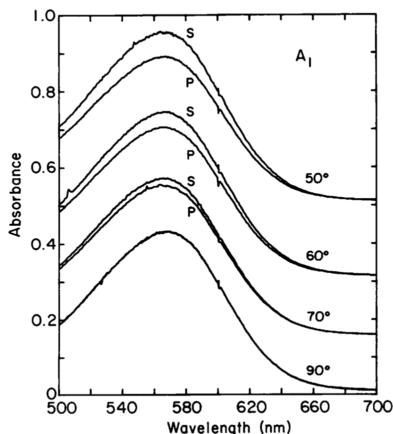

FIGURE 3 Absorption spectra of a purple membrane film containing native A_1-retinal for P- and S-polarized incident beams at α = 90°, 70°, 60°, and 50°.

close to the plane of the film. At the 90° tilt angle, the two absorption spectra are identical because the P and S polarization directions are equivalent. The overlap of the two spectra confirms that the sample plane is aligned perpendicular to the incident beam at α = 90° for both A_1- and A_2-films. As the sample is rotated about an axis perpendicular to the plane of incidence in Fig. 2, the absorbance of P-polarized light is attenuated because its projection on the sample plane is decreased. In contrast, the relative orientation between the S-polarization vector and the sample plane remains constant as the tilt angle is changed. In addition, the dichroic ratio is wavelength independent within the absorption band (not shown). This substantiates our assumption that anomalous dispersion

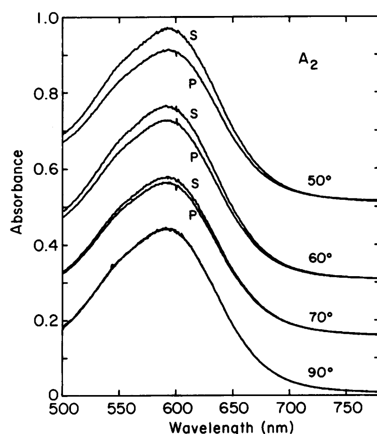

FIGURE 4 Absorption spectra of a purple membrane film bleached and regenerated with A_2-retinal for P- and S-polarized incident beams at α = 90°, 70°, 60°, and 50°.

effects are negligible in both samples (Heyn et al., 1977).

Fig. 5 presents values of D-1 in the neighborhood of 568 nm and 595 nm plotted as a function of $\cos^2\alpha$ for A_1- and A_2-films, respectively. Both plots display the predicted linear dependence. The values of $P_2(\Phi)$ computed from the slopes are -0.327 ± 0.006 for A_1-retinal and -0.276 ± 0.006 for A_2-retinal. Correcting for the mosaic spread via Eq. 2, we find that θ_{A1} equals 70.3° ± 0.4° and θ_{A2} equals 67.8° ± 0.4°. The $P_2(\Phi)$ value of the control regenerated A_1-film is -0.326 ± 0.010. This corresponds to a transition moment angle of 70.3° ± 0.6°. The agreement between the angles of native and regenerated A_1-retinal samples shows that the bleaching and regeneration procedures do not significantly modify either the protein structure or mosaic ordering. The angle of the A_1-transition moment from our experiment is consistent with earlier measurements which ranged between 68° and 71° (Heyn et al., 1977; Clark et al., 1980; Bogomolni et al., 1977; Korenstein and Hess, 1978). In addition, the 2.5° difference between θ_{A1} and θ_{A2} is close to the 3.4° predicted by QCFF/pi calculations.

To understand the linear dichroism results, it is important to realize that *a priori,* there are four possible orientations of the chromophore in the membrane—the retinal can be tilted "up" (toward cytoplasm in Fig. 6 A) or "down" (toward exterior in Fig. 6 B) and for each tilt, the Schiff base proton can also point up or down. Only two of the four are consistent with the result that θ_{A1} is larger than θ_{A2} and these are shown in Fig. 6.

DISCUSSION

The goal of this experiment is to investigate the orientation of the Schiff base group in BR_{568} by comparing the chromophore transition moment angles of pigments con-

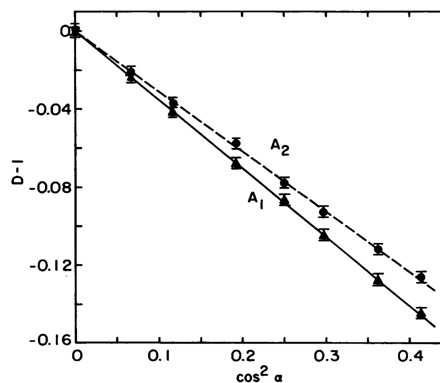

FIGURE 5 Plot of D-1 vs. $\cos^2\alpha$ for A_1 (———) and A_2 (-----) purple membrane films.

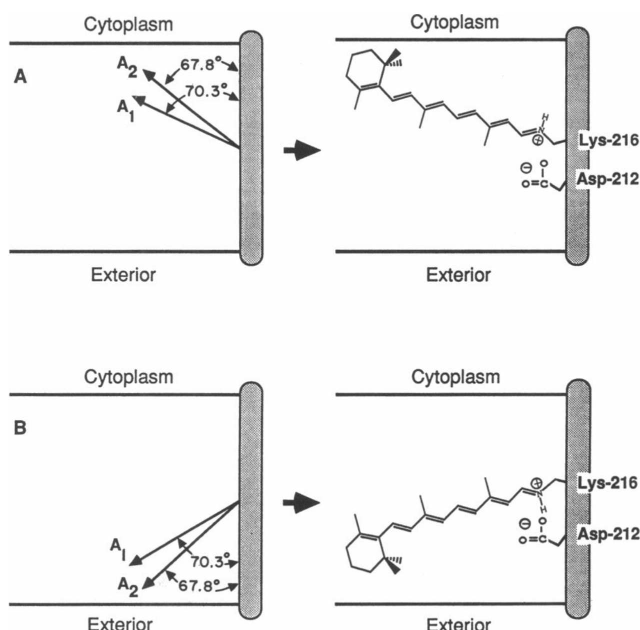

FIGURE 6 The two orientations of the Schiff base in BR_{568} that are permitted by the linear dichroism results.

taining A_1- and A_2-retinals. The linear dichroism experiments show that the transition moment angle of A_2-retinal in the purple membrane is ~2.5° closer to the membrane normal than that of A_1-retinal. This result is close to the 3.4° value predicted by theoretical calculations. This agreement is consistent with the idea that the plane of the chromophore is perpendicular to the membrane plane as has been suggested on the basis of polarized resonance Raman (Ikegami et al., 1987) and polarized FTIR experiments (Earnest et al., 1986). The fact that the A_2-transition moment angle is closer to the membrane normal constrains the possible Schiff base orientations to the two cases presented in Fig. 6. Our experiment establishes that the $N \rightarrow H$ bond and the $N \rightarrow C_5$ vector point toward the same membrane surface. The precision of our experiment is sufficient to unambiguously exclude the possibility that the $N \rightarrow H$ and the $N \rightarrow C_5$ vectors point toward opposite surfaces since this would require an A_2-transition moment orientation that differs from our present result by +5.0°. Grzesiek et al. (1989) have also recently concluded that the $N \rightarrow H$ and the $N \rightarrow C_5$ vectors point toward the same membrane surface based on neutron diffraction experiments on purple membrane (PM) films regenerated with deuterated retinals. To decide whether the Schiff base group points toward the cytoplasm or the exterior, we must know the absolute orientation of the chromophore. Experiments on this subject have yielded conflicting results, so they will now be examined in detail.

The primary evidence that the chromophore tilts toward the cytoplasm comes from fluorescence energy transfer studies on oriented PM films. Otomo and coworkers (1988) observed that energy transfer between positively charged donors/acceptors on the cytoplasmic membrane surface and the retinal was more efficient than energy transfer with the donors/acceptors on the exterior surface. From the initial fluorescence decay rate, they calculated a distance of 10 Å ± 3 between the center of the chromophore and the cytoplasmic surface. Most models of the BR folding pattern place Lys-216 near the middle of the membrane and since the retinal has a 20° tilt, the data of Otomo et al. (1988) suggests that the chromophore is tilted toward the cytoplasm (Fig. 6 *A*).

Recent experiments argue instead that the chromophore tilts toward the exterior. Leder et al. (1989) have also performed energy transfer experiments on BR in cell envelope vesicles. Analysis of the fluorescence decay rate obtained in the rapid-diffusion limit with donors in the external medium shows that the center of the chromophore is 10 Å ± 2 from the exterior surface, indicating that the chromophore is tilted toward the exterior. Leder and co-workers attribute the discrepancy between their results and those of Otomo et al. (1988) to the use of positively charged donor/acceptors in the oriented film studies. Because the cytoplasmic side of the membrane is more negatively charged, the donors/acceptors would adsorb preferentially onto the cytoplasmic surface and cause an increase in the energy transfer rate. The conclusions of Leder et al. (1989) are confirmed by Huang and Lewis (1989) who performed second harmonic generation experiments on oriented PM film. Interference of the second harmonic signals generated by the film and a reference quartz slide allowed them to determine that the ionone-ring end of the chromophore is tilted toward the exterior surface. Both studies affirm earlier conclusions from photoaffinity labeling experiments (Huang et al., 1982). Because the majority of experiments support the idea that the chromophore tilts toward the exterior, we will assume that this is the correct assignment of the tilt direction. Based on this assignment, we conclude from our linear dichroism experiment that the $N \rightarrow H$ bond points toward the exterior surface in BR_{568} (Fig. 6 *B*).

Structural model for bacteriorhodopsin

To construct a model for the active site in BR_{568}, we first assume the same folding pattern and secondary structure proposed by several authors (Huang et al., 1982; Engelman et al., 1986; Ikegami et al., 1987). Second, we assume the preferred helix assignments of Engelman et al. (1980) where helices 1–7 on the diffraction map correspond to helices A–G–F–E–D–C–B of the protein sequence. Projected density maps of purple membrane

obtained by electron microscopy (Baldwin et al., 1988; Hayward and Stroud, 1981; Henderson and Unwin, 1975) and neutron diffraction (Heyn et al., 1988) were used to position the seven α-helices as well as the chromophore. Helix *G* and the side chains of Lys-216 and Asp-212 were oriented to place Asp-212 just below the retinal Schiff base. Since the $N \rightarrow H$ bond then points toward the exterior and Asp-212, we make the reasonable inference that Asp-212 is the principal Schiff base counterion. FTIR experiments on aspartate mutants have indicated that Asp-212 is ionized in BR_{568} (Braiman et al., 1988*a*). A direct hydrogen-bonding interaction between Asp-212 and the Schiff base *N—H* group is consistent with the perturbation of *C═N* stretch frequency (Braiman et al., 1988*a*) and the absorption maximum (Mogi et al., 1988) in Asp-212 mutants. This is not surprising since Asp-212 is located four residues (~6 Å) below Lys-216. Experiments also support interaction of the Schiff base with Tyr-185 and Asp-85. Mutants involving these residues exhibit shifts in the *C═N* frequency (Braiman et al., 1988*a*, *b*) and the absorption maximum (Mogi et al., 1987, 1988). Thus, helices *C* and *F* were rotated to orient Asp-85 and Tyr-185 toward the retinal Schiff base.

In Fig. 7, we present a molecular graphics model of BR_{568}. The ionone ring end of the chromophore is tilted at an angle of ~20° toward the exterior and the $N \rightarrow H$ bond points toward the exterior. This places the carboxylate of Asp-212 ~3 Å below the Schiff base which is a reasonable distance for an ion-pair interaction. Tyr-185 is also sufficiently close to interact with Asp-212 and may be involved in a dipolar stabilization of the Schiff-base/Asp-212 ion pair. Low temperature FTIR experiments have argued that Tyr-185 is ionized in BR_{568} (Lin et al., 1987; Roepe et al., 1987; Braiman et al., 1988*b*). However, this prediction is in disagreement with recent solid-state ^{13}C-nuclear magnetic resonance results (Griffin et al., 1989). If Tyr-185 is not ionized, the FTIR and mutant data indicate that it must be in an unusual hydrogen-bonding environment as in Fig. 7. Extended interactions between Asp-212, Tyr-185, and the Schiff base may contribute to the weak hydrogen-bonding of the Schiff base that is observed in BR_{568} (Harbison et al., 1983).

The main difference between our model of the active site in BR_{568} and that of Braiman et al. (1988*a*) is the *N—H* bond orientation. We have oriented the $N \rightarrow H$ bond toward the exterior as required by the linear dichroism results. Braiman and co-workers adopted the opposite $N \rightarrow H$ orientation and postulated a direct interaction of the *N—H* group with the aromatic ring of the ionized Tyr-185. Such an arrangement of the interacting groups is not possible with the $N \rightarrow H$ bond pointing toward the exterior. However, many of the other features of the model in Fig. 7 such as the chromophore pocket formed by Trp-182, and Trp-189, and the relative locations of Asp-85, Asp-212, and Tyr-185 are very similar to those originally proposed by Braiman et al. (1988*a*).

Now that a more accurate structural model for the active site in BR is available, it can be examined to develop an hypothesis for the mechanism of the proton pump. In particular we will use this structure along with results from the elegant FTIR studies on BR mutants (Braiman et al., 1988*a*, *b*) to suggest identities for the residues in the recently proposed "*C—T* Model" for the

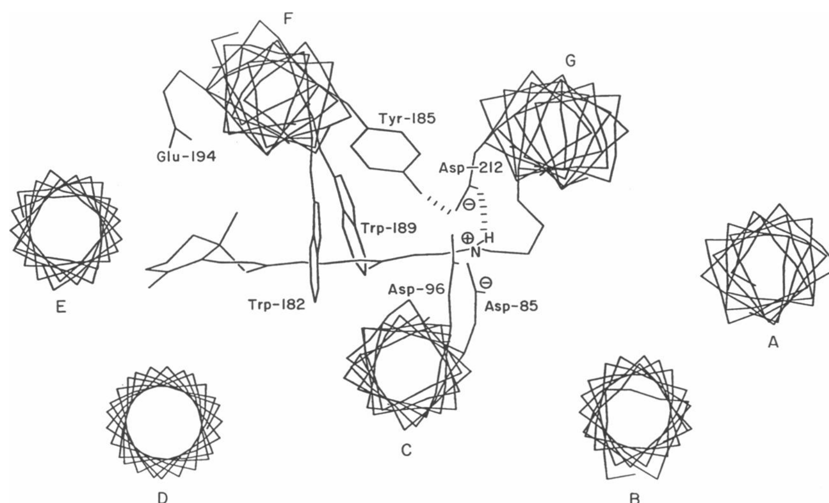

FIGURE 7 View from the cytoplasmic side of a molecular graphics model of BR_{568}. The ionone ring end of the chromophore is tilted ~20° toward the exterior and the $N \rightarrow H$ bond points to the exterior and Asp-212. Asp-85 is on the opposite side of the chromophore binding pocket from Asp-212. Tyr-185 can hydrogen-bond to Asp-212 while Asp-96 is too far up helix *C* to interact directly with the Schiff base.

proton pump (Fodor et al., 1988). In BR_{568}, the chromophore is hydrogen-bonded to its counterion (A_1^-) which is assigned to Asp-212. Upon light absorption, the retinal isomerizes to its 13-*cis* structure. Assuming that the position of the ring end of the chromophore is fixed, the photoisomerization carries the Schiff base away from Asp-212 and breaks the Tyr-185/Asp-212/Schiff-base interaction. This may account for the changes seen in the Tyr-185 FTIR signals in the BR $\rightarrow$ K transition (Lin et al., 1987; Roepe et al., 1987; Braiman et al., 1988 *b*). It is not known in which clockwise sense the isomerization occurs. The N—H group could swing toward helix G (and Tyr-185) or toward helix C and Asp-85. Reorientation of the Schiff base toward Asp-85 is supported by observations that substitution of Asp-85 by an asparagine abolishes proton pumping activity (Mogi et al., 1988) and that Asp-85 is protonated in the M state (Braiman et al., 1988*a*). In the K and L intermediates the Schiff base is hydrogen-bonded to Asp-85 which is assigned as A_2^- in the C—T model. In the $L \rightarrow M$ transition the Schiff base proton is transferred to A_2^- (Asp-85) as suggested by Braiman et al. (1988*a*). The key feature of the C—T model is the use of an isomerization-induced protein conformational change ($T \rightarrow C$) to permit the reprotonation of the Schiff base by a residue other than Asp-85. Direct reprotonation of the Schiff base by Asp-212 seems unlikely since the isomerization has just carried the N—H group away from Asp-212 and oriented it toward the cytoplasm. Thus, we propose that the $T \rightarrow C$ conformational change reorients or shifts the unprotonated Schiff base group away from Asp-85 and toward the cytoplasm so that it can pick up a proton to form the N intermediate (Fodor et al., 1988). It is likely that Asp-96 is one of the residues that feeds a proton to the Schiff base because mutation of Asp-96 to asparagine retards M decay (Holz et al., 1989) and suppresses proton-pumping (Mogi et al., 1988). However, because Asp-96 is quite distant from the chromophore this protonation must be facilitated by intervening residues such as Thr-89 or perhaps Tyr-185. After reprotonation, a thermal 13-*cis* $\rightarrow$ 13-*trans* isomerization occurs in a concerted fashion with the reversion of the protein from the C-form to the T-form in the $N \rightarrow O$ transition. The isomerization swings the Schiff base back toward Asp-85 which may be the counterion in O. Additional experiments are needed to identify the chromophore counterion in the N and O intermediates.

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