Distorted Structure of the Retinal Chromophore in Bacteriorhodopsin Resolved by ^{2}H-NMR[†]

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ABSTRACT: Structural details about the geometry of the retinal chromophore in the binding pocket of bacteriorhodopsin are revealed by measuring the orientations of its individual methyl groups. Solid-state ^{2}H-NMR measurements were performed on macroscopically oriented samples of purple membrane patches, containing retinal specifically deuterium-labeled at one of the three methyl groups along the polyene chain (C_{18}, C_{19}, C_{20}). The deuterium quadrupole splitting of each "zero-tilt" spectrum is used to calculate the orientation of the corresponding $C-CD_3$ bond vector with respect to the membrane normal; however, two possible solutions may arise. These ambiguities in angle could be resolved by recording a tilt series of spectra at different sample inclinations to the magnetic field and analyzing the resulting complex line shapes with the aid of computer simulations. The angles for the C_{18}, C_{19}, and C_{20} group are found to be 37 ± 1°, 40 ± 1°, and 32 ± 1°, respectively. These highly accurate values imply that the polyene chain of the retinal chromophore is not straight but rather has an in-plane curvature and possibly an out-of-plane twist. Together with the angles of the remaining methyl groups on the cyclohexene ring that have been measured previously, an overall picture has thus emerged of the intramolecular conformation and the three-dimensional orientation of retinal within bacteriorhodopsin. The deduced geometry confirms and refines the known structural information on the chromophore, suggesting that this ^{2}H-NMR strategy may serve as a valuable tool for other membrane proteins.

Integral membrane proteins are not routinely amenable to crystallization studies or multidimensional high-resolution NMR methods. Alternative strategies are therefore required to resolve structural details about an active site or other region of interest. Here, a solid-state deuterium NMR (^{2}H-NMR)[1] approach has been developed with the aim of determining the structure of a specifically deuterated ligand or prosthetic group within a membrane protein. The selective deuterium labeling constitutes an entirely nonperturbing approach, since the isomorphic replacement does not affect the structure or function of the system. The spectral analysis is based on the known anisotropy of the deuterium nucleus, from which the orientation of the carbon–deuterium bond vector can be calculated with respect to the normal direction of a uniaxially oriented membrane sample. From a set of several such bond vectors, it should be possible to resolve the three-dimensional structure of the complete molecule in the binding site, without recourse to any other structural information on the system.

The ^{2}H-NMR method has been developed around, and is now being applied to, the retinal chromophore of bacteriorhodopsin (BR) from the purple membrane of *Halobacterium salinarium*, which functions as a light-driven proton pump. In this investigation, the three methyl group along the polyene chain of retinal were individually deuterium-labeled and their

$C-CD_3$ orientations determined from the ^{2}H-NMR spectra of oriented samples. The structural details obtained by this method are thus complementary to the results from neutron scattering experiments, which have localized the positions of selectively deuterated retinal segments in the membrane (Seiff et al., 1985, 1986; Heyn et al., 1988; Hauss et al., 1990; Hauss, 1993). A three-dimensional model of the protein is available from electron microscopy to a resolution of 2.7 Å in-plane (Henderson et al., 1990), and other biophysical methods have provided various structural details about the chromophore (Heyn et al., 1977; Earnest et al., 1986; Lin & Mathies, 1989; Copie et al., 1990; Creuzet et al., 1991; Fahmy et al., 1991). It is thus possible to compare the ^{2}H-NMR results with the known information on the system, in order to demonstrate the reliability of the method and assess its particular strengths and shortcomings.

The angles of the three methyl groups on retinal obtained from the ^{2}H-NMR measurements here (37 ± 1°, 40 ± 1°, and 32 ± 1°, for C_{18}, C_{19}, and C_{20}) are in a range of high accuracy, in which the quadrupole splitting is very sensitive to small changes in orientation. Since these three methyl groups are found to be not parallel to one another, it is concluded that the polyene chain of retinal must be distorted by an in-plane curvature and possibly an out-of-plane twist. The present results follow our initial studies of a multiply labeled cyclohexene ring (Ulrich et al., 1992a), a preliminary investigation on C_{20} (Ulrich et al., 1992b), and the development of the necessary line-shape analysis program (Ulrich & Watts, 1993), thus concluding the complete structure determination of retinal in the dark-adapted ground state of BR. The same ^{2}H-NMR approach should further constitute a promising method to monitor the light-induced small changes in the orientation of the chromophore which are expected to occur in the course of the photocycle (Hauss et al., 1993).

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[1] Abbreviations: ^{2}H-NMR, deuterium nuclear magnetic resonance; BR, bacteriorhodopsin; PM, purple membrane; $\Delta\nu_Q$, quadrupole splitting.

METHODS

The synthesis and characterization of retinal analogues that are selectively deuterated in one of the C_{18}, C_{19}, and C_{20} methyl groups (see Figure 4 for numbering scheme) have been described (Lugtenburg, 1985; Heyn et al., 1988; Groesbeek, 1993). Incorporation into BR was achieved by growing a retinal-deficient strain of *H. salinarium* in the presence of the synthetic retinal (Seiff et al., 1985). Macroscopically oriented protein samples were prepared on small glass supports, by controlled evaporation at 85% humidity of a concentrated purple membrane (PM) suspension in deuterium-depleted water (Seiff et al., 1986; Ulrich et al., 1992a; Hauss, 1993). In these multilayer membrane films, the PM patches are oriented parallel to the support and are randomly distributed within the plane. Diffraction measurements have revealed a narrow mosaic spread (typically ±7° for a single plate) for the distribution of the local membrane normals around the support normal (Seiff et al., 1985, 1986; Heyn, 1988; Hauss et al., 1990). In such uniaxially oriented samples, all the C–CD$_3$ bond vectors are uniformly distributed on a cone which is characterized by a certain opening angle around the support normal as the cone axis. For the NMR measurements a stack of 14–18 of these sample plates was fitted longitudinally into a 10-mm NMR tube and maintained at 85% humidity by a small reservoir of saturated KCl solution, and a few control experiments were also carried out at 75% (NaCl solution) and 98% humidity (K$_2$SO$_4$ solution). The angle between the stack of plates and the spectrometer magnetic field could be varied by rotating the NMR tube manually in the horizontal solenoid. All ^{2}H-NMR spectra were recorded at 61.4 MHz on a Bruker MSL 400 spectrometer, using a quadrupole-echo pulse sequence with a $\pi/2$ pulse width of around 7 μs, echo delay times of 20–30 μs, and a repetition time of 200 ms. Typically, spectra were acquired over 14 h, processed with an exponential multiplication of 2 kHz, and symmetrized in order to improve the signal-to-noise ratio.

Immobilized proteins in uniaxially oriented membrane samples give rise to highly characteristic ^{2}H-NMR spectra, when labeled at one specific position such as an individual methyl group. The deuterium quadrupole splitting and the spectral line shapes contain information about the bond vector that can be used for the structure determination (Lee & Oldfield, 1982; Opella & Stewart, 1989; Ulrich & Watts, 1993). Note that, due to the rapid spinning of the methyl group in the otherwise immobilized sample, the orientation of the three deuterium atoms are time-averaged and the effective bond vector corresponds to the methyl-rotor axis. Generally, there exists a simple relationship between the spectral quadrupole splitting $\Delta\nu_Q$ and the angle θ of a C–CD$_3$ bond vector with respect to the magnetic field direction (Seelig, 1977):

$$\Delta\nu_Q = (3\cos^2\theta - 1)(40 \text{ kHz}) \qquad (1)$$

The scaling factor of 40 kHz was independently determined at –60 °C from the powder spectrum of a random dispersion of a deuterated PM sample (Ulrich et al., 1992a). This value was furthermore confirmed in the simulations of the ^{2}H-NMR tilt series for each of the three labeled methyl groups, according to the total spectral width and line-shape arguments discussed in Ulrich and Watts (1993).

Simple ^{2}-NMR spectra that can be readily interpreted are observed when the uniaxially oriented sample is aligned with its normal parallel to the spectrometer magnetic field direction ("zero-tilt" spectrum). These spectra consist of a pair of resonances, separated by a certain quadrupole splitting which is determined by the orientation of the methyl group on the protein. In this experimental geometry, all the labeled bond vectors make the same angle θ with the sample normal and therefore also with the spectrometer field direction. All deuteriomethyl groups thus contribute the same quadrupole splitting $\Delta\nu_Q$ to the overall spectral line shape. In practice, when the alignment of the membrane fragments in the sample plane is less than perfect, the mosaic spread of the sample gives rise to a certain degree of spectral line broadening (Ulrich & Watts, 1993).

From the quadrupole splitting in the zero-tilt spectrum of a single labeled methyl group it should be straightforward to calculate the angle of that group relative to the membrane normal, using eq 1. However, only the absolute value and not the sign of the quadrupole splitting is measured from the spectrum, and positive and negative splittings cannot be discriminated. Therefore, two solutions to eq 1 are obtained whenever $\Delta\nu_Q < 40$ kHz, which corresponds to the range of angles between 35° and 90°. To resolve this ambiguity, we have recently developed a strategy by which the value of the angle θ can be extracted uniquely from a series of measurements of the oriented sample at different inclinations to the spectrometer field (Ulrich & Watts, 1993). The line shapes of such a tilt series are considerbly more complex than the simple zero-tilt spectrum described above, because the bond vectors are distributed over a range of different angles. Consider a cone of methyl group bond vectors being progressively tilted with respect to the reference direction of the spectrometer field. Depending on the cone opening angle θ, at any particular alignment relative to the field, the bond vectors then assume a discrete range of angles. They all contribute their respective quadrupole splitting to the overall spectral line shape, which can be computed by summing up these overlapping contributions weighted by their corresponding probability density (Ulrich & Watts, 1993). A full tilt series can thus be analyzed quantitatively with the aid of computer simulations or qualitatively by eye, revealing both the value and the sign of the quadrupole splitting, from which the angle is unambiguously defined between 0° and 90° with respect to the membrane normal.

RESULTS

The ^{2}H-NMR spectra from three different BR samples (approximately 100 mg each, dark adapted) are shown in Figure 1, where the retinal is selectively deuterated in the C_{18}, C_{19}, or C_{20} methyl group, respectively. The uniaxially oriented PM patches were measured with the membrane normal parallel to the spectrometer magnetic field, at –60 °C. Measurements at room temperature gave essentially the same spectra but with a poorer signal-to-noise ratio, and the line shapes were obscured by an isotropic resonance from the residual deuterium in the water. In each of the zero-tilt spectra in Figure 1, two broad resonances are seen with a respective quadrupole splitting (absolute value) of 36 kHz (for C_{18}), 30 kHz (for C_{19}), and 46 kHz (for C_{20}). The initial step in the analysis now consists of calculating the corresponding value(s) for θ from eq 1. In the case of the C_{20}-labeled retinal with a (46 ± 1)-kHz splitting, the angle between the C–CD$_3$ vector and the membrane normal is found to be $\theta = 32 \pm 1°$. This solution is unambiguous, because the quadrupole splitting is larger than 40 kHz and must therefore be positive. The other two quadrupole splittings of 36 and 30 kHz from the C_{18} and C_{19} deuteriomethyl groups, on the other hand, each give rise to two possible bond angles, depending on the sign of the splitting
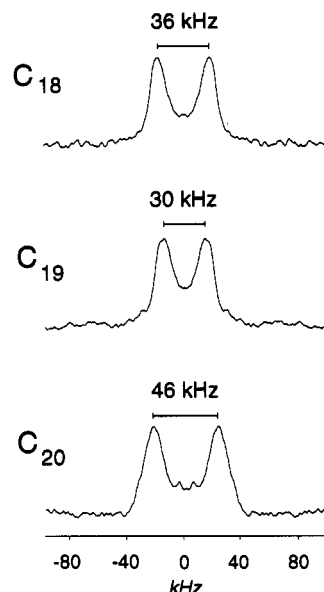
FIGURE 1: ^{2}H-NMR spectra from three different BR samples containing retinals with the individually deuterium-labeled methyl groups C_{18}, C_{19}, and C_{20}. The macroscopically oriented membrane samples were measured at −60 °C with their normal parallel to the spectrometer magnetic field, so that all the labeled bond vectors are uniformly distributed on a cone around the field direction. The quadrupole splittings of these zero-tilt spectra are then used for evaluation of the methyl group orientations.

which is not known. That is, with a measured $\Delta\nu_Q$ of 36 kHz a bond angle of $\theta = 37°$ or $79°$ is calculated for the C_{18} group, and a $\Delta\nu_Q$ of 30 kHz for C_{19} gives $\theta = 40°$ or $73°$, for positive or negative splittings, respectively.

The correct bond angle can be discriminated by measuring a tilt series of ^{2}H-NMR spectra, as illustrated in Figure 2. The experimental data from the C_{19} deuteriomethyl group are given in the middle column, at seven different sample inclinations (α, angle between sample normal and spectrometer field direction) from 0° to 90° in the spectrometer. For comparison, the line shape predicted by computer simulation are shown on either side of the experimental tilt series, with the simulation for $\theta = 40°$ in the left-hand column and for $\theta = 73°$ in the right-hand column. Note that only the zero-tilt simulations at $\alpha = 0°$ in the top row are indistinguishable, regardless of the sign of the 30-kHz quadrupole splitting, which is positive in the left column ($\theta = 40°$) and negative in the right column ($\theta = 73°$). Across the whole tilt series in Figure 2, a close resemblance is seen between the experimental spectra in the middle column with the simulated line shapes on the left ($\theta = 40°$) but not with those on the right ($\theta = 73°$). Therefore, the angle θ is uniquely identified to be $40 \pm 1°$ for the deuterated C_{19} group on retinal. A similar analysis for the C_{18} group ($\Delta\nu_Q = 36$ kHz) yields an angle of $37 \pm 1°$, and the other possible value of $79°$ is rejected. The experimental spectra of that tilt series plus those of C_{20} ($\Delta\nu_Q = 46$ kHz, $\theta = 32°$, see above) are shown in Figure 3, together with their best-fit line-shape simulations superimposed.

The analysis of a full tilt series of ^{2}H-NMR spectra not only allows the determination of the unique bond angle for a deuterated methyl group but also provides an internal check for the consistency of the spectral interpretation. In particular, simulations provide a means for the analysis of line-broadening effects, which arise from the sample mosaic spread as well as the intrinsic line width of the nuclear transition and instrumental factors. When line shapes are fitted to a full tilt series of spectra in a concerted manner and are also compared with
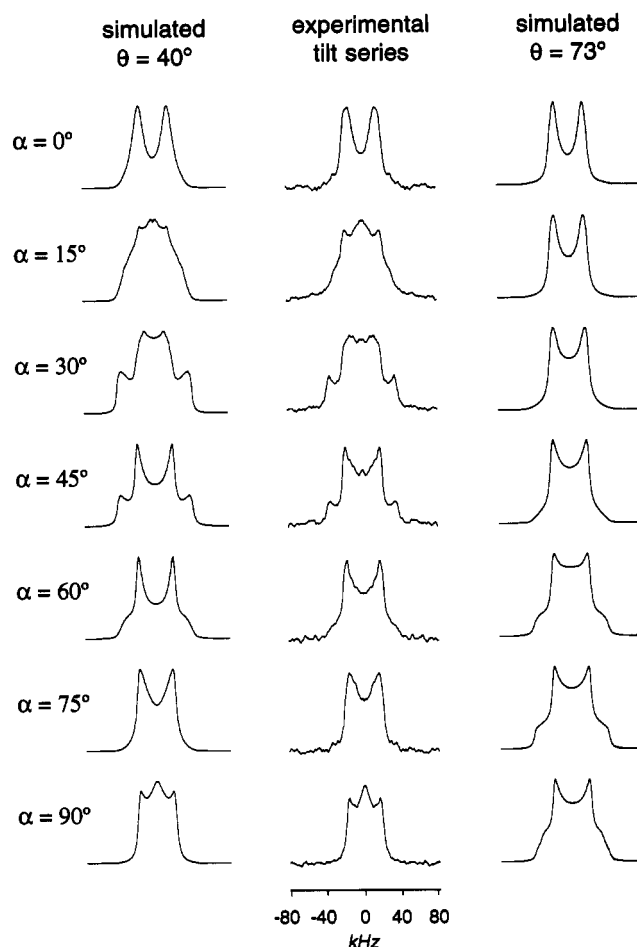

FIGURE 2: Tilt series of ^{2}H-NMR spectra from the C_{19} deuteriomethyl group of retinal in oriented purple membranes, recorded at seven different inclinations (α) of the sample normal relative to the spectrometer field. Both the experimental data (middle column) and the two simulated series (outer columns) are characterized by an absolute quadrupole splitting of 30 kHz in the zero-tilt spectrum ($\alpha = 0°$, top row). The simulations on the left are based on a methyl group angle of $\theta = 40°$ which corresponds to a positive splitting, while the simulations on the right are based on $\theta = 73°$ with a negative quadrupole splitting. From the close similarity of the left column with the experimental line shapes, it is concluded that the correct angle for the C_{19} methyl group is $40 \pm 1°$, while the other possible solution is rejected.

the powder spectrum of an unoriented sample, the different contributions can be discerned (Ulrich & Watts, 1993). In that way an intrinsic line width of around 2 kHz is found for the spectra shown here, together with a mosaic spread between $\pm 8°$ and $\pm 10°$ for the three samples. The mosaic spread was independently verified by ^{31}P-NMR measurements of the phospholipid component in one of the oriented ^{2}H-NMR samples (Ulrich et al., 1992a), and it compares well with the results from neutron diffraction experiments on similarly prepared PM films (Seiff et al., 1985, 1986; Heyn, 1988; Hauss et al., 1990). On the basis of these independent values of the two line-broadening parameters, the simulated line shapes shown in Figures 2 and 3 were generated by varying only the sample inclination α. From the good fit to the experimental spectra it is thus evident that the one characteristic angle θ describes the whole tilt series and that the underlying cone model for the bond vectors is consistent. The accuracy of the angles determined here is estimated to lie within $\pm 1°$, since the range around 45° is particularly sensitive as is seen from eq 1. A small change of 1° in θ would lead to a significant change in the quadrupole splitting of around 2 kHz in the zero-tilt spectrum. When the labeled segment

C$_{18}$ C$_{20}$

$\alpha = 0°$

$\alpha = 15°$

$\alpha = 30°$

$\alpha = 45°$

$\alpha = 60°$

$\alpha = 75°$

$\alpha = 90°$

-80 -40 0 40 80   -80 -40 0 40 80
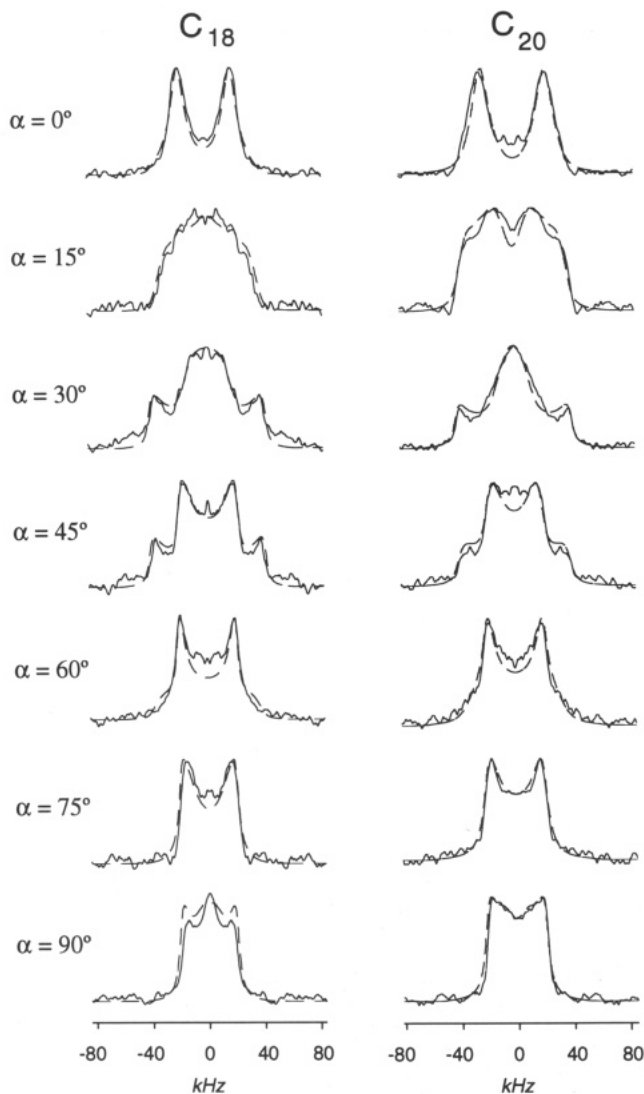
kHz   kHz

FIGURE 3: Tilt series of ^{2}H-NMR spectra from the deuteriomethyl groups C$_{18}$ (left column) and C$_{20}$ (right column) of retinal in oriented purple membranes, at seven different sample inclination (α) in the spectrometer field. The line-shape simulations are shown superimposed over the experimental spectra to illustrate the good line fit obtained by the prediction method, which utilizes two discrete line-broadening parameters to account for the mosaic spread and the intrinsic line width. The angles of the C$_{18}$ and C$_{20}$ methyl groups with respect to the membrane normal are found to be 37 ± 1° and 32 ± 1°, respectively, and the mosaic spread lies within ±10°.

is undergoing any small oscillations with a correlation time of less than $1/\Delta\nu_Q \approx 10^{-5}$ s, the angle obtained represents the time-averaged orientation of the methyl group.

DISCUSSION

The angles that have been determined for the C$_{18}$, C$_{19}$, and C$_{20}$ methyl groups on retinal provide information about the orientation of the polyene chain in the protein. The methyl groups on the cyclohexene ring have been studied previously, using a sample labeled simultaneously at C$_{16}$, C$_{17}$, and C$_{18}$ (Ulrich et al., 1992a). In particular, it was found that the two geminal methyl groups, C$_{16}$ and C$_{17}$, lie approximately horizontally in the membrane plane, with angles θ around 95° and 75°. The C$_{18}$ methyl group had also been measured as part of the multiply labeled sample (with an intensity of only two out of eight deuterons) by deconvolution of the overlapping spectral line shapes. The angle initially estimated for C$_{18}$ of around 46° deviates noticeably from the correct value of 37 ± 1° obtained by the direct measurement presented here.
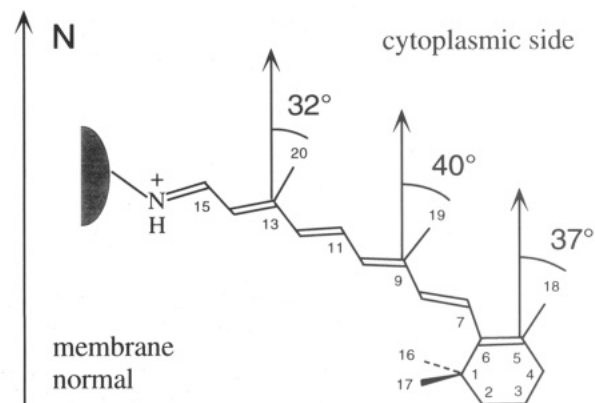

FIGURE 4: Orientation and conformation of retinal in BR, constructed from the individual methyl group orientations that have been determined by solid-state ^{2}H-NMR. The θ angles of the C–CD$_3$ bond vectors with respect to the membrane normal (N) were evaluated for C$_{18}$ (37°), C$_{19}$ (40°), and C$_{20}$ (32°) from the zero-tilt spectra shown in Figure 1 and with the aid of line-shape simulations of the tilt series in Figure 2 and 3. The 6(S)-*trans* structure of the polyene chain relative to the cyclohexene ring is evident. The difference between the angles of the three methyl groups along the polyene chain indicates that the chromophore possesses a curvature within the plane of the conjugated system and possibly a twist. The roughly horizontal orientations of the two methyl groups C$_{16}$ and C$_{17}$ make the molecular plane of the chromophore approximately vertical, which in turn defines the average inclination of the molecular long axis of retinal in BR.

Despite the intrinsic reliability of the ^{2}H-NMR approach, and as a direct consequence of its high angular sensitivity, this discrepancy arose from the underlying assumption in the initial study of a perfectly tetrahedral or trigonal geometry around the sp^3- or sp^2-hybridized carbon atoms. On the basis of this assumption, the line-shape deconvolution and the modeling of the initial cyclohexene ring structure had been performed with due regard for the ring puckering, but any distortion of the local carbon geometries would not have been reflected in the analysis. Therefore, for an exact structural characterization of any molecular framework, even a rigid one, it is essential that methyl groups are *individually* labeled and analyzed as has been done here. As an overall control, the complete procedure with the C$_{18}$-labeled retinal was repeated with a second sample, and in both independent experiments a value of 37 ± 1° was obtained, suggesting a high reproducibility.

Figure 4 illustrates how all the different methyl group orientations with respect to the membrane normal N are accommodated in space by the proposed structure of retinal within BR. This picture is clear from the measured values of θ, which are indicated as labels to the individual methyl groups. The roughly parallel orientations of the two methyl groups, C$_{18}$ (θ = 37°) and C$_{19}$ (θ = 40°), demonstrate that retinal must have a 6(S)-*trans* conformation rather than a 6(S)-*cis* when bound to bacteriorhodopsin. That is, a 180° rotation around the C$_6$–C$_7$ bond, which has a substantially lowered energy barrier compared to the other single bonds of the conjugated system, would produce a structure that is incompatible with the measured angles. This conclusion confirms previous solid-state NMR studies that have proposed a 6(S)-*trans* chromophore from comparison with crystalline model compounds (Copie et al., 1990; Creuzet et al., 1991). Focusing now on the specific angles of the three methyl groups, C$_{18}$, C$_{19}$, and C$_{20}$, along the polyene chain, it is apparent that the chromophore backbone cannot be perfectly straight. In an undistorted system of conjugated double bonds, these methyl groups would be expected to lie exactly parallel; however, in this case their individual orientations are not the same with

respect to the membrane normal. In particular, the two neighboring methyl groups, C_{19} ($\theta = 40°$) and C_{20} ($\theta = 32°$), show that the carbon framework of the polyene chain must be distorted by an in-plane curvature and possibly an out-of-plane twist. The fact that the two methyl groups, C_{18} ($\theta = 37°$) and C_{19} ($\theta = 40°$), are not entirely parallel may be attributed partially, but not wholly, to the additional rotational flexibility around the C_6–C_7 bond. It thus appears that the observed in-plane curvature, and possibly an out-of-plane twist, relieves the steric crowding of the three methyl groups (C_{18}, C_{19}, and C_{20}) along the retinal chain, as well as the interference of the *gem*-methyl groups (C_{16} and C_{17}) on the ring with the proton on C_8. A more refined picture of the chromophore in terms of the individual bond and torsion angles could be obtained by computer modeling of the molecular framework to the set of geometrical constraints, i.e., to the measured methyl group orientations. However, since the in-plane and out-of-plane distortional modes are interdependent, it is not possible to quantify the contribution of each, and a family of plausible retinal structures would emerge that are all compatible with the ²H-NMR results.

A significant distortion, qualitatively and quantitatively very similar to the one described above, is also observed in the high-resolution structure of a crystalline *all-trans* retinal Schiff base model compound (Santarsiero et al., 1990). In this virtually planar molecule, the C–C–C bond angles of the conjugated system are significantly enhanced at C_7, C_{10}, and C_{12}, while they are compressed at C_6, C_9, and C_{13} opposite the bulky substituents. The similarity of the relative methyl group orientations in the retinal Schiff base model compound and the BR chromophore may imply that the protein framework of bacteriorhodopsin does not necessarily impose any severe steric constraints on the chromophore leading to the distortion observed here by ²H-NMR. For some further insight into the interactions of retinal with the protein binding pocket, an energy minimization and short molecular dynamics calculation was performed on bacteriorhodopsin (G. Worth, personal communication), using the CHARMm22 all-atom force field.[2] Starting with the published model of the protein framework resolved by electron microscopy into which the chromophore had been placed according to other experimental constraints (Henderson et al., 1990), the ends of the helices were fixed and structures calculated over a 20-ps NVT simulation after an initial equilibration of the system at 300 K. The time-averaged orientations of the three methyl groups, C_{18}, C_{19}, and C_{20}, with respect to a common membrane normal showed the same range of angles as the experimental ²H-NMR results to within ±2°. The effect of thermal motion, nevertheless, was seen to induce fluctuations with some notable out-of-plane twisting. Populations of twisted chromophores have been proposed as an explanation for the biphasic band shape of the CD spectrum of BR in the visible region (Wu & El-Sayed, 1991), while polarized FT-IR experiments have suggested that in dark-adapted BR the only significant twist is located at the C_{14}–C_{15} bond (Fahmy et al., 1991).

For the comprehensive description of the overall chromophore orientation within the protein binding pocket as it is illustrated in Figure 4, the measured angles θ of the three methyl groups, C_{18}, C_{19}, and C_{20}, alone are not sufficient. While they have provided information about the relative conformational distortion of the retinal backbone, the average tilt angle of its long axis and that of the conjugated plane are not yet determined with respect to the membrane plane. Further constraints on the molecule are thus required, namely, the angles of the geminal C_{16} and C_{17} methyl groups on the retinal ring which have been measured previously by ²H-NMR (Ulrich et al., 1992a). From their roughly horizontal orientations it had been concluded that the plane of the puckered cyclohexene ring must sit nearly upright within the membrane. Together with the 6(S)-*trans* conformation between the ring and chain (see above), this also makes the molecular plane of the conjugated chain roughly vertical to within an approximately ±20° range. The average end-to-end tilt angle of the chromophore long axis may then be defined through the orientations of the methyl groups, C_{19} and C_{20}, which make a right angle to the local chain direction, as seen in Figure 4. If the conjugated plane was planar and aligned perfectly vertically, then the mean value of the two angles, $(40° + 32°)/2 = 36°$, could be used to estimate the average tilt of the chromophore long axis as $90° - 36° \approx 54°$ with respect to the membrane normal. In the case of a slightly twisted or slanted molecular plane, the average long-axis tilt angle would be somewhat larger than 54°. Optical spectroscopy has shown that the electronic transition moment of the conjugated system is inclined by about 67° relative to the membrane normal and that its plane lies approximately perpendicular to the membrane surface (Heyn et al., 1977; Earnest et al., 1986; Lin & Mathies, 1989; Fahmy et al., 1991). The ²H-NMR results are therefore in excellent agreement with the known information on BR and have provided further structural details with high accuracy.

The detailed analysis of the retinal structure presented in Figure 4 has been based on ²H-NMR data that were recorded at a temperature of −60 °C and a relative humidity of 85%. Under those conditions the chromophore might be expected to be comparatively more immobilized than in the physiological state of the protein, and the question arises as to whether its average conformation and orientation are modified. Further ²H-NMR experiments at room temperature and down to −120 °C (where the methyl groups are still undergoing fast rotation; Ulrich et al., 1992a; Copie et al., 1990) demonstrate that the quadrupole splittings remain virtually the same over that range (spectra not shown). Therefore, it appears that the chromophore conformation and its orientation are not significantly affected by freezing the protein, as had been found from diffraction studies. Control ²H-NMR experiments at a lower humidity of 75% (spectra not shown) gave no indication of any change in the methyl group orientations either, which implies that the retinal structure is relatively insensitive to the degree of protein hydration over that range. At a relative humidity of 98%, the swelling of the oriented PM films leads to the destruction of the uniaxial alignment and a loss of order, so that no comparisons could be drawn. Anyhow, it should be noted at this point that the high salt concentration of the natural habitat of *H. salinarium* leads to a substantially reduced water activity on the extracellular side of the membrane (thermodynamically equivialent to a relative humidity of approximately 70%). The details of the retinal struture presented here may thus be regarded relevant over a wide range of conditions for bacteriorhodopsin.

All ²H-NMR experiments were performed in the dark-adapted ground state of the BR photocycle, where there exists a 1:2 mixture of the *all-trans*- and the 13-*cis*,15-*syn*-retinal isomers in the protein. Another question concerns the simultaneous presence of these two populations. Each zero-tilt spectrum in Figure 1, however, is characterized by a single pair of resonances only, and the tilt series in Figures 2 and

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[2] QUANTA/CHARMm release 3.3, Molecular Simulations Inc., Waltham, MA.

3 could each be successfully simulated for just one methyl group orientation alone. The inhomogeneous deuterium line width could be attributed entirely to the microscopic disorder in the sample, since the mosaic spread estimated from the line-shape simulations is close to the value found by independent ^{31}P-NMR measurements (Ulrich et al., 1992a). The two retinal isomers therefore do not produce discrete shifted components, not even beyond the limited resolution of the relatively broad spectral lines. It is thus concluded that the thermal isomerization of the Schiff base terminus around the $C_{13}=C_{14}$ double bond, and simultaneously the $C_{15}=N$ bond, has no effect on the orientation of the methyl groups C_{18}, C_{19}, or C_{20} or on the retinal ring. While this local rearrangement appears to be readily accommodated by the protein binding pocket, the light-induced 13-*cis* isomerization in the photocycle, on the other hand, is expected to induce distinct structural changes of the protein in the course of the proton translocation. Any concomitant realignment of retinal might then be revealed by ^{2}H-NMR measurements of trapped intermediates, since the method is ideally sensitive to the small shifts in angle that are expected to occur during the photocycle (Hauss et al., 1993).

CONCLUSIONS

The individual orientations of the bonds connecting the three methyl groups, C_{18}, C_{19}, and C_{20} to the polyene chain in the retinal chromophore of bacteriorhodopsin were obtained with respect to the membrane normal, using solid-state ^{2}H-NMR. The three bonds are not parallel, indicating that the conjugated chain has an in-plane curvature and possibly an out-of-plane twist. Since the angles are in the range from 32° to 40°, the analysis of the deuterium quadrupole splittings and line shapes provides structural information of high accuracy. Together with the previous determinations of the C_{16} and C_{17} methyl groups, a comprehensive description of the molecular conformation and orientation of the chromophore in the protein binding pocket emerges. It is shown that ambiguities in angle from the analysis of the quadrupole splitting in the zero-tilt spectrum can be uniquely resolved by measuring the immobilized uniaxially oriented sample at a number of tilt angles between the sample normal and the spectrometer magnetic field. This ^{2}H-NMR method should be applicable to other membrane proteins as well, for which it would supply structural details on selected bond vectors, provided the group of interest can be specifically deuterated and oriented samples can be prepared in sufficient amounts.

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