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Structural Analysis and Dynamics of Retinal Chromophore in Dark and Meta I States of Rhodopsin from ^2H NMR of Aligned Membranes

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Summary

Rhodopsin is a prototype for G protein-coupled receptors (GPCRs) that are implicated in many biological responses in humans. A site-directed ^2H NMR approach was used for structural analysis of retinal within its binding cavity in the dark and pre-activated meta I states. Retinal was labeled with ^2H at the C5, C9, or C13 methyl groups by total synthesis, and was used to regenerate the opsin apoprotein. Solid-state ^2H NMR spectra were acquired for aligned membranes in the low-temperature lipid gel phase *versus* the tilt angle to the magnetic field. Data reduction assumed a static uniaxial distribution, and gave the retinylidene methyl bond orientations together with the alignment disorder (mosaic spread). The ^2H NMR structure of 11-*cis*-retinal in the dark state revealed torsional twisting of the polyene chain and the β -ionone ring. The distorted retinylidene ligand undergoes restricted motion, as evinced by order parameters of ≈ 0.9 for the rapidly spinning $\text{C}-\text{C}^2\text{H}_3$ groups, with off-axial fluctuations of $\approx 15^\circ$. Retinal is accommodated within the rhodopsin binding pocket with a negative pre-twist about the C11=C12 double bond, which explains its rapid photochemistry and indicates the trajectory of the 11-*cis* to *trans* isomerization. For the cryotrapped meta I state, the ^2H NMR structure showed a reduction of the polyene strain, whereas the β -ionone ring maintained its torsional twisting. Strain energy and dynamics of retinal are interpreted with regard to substituent control of receptor activation. Steric hindrance between *trans* retinal and Trp²⁶⁵ can trigger formation of the subsequent activated meta II state. Our results are pertinent to quantum and classical molecular mechanics simulations, and show how ^2H NMR can be applied to ligands bound to GPCRs in relation to their characteristic mechanisms of action.

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Keywords

G protein-coupled receptors; membranes; solid-state NMR; retinal; rhodopsin; signal transduction; vision

Introduction

Apart from its role in the visual process, rhodopsin is a paradigm for membrane receptors that detect stimuli from a large variety of hormones, neurotransmitters, chemokines, and other factors^{1,2}. The chromophore of rhodopsin is an 11-*cis*-retinylidene prosthetic group, which functions as an inverse agonist, and is bound to Lys²⁹⁶ by a protonated Schiff base with Glu¹¹³ as its counterion^{3–6}. Absorption of a photon initiates 11-*cis* to *trans* isomerization of the retinylidene ligand, giving an equilibrium between metarhodopsins I and II that depends on both the protein^{7–9} and lipid environment^{10–12}. Knowledge of the crystal structure of rhodopsin^{13–17} is essential to unraveling how the visual process is stimulated by light. Yet even at 2.2 Å resolution¹⁷ there may be ambiguity in the retinal structure within the rhodopsin binding cleft^{18–20}. Besides the dark state, an electron crystallographic structure is available for 2-D crystals of a photostationary mixture enriched in metarhodopsin I (meta I)²¹. An important aspect is the role of ligand flexibility in the spectral and pharmacological properties of the receptor. A current picture is that photoisomerization of retinal gives helical movements that affect the cytoplasmic loops of rhodopsin and expose binding sites for transducin^{4,22}, which in turn stimulates an effector phosphodiesterase^{3,4}. Conformational strain of the bound ligand is implicated in the mechanism of receptor activation through interactions involving the protein and the membrane bilayer, all of which are tightly coupled¹².

Knowledge of the orientation and stereochemistry of retinal in the various photointermediate states is vital to understanding the mechanism of light activation of rhodopsin²³. In this work, we used solid-state ²H NMR spectroscopy^{24,25} to address information about the retinal structure and dynamics in the dark and meta I states of the photoreceptor. We asked the following questions: (i) what is the basis for the phenomenal stability of 11-*cis* retinal in the dark state of rhodopsin? (ii) How does light instantly transform retinal to a labile species that undergoes 11-*cis* to *trans* isomerization with a high quantum yield? (iii) What are the structural changes yielding activation of the receptor? Our work entailed total organic synthesis of ²H isotopically-labeled retinals, together with biophysical ²H NMR techniques and theoretical lineshape calculations²⁶. Angular constraints were provided by ²H NMR quadrupolar couplings^{24,26,27} and distances from complementary rotational resonance ¹³C NMR studies^{25,28,29}. This approach is analogous to solution NMR, where residual dipolar couplings are used with distance restraints from nuclear Overhauser effect (NOE) spectroscopy. A highly distorted structure is found for retinal in the dark state, consistent with vibrational^{9,30–32} and circular dichroism (CD) spectroscopy^{30,31}, and with bioorganic studies of rhodopsin^{33,34}. Despite constraints of the binding cavity the ligand undergoes restricted motion, as shown by the rapidly spinning methyl groups, with significant off-axial fluctuations. We find that 11-*cis* to *trans* isomerization of retinal rotates the Schiff base end of the ligand while maintaining the β-ionone ring within its hydrophobic pocket. Our results

are pertinent to how energy is transduced and dissipated in the membrane-bound receptor yielding its activation. Further, we demonstrate the use of solid-state ^2H NMR for investigating conformational changes of ligands bound to membrane proteins in conjunction with their functional mechanisms.

Results

Powder-type ^2H NMR spectra of retinal bound to rhodopsin

We applied solid-state ^2H NMR spectroscopy to investigate the conformation and orientation of the retinylidene chromophore within the ligand-binding cavity of rhodopsin. Figure 1(a)–(c) shows representative ^2H NMR spectra from -100 kHz to $+100$ kHz for 11-*Z*-[9- C^2H_3]-retinylidene rhodopsin in randomly oriented POPC membranes over a broad temperature range of 120 °C. Powder-type ^2H NMR spectra at temperatures of -30 , -60 , and -150 °C are similar and exhibit discontinuities at ∓ 19.5 kHz and ± 39.0 kHz, due to the $\theta = 90^\circ$ and $\theta = 0^\circ$ orientations of the largest principal value of the electric field gradient (EFG) tensor relative to the main magnetic field²⁴. Below the order-disorder phase transition temperature of POPC ($T_M = -4$ °C) the ^2H NMR spectra correspond to the lipid gel state, where rotational and translational diffusion of both the protein³⁵ and lipids are diminished on the ^2H NMR time scale (< 10 μs for rotating methyls). Weak signals from the residual $^1\text{HO}^2\text{H}$ of the solvent are subtracted at $T = -30$ and -60 °C (not shown). The lowest temperature studied, $T = -150$ °C, is close to the cryogenic temperatures employed in X-ray crystallography of rhodopsin^{13,15–17}. At higher temperatures, above T_M , the large $^1\text{HO}^2\text{H}$ peak dominates the ^2H NMR spectra, and distinct quadrupolar splittings from the ^2H -labeled retinal are unobservable (not shown).

For rhodopsin, the powder-type ^2H NMR spectra correspond to methyl groups of the retinylidene ligand undergoing fast axial rotation (< 10 μs). In ^2H NMR the static EFG is parallel to the $\text{C}-^2\text{H}$ bond, and for rapidly rotating methyls the residual EFG is projected onto the three-fold (C_3) axis. The ^2H NMR spectra were simulated using the theoretical expression for a Pake doublet due to a spherical distribution of EFG principal axes²⁴. In Figure 1(d) the spectral probability density $\rho(\xi_\pm)$ for the two $I=1$ spectral branches is graphed against the reduced frequencies $\xi_\pm$ where the quadrupolar coupling is divided by $(3/4)\chi_Q^{\text{eff}}$ (*cf.* Materials and Methods). For rotating methyls, the static quadrupolar coupling of $\chi_Q = 170$ kHz is scaled by $P_2(\cos 109.47^\circ) = -1/3$, giving an effective coupling constant of $\chi_Q^{\text{eff}} = 56.67$ kHz, assuming ideal tetrahedral geometry. Spectral discontinuities occur at frequencies of $\mp \frac{3}{8}\chi_Q^{\text{eff}} = \mp 20.875$ kHz and $\pm \frac{3}{4}\chi_Q^{\text{eff}} = \pm 41.75$ corresponding to $\theta = 90^\circ$ and $\theta = 0^\circ$, respectively. Off-axial motions further average the quadrupolar coupling, as described by the order parameter $S_{C_3} = \langle \chi_Q \rangle / \chi_Q^{\text{eff}}$ of the methyl three-fold axis, where brackets denote the time average. Simulated ^2H NMR spectra are superimposed in Figure 1 on the experimental data, and give the flat residuals plotted beneath each spectrum. Additional powder-type ^2H NMR spectra of 11-*Z*-[5- C^2H_3]-retinylidene rhodopsin and 11-*Z*-[13- C^2H_3]-retinylidene rhodopsin in POPC membranes (1:50) are shown in Figure 1(e) and (f), respectively, at $T = -100$ °C. Best-fit values of the quadrupolar splitting $\Delta \nu_Q^{\text{powder}}$, *i.e.* corresponding to $\theta = 90^\circ$, for the C5, C9, and C13 methyl groups are 38.0, 39.0, and 38.0 ± 1.0 kHz, with a linebroadening (Lorentzian FWHM) of 3.2–5.0 kHz. Comparison of

the residual quadrupolar couplings to the theoretical value for three-fold rotation yields order parameters²⁴ of $S_{C_3} = 38/41.75 \approx 0.9$ for the methyl groups of the retinylidene ligand within the receptor binding cavity, corresponding to off-axial fluctuations of $\approx 15^\circ$. Although the retinal chromophore is held tightly within its binding pocket, restricted motions of appreciable amplitude occur. No significant differences are observed among the methyl substituents from -30°C down to -150°C .

Deuterium NMR lineshapes for aligned membranes

Simulations of experimental ^2H NMR spectra employed a lineshape treatment for a static uniaxial distribution²⁶, as applied previously to bacteriorhodopsin (bR) in purple membranes³⁶ and to aligned DNA fibers³⁷. By aligning the recombinant POPC membranes containing rhodopsin on planar substrates, we determined the orientation of the ^2H quadrupolar coupling tensor relative to the membrane frame. Referring to Figure 2, the experimental arrangement involves a stack of supported membranes containing rhodopsin labeled at the C5, C9, or C13 methyl groups. Rhodopsin is depicted by the seven transmembrane helices, together with the van der Waals surface of the receptor. An expansion of the retinylidene ligand is provided at top right to illustrate the structure determination. We established the orientation θ_B of the $\text{C}-\text{C}^2\text{H}_3$ bond axis, relative to the local membrane frame, by theoretically simulating the ^2H NMR lineshape as a function of the tilt angle θ between the average membrane normal $\mathbf{n}_0$ and the main magnetic field $\mathbf{B}_0$ (tilt series). First, we need to consider the uniaxial distribution (angle ϕ) of the methyl $\text{C}-\text{C}^2\text{H}_3$ bond axis about the local membrane normal $\mathbf{n}$ in the low-temperature lipid gel state. Due to the alignment disorder (Euler angle θ') the local membrane normal $\mathbf{n}$ does not coincide with the average normal, $\mathbf{n}_0$, so it too is azimuthally distributed (angle ϕ') within the membrane stack. Angles for the overall transformation from $\mathbf{n}$ to $\mathbf{B}_0$ are θ'' and ϕ'' . Hence, we must integrate over a double static uniaxial distribution (angles ϕ and ϕ') to evaluate the bond orientations relative to the applied magnetic field $\mathbf{B}_0$. We have shown previously for aligned samples how the extent of 3-D alignment disorder can be taken into account to properly simulate the solid-state NMR spectra²⁶. Essentially we map the semi-random distribution of $\text{C}-\text{C}^2\text{H}_3$ bond orientations from the angular space into the frequency space of the ^2H NMR spectrum. (In effect, the membrane stack corresponds to a 1-D quasi-crystal with alignment disorder in the longitudinal z -direction, whereas it is analogous to a random 2-D powder in the transverse xy -plane.) In this way, the ^2H NMR spectra allow us to deduce the bond axis orientation θ_B together with the standard deviation σ of the 3-D alignment disorder (mosaic spread; *cf.* Materials and Methods)²⁶.

Angular-dependent ^2H NMR spectra for dark and meta I states

We acquired ^2H NMR spectra of rhodopsin regenerated with ^2H -labeled retinal in membranes that were aligned by isopotential ultracentrifugation³⁸. Both the dark and meta I states of the photoreceptor were investigated. The quality of the membrane orientation was investigated by ^{31}P NMR spectroscopy (not shown). Compared to previously published data³⁸, we did not obtain complete membrane alignment, but rather aligned fractions of $\approx 90\%$ were typical for rhodopsin in POPC or DMPC bilayers. For the dark state, full tilt sequences of ^2H NMR spectra acquired at $T = -150^\circ\text{C}$ are shown in Figures 3(a)–(c) for aligned rhodopsin/POPC (1:50) membranes, with retinal ^2H -labeled at the C5, C9, or C13

methyl groups, respectively. These data confirm and extend our preliminary results²⁷, where characteristic changes in the ^2H NMR spectral lineshapes are evident as a function of tilt angle (θ), which manifest the different $\text{C}-\text{C}^2\text{H}_3$ bond orientations relative to the membrane plane. Similar studies were conducted at temperatures of $T = -60$ and -30 °C (not shown). The ^2H NMR spectra exhibit little temperature dependence over a broad range of 120 °C. Theoretical simulations assume a static uniaxial distribution of the rhodopsin molecules with rotating methyl groups, and are superimposed on the experimental ^2H NMR data (*cf.* Materials and Methods, Eqs. A5–A7). In agreement with the data for powder-type samples (*cf.* Figure 1), we find off-axial order parameters for the methyl groups of $S_{\text{C}_3} \approx 0.9$.

Moreover, the meta I photointermediate was cryotrapped in planar supported bilayers to investigate changes in the retinal conformation and orientation induced by light absorption. Figure 4(a)–(c) displays a full tilt series of ^2H NMR spectra acquired at -100 °C for macroscopically aligned POPC membranes. The tilt series confirms our preliminary data for metaI³⁹, and substantiates that acquiring ^2H NMR spectra at values of $\theta = 0^\circ$ and 90° may suffice to estimate the methyl orientation when signal strength is limiting. This is because ^2H NMR spectra for $\theta = 0^\circ$ are most sensitive to the bond orientation for samples with a large mosaic spread. Theoretical ^2H NMR spectra are superimposed on the experimental data in Figure 4 (*cf.* Eqs. A5–A7). Comparing the meta I state to the dark state, the largest differences in the ^2H NMR spectra were found for the $\text{C}_9-\text{C}^2\text{H}_3$ group, with smaller variations for the $\text{C}_5-\text{C}^2\text{H}_3$ and $\text{C}_{13}-\text{C}^2\text{H}_3$ groups. These differences manifest the bond orientation θ_B as well as the mosaic spread σ , as further described below. As for the dark state, the ^2H NMR spectra indicate that the retinylidene methyl substituents undergo rapid rotation, with order parameters for off-axial fluctuations of ≈ 0.9 within the meta I binding cavity.

Global fitting of ^2H NMR lineshapes

Figure 5(a)–(c) shows the results of global fitting of the ^2H NMR lineshapes for the C_5 , C_9 , or C_{13} methyl groups of rhodopsin/POPC membranes in the dark state, respectively. The root mean square deviation (RMSD) of the calculated ^2H NMR spectra from the experimental data for the entire tilt series is graphed as a function of θ_B and σ , and differs significantly for the various labels. In Figure 5(d)–(f), we display cross-sections through the RMSD surfaces at the global minimum for the C_5 , C_9 , and C_{13} methyl groups, respectively. Accurate bond orientations of the ^2H -labeled methyl groups are obtained, despite the appreciable mosaic spread ($\sigma = 18\text{--}21^\circ$; *cf.* Table 1). Values for the mosaic spread obtained from the ^2H NMR lineshape simulations are larger than in our previous studies of aligned purple membranes containing bR³⁶. This may be due to the fact that rhodopsin ($M_r = 40$ kDa) is a larger molecule than bR ($M_r = 26$ kDa), with substantial extramembraneous domains⁴⁰. We determined values of the $\text{C}-\text{C}^2\text{H}_3$ bond orientations of $70 \pm 3^\circ$, $52 \pm 3^\circ$, and $68 \pm 2^\circ$ for the C_5 , C_9 , and C_{13} methyl groups of retinal in the dark state of rhodopsin, in agreement with earlier results²⁷. Our values differ significantly from previously published work⁴¹, yet they are in good agreement with X-ray crystallography^{14,17} as summarized in Table 1. The data are interpreted in terms of average bond orientations and average torsion angles of the retinylidene ligand. The nonsymmetric error surface for the C_5 methyl, *cf.* Figure 5(a), may imply conformational heterogeneity or mobility of retinal¹⁸ as distinct

from the mosaic spread. Restricted mobility of the β -ionone ring could affect the ^2H NMR angles and distances from ^{13}C NMR rotational resonance studies²⁹.

The RMSD surfaces for the meta I state are displayed in Figure 6(a)–(c). For the 9- C^2H_3 and the 13- C^2H_3 groups clear minima are evident; however the error surface for the 5- C^2H_3 group is less well-defined. As noted above for the dark state, this may indicate conformational heterogeneity or flexibility of the β -ionone ring, which is not explicitly considered in the lineshape simulation. Cross-sections through the RMSD surfaces at the global minima are shown in Figure 6(d)–(e). The minima for the C5, C9, and C13 methyl substituents correspond to bond orientations θ_B of $72\pm 4^\circ$, $53\pm 3^\circ$, and $59\pm 3^\circ$, respectively (Table 1). Due to the larger mosaic spread of meta I ($\sigma = 22\text{--}25^\circ$) compared to the dark state ($\sigma = 18\text{--}21^\circ$), the ^2H NMR spectra for the C9 methyl differ whereas the bond orientations are similar. By contrast, for the C13 methyl group a smaller spectral difference is evident, despite the larger change in bond orientation. Clearly, a proper treatment of the alignment disorder²⁶ is needed to compare ^2H NMR data for the photointermediates of rhodopsin. The fitting parameters for meta I are compared to the dark state in Table 1.

Modeling retinal conformation in dark state

For analysis of the retinal conformation, we applied a simple model with three planes of unsaturation (Figure 7), encompassing the β -ionone ring and the polyene chain to either side of the C12–C13 bond^{23,26,27,34,42}. Relative orientations of pairs of ^2H -labeled methyl substituents are used to calculate effective dihedral angles for the planes of unsaturation. Angular restraints are provided by the orientations of the three-fold axes of the C5, C9, and C13 methyl groups to the local membrane normal $\mathbf{n}$, as obtained from ^2H NMR of aligned membranes^{27,39}. These in turn specify the C6–C7 and C12–C13 dihedral angles of the retinylidene chromophore, as shown in Figure 7(a). Moreover, the ^2H NMR data for aligned samples allow one to investigate the orientation of the retinal ligand within the binding cavity of rhodopsin.

Each plane (designated A, B, or C) is defined by two vectors, and requires two angular constraints (degrees of freedom) to specify its spatial orientation. The three molecular planes share two bonds in common, so a total of four independent parameters (degrees of freedom) is needed to define the retinal structure. Since data for the C1 methyl group are unavailable, the electronic transition dipole moment of the retinylidene chromophore from linear dichroism measurements is introduced as a fourth orientational restraint^{43–46}. Circular dichroism (CD) data are also available for the dark state of rhodopsin, which yield information on the enantiomeric selectivity of the protein binding pocket^{33,34}. Hence, the potentially large number of degrees of freedom is made smaller, so that we can carry out the conformational analysis with the relatively sparse angular restraints from ^2H NMR data. Furthermore, distances obtained from solid-state rotational-resonance ^{13}C NMR studies^{28,29} can be introduced. Yet by themselves, these do not pin down the conformation, on account of the relatively weak dependence of the carbon-carbon distances on torsion angles. Rather, it is most accurate to combine angular and distance restraints, in analogy with solution NMR spectroscopy.

Symmetry-related aspects of the molecular modeling likewise need to be considered, which are often overlooked. The nuclear spin interactions in solid-state ^2H NMR²⁴ as well as the electronic transition dipole moment⁴⁶ transform by the totally symmetric representation of the D_{coh} point group. They are invariant to the symmetry operations of inversion, rotation, or reflection as found in standard character tables. Considering two vectors to define a plane, (i) both can point either up or down with respect to the membrane normal, or (ii) one of the vectors can point up and the other down (the parity is even). In the first case, rotation of the two vectors on the rim of a unit cone yields two relative orientations for the vectors corresponding to a given retinal isomer. The second case gives two additional solutions, so that four orientations of a given molecular fragment are possible. It follows that $4^2 = 16$ combinations of the orientations of the two planes exist for a given dihedral angle, which gives a maximum of $4^3 = 64$ combinations for the relative orientations of the A, B, and C planes. Moreover, the rhodopsin molecules can point either up or down, yielding a total of $2 \times 64 = 128$ possible combinations. Most solutions can be eliminated by taking into consideration the molecular geometry, as well as solid-state ^{13}C NMR rotational resonance distances^{28,29}, and CD data where available^{33,34}.

Calculation of effective torsional angles

How can one use the ^2H quadrupolar coupling tensors of the methyl substituents to characterize the retinal orientation and conformation within its binding cavity? Here we shall consider a pair of methyl groups attached to consecutive planes of unsaturation of retinal, whose effective torsion angle $\chi_{i,k}$ is given by²⁷:

$$\chi_{i,k} = \cos^{-1} \left(\frac{\cos\theta_i \cos\theta_B^{i,k} - \cos\theta_B^i}{\sin\theta_i \sin\theta_B^{i,k}} \right) - \cos^{-1} \left(\frac{\cos\theta_k \cos\theta_B^{i,k} - \cos\theta_B^k}{\sin\theta_k \sin\theta_B^{i,k}} \right) \quad (1)$$

Orientations of the individual methyl group axes *versus* the local membrane normal $\mathbf{n}$ are given by θ_B^i and θ_B^k (*cf.* Table 1), and θ_i and θ_k are angles of the two methyl axes to the $\text{C}_i\text{--C}_k$ bond with torsion angle $\chi_{i,k}$, such as the C6–C7 or the C12–C13 bond. Analogous electronic transition dipole (TD) orientations are denoted by θ_B^{TD} and θ_{TD} . The $\text{C}_i\text{--C}_k$ bond angle to the local membrane normal is $\theta_B^{i,k}$, and is calculated from the C9 methyl the orientation, together with the transition dipole in either the dark^{44,46} or meta I⁴³ state. According to Eq. (1) the sense (sign) of the torsion angle cannot be distinguished, so that we cannot decide between twisted *s-cis* and *s-trans* conformers, which deviates from an earlier report⁴¹. Although the bond orientations from ^2H NMR can be used to derive effective torsion angles, there is not a one-to-one correspondence.

For absolute configurations, we use the standard convention for the dihedral (torsion) angle⁴⁷ following the right-hand rule, where the *cis* conformation is $\chi_{i,k} = 0^\circ$. Our calculations assume either ideal orbital geometry, or they include a distorted C11=C12–C13 bond angle of 130° as shown by the crystal structure of 11-*cis*-retinal⁴⁸. Returning to Figure 7(a), we first consider the polyene chain, where the C12–C13 dihedral angle manifests the torsional twist of the C6–C13 plane (B) relative to the C12–C15 plane of unsaturation (C).

The relevant geometric angles are $\theta_{TD} = 104^\circ$ (114°), $\theta_B^{TD} = 74^\circ$, $\theta_{13} = 60^\circ$ (60°), and $\theta_B^{13} = 68^\circ$. Ideal values are listed first, followed by the results for nonideal orbital geometry⁴⁸ in parentheses. The bond tilt of $\theta_B^{12,13} = 52^\circ$ (58°) is obtained directly from the ^2H NMR result for the C9 methyl group for ideal hybridization, or alternately the C9 methyl group and transition dipole for nonideal geometry as in crystalline 11-*cis*-retinal^{46,48}. Applying Eq. (1) we obtain $\chi_{9,13} = \pm 2$ (± 5), ± 42 (± 50), ± 54 (± 18 , ± 79), ± 98 , ± 150 (± 147), and ± 166 (± 175)°. The allowed solutions can be further reduced by including interatomic distance constraints from rotational resonance ^{13}C NMR dipolar couplings²⁸. Only the latter two solutions agree with the C10-to-C20 and C11-to-C20 distances for the polyene chain²⁸. The last is stereochemically disallowed by the small distance between the C13 methyl group and hydrogen H10; whereas CD studies of locked retinoids imply the twist is positive³⁴, leaving $\chi_{9,13} = +150 \pm 4^\circ$ ($+147 \pm 4^\circ$) (Table 1). Only random errors are considered, although possible systematic errors in the model mean the accuracy may be less. The physical solution is shown in Figure 7(a) and represents a positively twisted, 12-*s-trans* conformation, where the C13 methyl group and the transition dipole point in the same direction from the membrane plane, and the C9 methyl group points oppositely. The effective torsion angle of the C12–C13 bond is in agreement with our earlier work²⁷ and with the rhodopsin X-ray structure^{14,17} (Table 1).

Next we come to the β -ionone ring, which plays an important role in the activation mechanism of rhodopsin^{8,18,23,49–51}. Referring once again to Figure 7(a), the C6–C7 dihedral angle is given by the orientations of the C5 and C9 methyl groups, which describes the ring twisting *versus* the polyene chain, *i.e.* the C5–C7 plane of unsaturation (A) relative to the C6–C13 plane (B). The geometrical angles are $\theta_5 = \pm 60^\circ$, $\theta_B^5 = 70^\circ$, $\theta_9 = \pm 60^\circ$, and $\theta_B^9 = 52^\circ$, where ideal hybrid orbital geometry is assumed. The C6–C7 bond tilt is $\theta_B^{6,7} = 45^\circ$, which is obtained from the C9 methyl bond orientation and the electronic transition dipole orientation. Applying Eq. (1), we calculate possible solutions of $\chi_{5,9} = \pm 5$, ± 24 , ± 53 , ± 65 , ± 111 , ± 154 , and $\pm 159^\circ$, corresponding to the dihedral angle of the A and B planes. Thus, the β -ionone ring conformation can be either (twisted) 6-*s-cis* or twisted 6-*s-trans*, which cannot be distinguished based on solid-state ^2H NMR measurements alone²⁷. Moreover, positional restraints can be introduced from ^{13}C NMR²⁹ for the C8-to-C18 and C8-to-C16/C17 distances from the polyene chain to the β -ionone ring. Together with the chirality from CD studies³³, we find that the physical solution is $\chi_{5,9} = -65 \pm 6^\circ$, representing a negatively twisted 6-*s-cis* conformation as depicted in Figure 7(a) (Table 1). Due to systematic errors, the accuracy may be less than obtained by considering only random errors. This result agrees with our earlier study²⁷ and with previous ^{13}C NMR chemical shift tensor measurements⁵².

To further evaluate the 11-*cis*-retinylidene structure derived from ^2H NMR, we inserted it into the binding pocket of the X-ray structure of rhodopsin in the dark state with the highest currently available resolution (2.2 Å; PDB accession code 1U19)¹⁷. The Schiff base end was superimposed with the nitrogen of Lys²⁹⁶, thus maintaining the carbon atoms from C12 to C15 very close to the X-ray coordinates. For the simple three-plane model^{23,27}, however, this led to a steric clash of retinal with the side chains of Tyr¹⁷⁸ and Cys¹⁸⁷ in extracellular

loop E2, as well as Met²⁰⁷, Tyr²⁶⁸, and Ala²⁹² in helices H5, H6, and H7, respectively. We resolved this difficulty by introducing an additional twist of the C11=C12 bond of the polyene chain. In the extended three-plane model, the C11=C12 torsion angle was determined by fitting the C10-to-C20 and C11-to-C20 distances to ¹³C NMR rotational resonance data²⁸. We find that the C11=C12 torsion angle is $-25 \pm 10^\circ$ and the C12–C13 torsion angle is $176 \pm 6^\circ$ (Table 1), where the twist is predominantly localized to the C11=C12 bond. The C10-to-C20 and C11-to-C20 distances are relatively insensitive to the C11=C12 torsion angle, giving somewhat large errors. The corresponding structure for retinal is indicated in Figure 7(b), where the rhodopsin N-terminus is at top (extracellular side) and the C-terminus is at bottom (cytoplasmic side). As further discussed below, pre-twisting of the C11=C12 *cis*-double bond better allows the retinal structure to fit the rhodopsin binding cleft.

Light-induced changes for retinal in meta I state

For the retinylidene cofactor in meta I with an all-*trans* polyene chain, our approach was essentially the same as described above for the dark state (Figure 8). Besides the C5, C9, and C13 methyl bond orientations, the electronic transition dipole moment was introduced as a further orientational restraint, corresponding to the main absorption band in meta I⁴³. Considering first the polyene chain, in the meta I state the relevant geometrical angles are $\theta_{TD} = 134^\circ$, $\theta_B^{TD} = 74^\circ$, $\theta_{13} = 60^\circ$, $\theta_B^{13} = 59^\circ$, and lastly $\theta_B^{12,13} = 55^\circ$, where ideal orbital geometry is assumed. Applying Eq. (1), we obtain possible solutions for the torsion angle of $\chi_{9,13} = \pm 173^\circ, \pm 72^\circ, \pm 45^\circ, \pm 99^\circ$, and $\pm 1^\circ$. Only $\chi_{9,13} = \pm 173 \pm 4^\circ$ agrees with experimental ¹³C NMR rotational resonance data²⁸ for the C10-to-C20 and C11-to-C20 distances, which is consistent with a nearly fully relaxed 11-*trans* conformation in the meta I state^{28,53}, where random errors are considered as for the dark state. Turning next to the β -ionone ring, the fixed geometrical angles are $\theta_5 = 60^\circ$, $\theta_B^5 = 72^\circ$, $\theta_9 = 60^\circ$, and $\theta_B^9 = 53^\circ$. In addition, the C6–C7 bond tilt is $\theta_B^{6,7} = 55^\circ$, as obtained from the C9 methyl bond orientation and transition dipole moment⁴³. Applying the analytical formula, Eq. (1), we obtain $\chi_{5,9} = \pm 15^\circ, \pm 28^\circ, \pm 32^\circ, \pm 57^\circ, \pm 96^\circ, \pm 116^\circ, \pm 155^\circ$, and $\pm 158^\circ$ as possible solutions. According to rotational resonance ¹³C NMR data⁵⁴, the experimental C8-to-C18 distance is 2.95 ± 0.15 Å in meta I, which implies a 6-*s-cis* conformation⁵⁴. Hence the only possibilities for the β -ionone ring are $\chi_{5,9} = \pm 32^\circ$ and $\pm 57^\circ$. The electron density map for meta I shows that the β -ionone ring is strongly immobilized²¹. Moreover, its position relative to the retinylidene Schiff base does not change appreciably *versus* the dark state. It follows that only two solutions are possible, as shown in Figure 8(a)–(b), with the N-terminus of rhodopsin (extracellular side) at top. The effective torsion angle for the C6–C7 bond is $\chi_{5,9} = \mp 32^\circ$ whereas for the C12–C13 bond it is $\chi_{9,13} = \pm 173^\circ$. Due to the absence of CD data for meta I the enantiomers cannot be distinguished; in either case a twisted 6-*s-cis* conformation of the C6–C7 bond is found. Although the polyene chain is relaxed in meta I, the β -ionone ring retains its torsional strain as in the dark state. The calculated meta I structures were further restrained by inserting them into the dark-state rhodopsin structure¹⁷. It was assumed that the shape of the retinal binding cavity was nearly the same in meta I as in the dark state. The structure in Figure 8(a) with $\chi_{5,9} = -32^\circ$, and $\chi_{9,13} = 173^\circ$ fits best within the binding cleft, having its only close contact with the side chain of Trp²⁶⁵, which supports our earlier conclusions³⁹. By contrast,

the structure in Figure 8(b) with $\chi_{5/9} = 32^\circ$, and $\chi_{9/13} = -173^\circ$ makes close contacts with the side chains of Glu¹²², Trp²⁶⁵, Tyr²⁶⁸, and Ala²⁹², and is deemed less likely.

Discussion

We have inferred the retinal conformation of rhodopsin in the dark state using solid-state NMR spectroscopy, and have investigated light-induced changes in the metarhodopsin I intermediate. Twisting of the retinal molecule obtained from the relative ^2H NMR tensor orientations²⁷ is in agreement with other biophysical studies^{14,23,27,29–31,34,46,52,55–60}, and is key to understanding its photoreaction dynamics^{32,61–63}. Of particular significance for rhodopsin is the nonplanarity of the conjugated polyene chain of retinal, together with the β -ionone ring²³. Nonplanar distortion of the retinal ligand is also shown by the presence of hydrogen-out-of-plane (HOOP) modes in resonance Raman^{32,62,64,65} and Fourier transform infrared⁵⁶ spectroscopy, and by CD studies^{30,31,34,55}. Compared to previous ^2H NMR studies^{38,41} and quantum chemical calculations¹⁹, we obtain different results for the retinylidene methyl group orientations that are consistent with X-ray crystallographic data^{14,17}. All of these aspects are significant for explaining how the photonic energy is stored, released, and focused or dissipated in the triggering of visual signaling by rhodopsin in photoreceptor membranes^{19,30,31,34,59,60}.

Torsional strain and deformation of retinal in dark state

The concept of torsional strain as an explanation for energy storage by rhodopsin is based on steric interactions of retinal within the ligand binding cavity, which influence the regioselectivity of the isomerization and the ultrafast photoreaction dynamics^{30,31,60,63}. Three interaction sites are considered for the retinal ligand by including the Schiff base and its associated counterion(s). The retinylidene 9- C^2H_3 group has a specific binding interaction with rhodopsin, and the β -ionone ring occupies a hydrophobic pocket^{16,66–68}; both are crucially important for rhodopsin activation^{42,56}. Studies of rhodopsin regenerated with modified retinals show that specific methyl sites are implicated in rhodopsin function⁴². Removal of the C9 methyl group leads to a relaxation of chromophore strain⁵⁶ giving a partial agonist^{9,50,69}, which shifts the meta I to meta II equilibrium towards the inactive meta I state, with diminished transducin activation⁵⁰. Similarly, deletion of the C13 methyl group yields less intense HOOP modes in vibrational spectroscopy^{9,30}, together with a decreased photoreaction rate and quantum yield⁷⁰. Deletion of the C13 methyl group also shifts the metarhodopsin equilibrium towards the inactive meta I state⁹. A diminution of chromophore strain in the dark state thus favors meta I and affects rhodopsin activation. Moreover, the β -ionone ring of the retinylidene ligand may be implicated in the spectral properties⁷¹ and activation of rhodopsin^{8,23,42,49,50,72}. The ring moiety of retinal may function through maintaining the activated meta II conformation of rhodopsin^{8,50,51}. Acyclic analogs of retinal lacking the β -ionone ring operate as partial agonists, and they shift the metarhodopsin equilibrium towards the inactive meta I state^{8,72}. A concomitant reduction of transducin activation occurs⁵⁰, evincing the key role played by the β -ionone ring in the visual mechanism. Retinal modifications^{8,9} and lipid modifications^{10–12} both function as partial agonists, and perturb the meta I to meta II equilibrium in a way that is attributable to strain energy (frustration) of the membrane-bound receptor⁷³.

For the dark state of rhodopsin, torsional twisting of the polyene chain entails a concerted deformation involving mainly the three consecutive bonds from C10 to C13^{28,30,32,53,62}. The torsional twisting arises from the binding cavity, which restrains the β -ionone ring within its hydrophobic pocket at one end of the molecule, and the Schiff base in close proximity to its counterion at the opposite end. Steric hindrance between the C13 methyl group and hydrogen H10 of the polyene chain may also contribute to the torsional deformation. From the relative methyl orientations, the polyene chain has a twisted 12-*s-trans* conformation, which manifests the stereochemical requirements of the binding site. The effective torsion angle includes twisting about the C11=C12 double bond, as further discussed below. By contrast, a twisted 12-*s-cis* conformation is found in the crystal structure of 11-*cis*-retinal⁴⁸. For the β -ionone ring the angular restraints from ²H NMR, together with ¹³C NMR rotational resonance distances²⁹ and CD studies³⁴, imply that it has a negatively twisted 6-*s-cis* conformation, on average, in agreement with solid-state ¹³C NMR chemical shift data⁷⁴. The distorted 6-*s-cis* conformation of retinal in rhodopsin^{27,42,52} and isorhodopsin⁷⁴ is distinct from the planar 6-*s-trans* conformation found in bR^{36,75}. Hence, the protein environment can modulate the β -ionone ring conformation substantially. The negative twist about the C6–C7 bond involves nonbonded interactions between the C5 methyl of the β -ionone ring and the H8 hydrogen of the polyene chain^{23,34}. However, the β -ionone ring may undergo torsional fluctuations about the C6–C7 bond, and may be less restricted than the polyene chain, which is consistent with molecular dynamics simulations⁷⁶. Nonbonded interactions involving the rapidly spinning C5 methyl may contribute to thermal fluctuations of the C6–C7 torsion angle. Mobility of the β -ionone ring within its hydrophobic binding pocket might explain the fact that it is more difficult to establish a unique conformation for this group^{14,18,27,29,41,76}.

Retinal conformation in pre-activated meta I state

A central question in visual signal transduction is the mechanism by which retinal is converted sequentially by light from an inverse agonist to an agonist that activates rhodopsin. The meta I and meta II states of rhodopsin correspond to the low-affinity and high-affinity forms of ligand-activated GPCRs. Control of reaction selectivity through substituents of the retinal ligand and the protein environment are of intense current interest. The 11-*cis* to *trans* isomerization leads to a selective relaxation of the polyene chain in the vicinity of the C10–C11=C12–C13 region of the molecule; whereas the β -ionone ring maintains its conformational distortion. According to ²H NMR, in meta I the orientation of the C5 and C9 methyl substituents is similar to the dark state. The largest change compared to the dark state involves the C13 methyl group closest to the retinylidene Schiff base end of the chromophore. Assuming a three-plane model, we calculate that the C11=C12 dihedral angle changes from -25° to $\approx 180^\circ$ in meta I *versus* the dark state. As noted above, the conformational strain is manifested by HOOP modes in resonance Raman and Fourier transform infrared spectroscopy^{56,62}. Following 11-*cis* to *trans* isomerization, a gradual reduction in HOOP mode intensity of the retinylidene chromophore is seen in the various rhodopsin photointermediates^{56,64,77}. A similar progressive diminution is evident in the visible CD of the rhodopsin intermediates⁵⁵, which agrees with the ²H NMR analysis.

Next we turn to the β -ionone ring, which is thought to undergo a repositioning in conjunction with helical movements leading to rhodopsin activation^{3,4,18,23,49,51,78}. At what stage of the photoactivation pathway do these changes occur? According to ^2H NMR, in meta I the β -ionone ring maintains a strained conformation, which is formally 6-*s-cis* and is similar to the dark state²⁷. Based on CD and bioorganic studies of locked retinoids, Nakanishi and coworkers^{33,34} have shown that the helicity about the 6-*s-cis* bond is negative in the dark state, persisting up to activation of the G protein, *viz.* the meta II state. Together with these data, ^2H NMR shows that the β -ionone ring remains negatively twisted from the dark state up to meta I, in contrast to the largely relaxed *trans* polyene chain. In addition, solid-state ^{13}C NMR chemical shift data indicate that the β -ionone ring is 6-*s-cis* in both rhodopsin and isorhodopsin⁷⁴; and it is proposed that it remains 6-*s-cis* in meta I⁵⁴. Application of a three-plane model yields that the C6–C7 torsion angle changes on average from -65° in the dark state to -32° in meta I. The difference is most likely attributable to a change in interaction between the β -ionone ring and the surrounding amino acid residues, due to its displacement towards helix H5. Based on these findings, we conclude that the average conformation about the 6-*s* bond is retained up to meta I in the steps leading to activation of rhodopsin.

Structural analysis for retinal cofactor

Figure 9 shows the structure of retinal obtained in this work inserted into the binding pocket of the rhodopsin X-ray structure¹⁷. The dark-state NMR structure is a refinement of our previous result²⁷. In Figure 9 (a) we compare the NMR structure of retinal deduced with the extended three-plane model (green) to the crystallographic structure¹⁷ (red) in the dark state. The retinylidene nitrogen atoms of the two structures are superimposed, which places the C12 to C15 atoms close to the crystallographic positions, and constrains the Schiff base end of the ligand. Relatively good agreement of the two structures is most likely within the uncertainties of the two methods. Torsional twisting of the polyene stems from localization of the β -ionone ring within its hydrophobic pocket at one end of the chromophore, together with the salt bridge of the retinylidene Schiff base at the other end. The absolute sense of the helical twists of the 12-*s-trans* and 6-*s-cis* conformations manifest the chiral selectivity of the binding site, which is related to the trajectory of the 11-*cis* to *trans* photoisomerization. In the dark-state structure, the C9 and C13 methyl groups point in opposite directions, *i.e.* toward the extracellular and cytoplasmic sides of the membrane, respectively. The distance from the C13 methyl to the closest carbon atom of the Trp²⁶⁵ indole ring is $\approx 4 \text{ \AA}$, suggesting that interactions of retinal with Trp²⁶⁵ help stabilize the dark state conformation⁵¹. Moreover, the configuration of the $-\text{C}=\text{NH}^+$ bond of the protonated Schiff base is *anti* and its hydrogen points oppositely from the C13 methyl, *i.e.* toward the extracellular side in the direction of the counterion Glu¹¹³.

The inset displays the retinal NMR structure in the dark state as obtained with the extended three-plane model (green) compared to the NMR structure calculated using the simple three-plane model (red)²⁷. The simple model leads to steric hindrance of retinal with the side chains of several amino acid residues in the binding site (Tyr¹⁷⁸ and Cys¹⁸⁷ in E2, Met²⁰⁷ in H5, Tyr²⁶⁸ in H6, and Ala²⁹² in H7; *cf.* Results). A negative twist of the C11=C12 double bond is necessary to avoid this steric clash, and maintains the position of the β -ionone ring

in a cavity formed by the surrounding amino acids, which may entail further twisting about the C12–C13 bond²⁷ (*cf.* Results). Twisting about single bonds is insufficient to avoid the steric clash, on account of the different geometry compared to the double bonds, *i.e.* the rotation axes point in a different spatial direction. In fact, such rotations would bring the β -ionone ring closer to the Schiff base, potentially coming into close contact with Trp²⁶⁵. On the other hand, twisting about either the C9=C10 double bond or the C13=C14 bond is analogous geometrically to twisting about the C11=C12 bond. But the distance of the Schiff base N atom from the rotation axis is smaller than for the C11=C12 bond, and would require a correspondingly larger torsional deformation. Hence, we conclude that twisting about the C11=C12 bond is energetically optimal to position the β -ionone ring within its hydrophobic cavity, while maintaining the retinylidene Schiff base near its counterion as in the dark-state rhodopsin structure¹⁷. Last, we note that the absolute sense of the twist about the C11=C12 bond is negative (counterclockwise); whereas the twist about the C12–C13 *s*-bond is positive (clockwise). The twist (strain) about the C11=C12 bond manifests the stereochemical requirements of the binding cleft, and suggests that the torque (stress) for the isomerization is in the negative direction. Similar conclusions regarding in-plane bonding and local twisting involving the C11=C12 and C12–C13 bonds have been reached from combined quantum mechanics/molecular mechanics simulations⁶⁰.

An interesting aspect is that pre-twisting about the 11-*cis* double bond can govern the chromophore movements upon photoexcitation, and thereby affect the ultrafast reaction dynamics. A negative direction of isomerization about the C11=C12 bond is consistent with hybrid quantum mechanics/molecular mechanics simulations, which indicate that steric interaction of the C13 methyl with Ala¹¹⁷ hinders rotation toward positive angles⁵⁹. The positive helical twist about the C12–C13 bond is in agreement with CD and bioorganic studies of locked retinoids³⁴ and with our previous results²⁷. It is noteworthy that the crystal structure of rhodopsin is used only to choose a suitable model for retinal within the binding pocket in the dark state. The geometrical parameters of the three-plane model are based exclusively on solid-state NMR and linear dichroism data. In this sense, the retinal NMR structure is to a certain degree X-ray independent. Obtaining additional NMR data would enable the development of a more sophisticated and accurate retinal model. On the other hand, refinement of the retinal structure by fitting crystallographic electron density profiles using the methyl bond orientations from solid-state ²H NMR with ¹³C distance data is attractive as a future avenue.

In Figure 9(b) we show the NMR structure proposed for retinal in the meta I state (red), as compared to the NMR structure of retinal in the dark state of rhodopsin (green). The retinal structure for meta I assumes a simple three-plane model^{23,34} (*cf.* Figure 8) and is inserted into the dark-state rhodopsin binding cavity. This is justified by the similarity of the electron densities of meta I and the dark state at low to medium resolution²¹. We aligned the Schiff base nitrogen atoms of both structures as a further positional restraint. As can be seen, the *trans* polyene chain is essentially relaxed and is nearly planar^{39,53}, while maintaining a significant twisting of the β -ionone ring. Moreover, the Schiff base end of the retinylidene chromophore is rotated about the polyene chain axis relative to the rest of the molecule, whereas the β -ionone ring is practically unchanged with respect to the dark state²¹. Isomerization is in the negative direction due to the helicity of the C11=C12 bond in the

dark state, together with the steric hindrance within the binding pocket (*vide supra*). Assuming a three-plane model, the Schiff base hydrogen would switch from facing the extracellular side in the dark state to the cytoplasmic side in meta I. This may have implications for a (possibly complex) counterion switch, as discussed elsewhere^{5,6,65}.

The beautiful game: visual function

Readers should note that NMR spectroscopy is exquisitely sensitive to molecular motions over a broad range of time scales²⁴. A chromophore with order parameters close to unity and negative pre-twisting of the C11=C12 bond may explain the dark-state stability of rhodopsin, together with the high regio- and stereoselectivity of the photoreaction. Light instantly converts retinal into a labile species that undergoes ultrarapid 11-*cis* to *trans* isomerization along the reaction coordinate, with a high quantum yield⁶¹. Following vertical excitation from the ground state, the torsional strain gives a steep gradient of the excited state potential energy surface away from the Frank-Condon region^{30,62}. The large driving force yields an acceleration along the reaction coordinate, which can facilitate ultrafast isomerization of the ligand within the rhodopsin binding cavity³⁰. A relatively small conformational change of the ligand in the initial photoproduct, as in the photorhodopsin (and bathorhodopsin) intermediate^{32,61,79}, favors a greater reaction rate. However, the reaction coordinate most likely does not correspond to a simple torsional twisting, but rather entails coupling to other degrees of freedom^{32,68,80}. The extended three-plane model treats the concerted twisting of the retinal polyene, and is compatible with a conical intersection as the minimum energy path between the excited state and ground state potential energy surfaces, with only a small barrier⁸⁰. Tuning or perturbation of different low lying conical intersections may underlie substituent control of photoreaction selectivity⁶³. The photochemical funnel allows for very efficient nonadiabatic decay to the photoproduct ground state in terms of the various molecular modes^{32,80}. In this context, the sensitivity of NMR to molecular motions and thermal fluctuations involving the protein environment may be instrumental in achieving greater insight.

The above conclusions regarding conformational strain are also significant with regard to larger-scale chromophore movements in the activation of rhodopsin^{23,49,51}. The stress due to torsional twisting (frustration) of retinal is appreciable^{34,59,63} in relation to the energy stored by photoisomerization of retinal ($\approx 140 \text{ kJ mole}^{-1}$)⁸¹. By comparison, an energy only $\approx 10 \text{ kJ mole}^{-1}$ is sufficient to shift the meta I to meta II equilibrium from reactant to product⁷³. Transduction of the photonic energy through relief of the torsional strain and other energetic contributions (electrostatic, van der Waals) drives conformation changes that govern receptor activation. The NMR structure indicates that in meta I the 11-*cis* to *trans* isomerization allows a selective relaxation of the torsional strain in the vicinity of the C11=C12 double bond, but not the β -ionone ring. Meta I can be thought of as a pre-activated state, which is distinct from the subsequent meta II intermediate where activation of rhodopsin occurs^{4,7,22,34,51}. Protein binding sites for the retinal ligand involve (*i*) the β -ionone ring within its hydrophobic pocket, (*ii*) the polyene chain, and (*iii*) the protonated Schiff base with its salt bridge. The C9 methyl can be viewed as the anchor point or handle for transmission of the initial impulse due to photoisomerization to the protein. According to our model, *cf.* Figure 9(b), in the pre-activated meta I intermediate the initial impulse most

likely includes a steric clash of retinal with Trp²⁶⁵ due to the chromophore straightening, which triggers outward reorientation of the cytoplasmic part of helix H6. The retinylidene Schiff base is deprotonated in the subsequent meta II state and the salt bridge to its counterion^{5,65} is extinguished, giving an impulse which is now transmitted in the direction of the β -ionone ring. Receptor activation may involve movement of the C9 methyl towards Trp²⁶⁵, due to a translation of the chromophore of ≈ 5 Å in the direction of helix H5⁵¹, together with reorientation of helix H6^{4,78}. This conclusion is in agreement with the model of Borhan et al.⁴⁹, where the β -ionone ring relocates away from Trp²⁶⁵ following light absorption. On the other hand, it differs from the proposal of Spooner et al.²⁰ that in meta II the β -ionone ring maintains its position as found in the dark state.

These findings demonstrate the use of solid-state ²H NMR spectroscopy to investigate the mechanism of light activation of the canonical GPCR rhodopsin. Comparison of meta I to the activated meta II state is expected to benefit our understanding of receptor activation in general. Our data illustrate how solid-state ²H NMR methods can be applied to investigate conformation changes of ligands bound to rhodopsin and other membrane proteins. Moreover, the dynamics of the ligand as revealed by ²H NMR can further illuminate interactions within the receptor binding pocket. Application of this technology to other family A GPCRs can be salutary with regard to ligand-based drug discovery, as well as new pharmaceutical development.

Material and Methods

Preparation of aligned membranes and cryotrapping of meta I

Rhodopsin-containing membranes were isolated from frozen bovine retinas (W. L. Lawson Co., Lincoln, NE) as described¹⁰. Suspensions of rod outer segment membranes in 5 mM HEPES buffer, pH 6.8, were bleached at 4 °C for ≈ 30 min in the presence of 100 mM hydroxylamine by illumination with yellow light (550 nm bandpass filter) using a 150-W light source (FOSTEC; Auburn, NY). Typically 70 mg of rhodopsin were bleached in 20 mL of the above buffer (0.9 μ M). The membrane suspension was centrifuged and 11-*Z*-[5-²H₃]-retinal, 11-*Z*-[9-²H₃]-retinal, or 11-*Z*-[13-²H₃]-retinal³⁹, *i.e.* synthetic 11-*cis*-retinal specifically labeled with ²H at the C5, C9, or C13 methyl groups, in EtOH was immediately added to the opsin-containing membranes in 1.5 molar excess (≈ 10 μ L/mL membrane suspension). Regeneration was carried out for 1.5 h at 37 °C. Rhodopsin was then dissolved in dodecylammonium bromide (DTAB) and recombined with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in a 1:50 molar ratio as described¹⁰. After dialysis, the rhodopsin-containing membranes were resuspended in 5 mM HEPES buffer, pH 6.8, containing 1 mM DTT and 1 mM EDTA prepared from ²H-depleted ¹H₂O. All final samples used for NMR had A_{280}/A_{500} ratios in the range 1.6–2.0 when solubilized in 100 mM DTAB. When not in use the samples were kept at –70 °C. Following the NMR experiments the A_{280}/A_{500} ratio was checked, and was the same as the initial value to within experimental error.

Planar supported membranes containing bovine rhodopsin were prepared by isopotential ultracentrifugation³⁸. Custom-machined inserts included a stainless steel isopotential surface at the base of a black delrin body, and were contained in polycarbonate centrifuge tubes with

an epoxy fill at the bottom. About 150 μL of a membrane suspension containing *ca.* 1 mg of rhodopsin were loaded into each insert, which contained an ultrathin glass plate ($16 \times 6 \times 0.07$ mm; Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany) supported by the stainless steel surface. Membranes were centrifuged at 21 000 rpm ($28\,000 \times g$) for *ca.* 2 hr. After slow evaporation at 4 °C, the membrane film was hydrated by isopiestic transfer using saturated NaBr in ^2H -depleted water with a relative humidity $\approx 63\%$ at 4 °C. As a final step, 25 glass plates were stacked and inserted in a cut-off 8×22 mm NMR tube and sealed with a Teflon plug. Metarhodopsin I was cryotrapped³⁹ by illuminating individual glass plates containing rhodopsin/POPC (1:50) oriented membrane films in 5 mM MES buffer at pH 7 for 1–2 min at 2 °C with green light (550–700 nm) from a 150-W tungsten–halogen light source (FOSTEC; Auburn, NY). The glass plates were immersed in liquid nitrogen, and UV–visible spectra were recorded at 0–2 °C using a locally constructed glass cryostat with quartz windows. The photolyzed samples gave a band at $\lambda_{\text{max}} \approx 479$ nm indicative of meta I, with no contribution due to nonphotolyzed rhodopsin ($<5\%$). Finally *ca.* 25 glass plates were stacked and inserted in an 8×22 mm NMR tube maintained at -70 °C and sealed with a Teflon plug. Samples used for ^2H NMR typically contained 20–25 mg of rhodopsin.

Solid-state ^2H NMR spectroscopy

^2H NMR spectra were acquired using a Bruker AMX-500 spectrometer with a narrow bore magnet (11.7 T). The low signal-to-noise placed demands on the electronics and hardware of the spectrometer system, especially the probe, which was optimized to minimize the temperature-dependent acoustical ringing. The ^2H NMR probe had an 8×12 mm long transverse solenoid as the radiofrequency coil with high-voltage capacitors, and provided a $4.2\ \mu\text{s}$ 90° pulse at 76.77 MHz. A composite-pulse, quadrupolar-echo sequence ($135^\circ_{-x}90^\circ_{+x}45^\circ_{-x}-\tau-135^\circ_{+y}90^\circ_{-y}45^\circ_{+y}-\tau$ -acquisition) was employed, and was phase-cycled to acquire the ^2H NMR signals. The effect of acoustical ringing of the radiofrequency coil was minimized by varying the delay τ from 40 to 70 μs , depending on the temperature. The recycle delay for the pulse sequence was typically 0.1–1.5 s in accord with the $T_{1\rho}$ relaxation time. Fourier transformation was initiated at the quadrupolar echo maximum using locally written data processing software (Matlab; The MathWorks, Inc., Natick, MA); both quadrature channels were employed with no first-order phase correction. Spectra were not symmetrized^{38,41}, which yields an increase in signal-to-noise by $\sqrt{2}$ but can lead to artifacts including virtual quadrupolar splittings⁸². Echo signals were multiplied by a decaying exponential, representing a linebroadening of 500 Hz in the frequency domain. Each ^2H NMR spectrum is the result of $\approx 0.2\text{--}1 \times 10^6$ scans ($\approx 1\text{--}14$ d acquisition). The membrane tilt angle was adjusted manually using a protractor. The presence of nonoriented bilayers was determined from ^{31}P NMR spectroscopy (typically 10%) and was taken into account by spectral subtraction. If necessary, the ^2H NMR signals from water and the natural ^2H abundance of the lipids were subtracted.

Spectral lineshape analysis for static axial distribution

Theoretical ^2H NMR spectra were calculated for the oriented samples by assuming a static uniaxial distribution about the average membrane normal²⁶. The theoretical lineshapes

depend on both the methyl bond orientation θ_B as well as the alignment disorder (mosaic spread), which was assumed to be Gaussian with a standard deviation of σ . In gel-state bilayers, membrane proteins do not undergo significant rotational diffusion³⁵ on the ^2H NMR time scale ($\approx 10 \mu\text{L}$). The quadrupolar frequencies of the two spectral branches read²⁴

$$\nu_Q^\pm = \pm \frac{3}{4} \chi D_{00}^{(2)}(\Omega_{XL}), \quad (\text{A1})$$

where χ is the coupling constant (static $\chi = \chi_Q = 170 \text{ kHz}$; or residual $\chi = \langle \chi_Q \rangle$); an axially symmetric coupling tensor, *i.e.* $\eta = 0$, is assumed. The lineshape of an oriented sample is expressed in terms of the quadrupolar splitting of the corresponding random (powder-type) sample, given by $\Delta \nu_Q^{\text{powder}} = \frac{1}{2}(\nu_Q^+ - \nu_Q^-)$. For a rotating methyl group, the static coupling constant is averaged to $\chi_Q^{\text{eff}} = \chi_Q (3 \cos^2 109.47^\circ - 1)/2 = -\frac{1}{3} \chi_Q = -56.67 \text{ kHz}$. Additional off-axial fluctuations are described by $S_{C_3} = \langle \chi_Q \rangle / \chi_Q^{\text{eff}}$ which is the order parameter of the methyl three-fold (C_3) axis, where the brackets denote a time average. The symbol $\mathbf{D}^{(j)}$ designates the Wigner rotation matrix of rank j , and $\Omega_{XL} = (\tilde{\phi}, \tilde{\theta}, 0)$ are the Euler angles which transform the irreducible components of the coupling tensor from its principal axes system (molecule fixed) to the laboratory coordinate frame, defined by the main magnetic field $\mathbf{B}_0$.

The solid-state NMR spectrum of a spin-1 nucleus such as ^2H is described by the distribution of the spectral intensity as function of the reduced frequencies $\xi_\pm$ for each of the spin $I=1$ spectral branches. For an axially symmetric coupling tensor $\xi_\pm \equiv \nu_Q^\pm / (3/4)\chi$, that is $\xi_\pm = \pm D_{00}^{(2)}(\tilde{\theta}) = \pm \frac{1}{2}(3 \cos^2 \tilde{\theta} - 1) \in [\pm \frac{1}{2}, \pm 1]$ ²⁴. The elements of the Wigner rotation matrix transform by²⁴:

$$D_{00}^{(j)}(\Omega_{XL}) = \sum_{m'=-2}^2 \sum_{m=-2}^2 D_{0m'}^{(j)}(\Omega_{XN}) D_{m'm}^{(j)}(\Omega_{ND}) D_{m0}^{(j)}(\Omega_{DL}). \quad (\text{A2})$$

The first set of Euler angles $\Omega_{XN} \equiv (0, \theta_B, \phi)$ includes the orientation of the principal axes system of the coupling tensor ($X \equiv$ static or residual) relative to the local membrane normal (N), where θ_B is the bond orientation and ϕ is the random azimuthal rotation about the local normal (*cf.* Figure 2). The second transformation $\Omega_{ND} \equiv (0, \theta', \phi')$ treats the disorder of the local membrane normal relative to the average normal (D) to the membrane surface in terms of θ' as well as the random azimuth ϕ' . The last transformation describes the inclination (tilt) of the average membrane normal to the laboratory (L) frame (main magnetic field). The analytical calculation is made simpler by considering rank-1 rotation matrix elements (poor man's closure), giving:

$$\cos\tilde{\theta} = \cos\theta_B \cos\theta'' - \sin\theta_B \sin\theta'' \cos(\phi + \phi''), \quad (\text{A3})$$

$$\cos\theta'' = \cos\theta' \cos\theta - \sin\theta' \sin\theta \cos\phi'. \quad (\text{A4})$$

Here the additional angles $\Omega_{NL} \equiv (\phi'', \theta'', 0)$ describe the overall rotation of the local frame to the laboratory, where θ'' is the tilt of the local normal and ϕ'' is a phase factor that links the two transformations²⁶.

The following general results are then obtained for the lineshape corresponding to the two $I=1$ spectral branches of the ^2H nucleus²⁶:

i. if $\alpha > \gamma > \delta > \beta$ or $\gamma > \alpha > \beta > \delta$ then:

$$|p(\xi_{\pm})| \propto \frac{1}{|\cos\theta|} \int_0^{\pi} \frac{1}{y} K\left(\frac{x}{y}\right) \exp\left(\frac{-\theta'^2}{2\sigma^2}\right) \sin\theta' d\theta', \quad (\text{A5})$$

ii. if $\gamma > \alpha > \delta > \beta$ or $\alpha > \gamma > \beta > \delta$ then:

$$|p(\xi_{\pm})| \propto \frac{1}{|\cos\theta|} \int_0^{\pi} \frac{1}{x} K\left(\frac{y}{x}\right) \exp\left(\frac{-\theta'^2}{2\sigma^2}\right) \sin\theta' d\theta'. \quad (\text{A6})$$

In the above expressions, $x \equiv \sqrt{(\gamma - \delta)(\alpha - \beta)}$ and $y \equiv \sqrt{(\alpha - \delta)(\gamma - \beta)}$, and the cosines of the sum and difference angles are defined as follows: $\alpha \equiv \cos(\tilde{\theta} - \theta_B)$; $\beta \equiv \cos(\tilde{\theta} + \theta_B)$; $\gamma \equiv \cos(\theta - \theta')$; and $\delta \equiv \cos(\theta + \theta')$. The kernel $K(k) = F(\pi/2, k)$ represents a complete elliptic integral of the first kind in the normal trigonometric form:

$$K(k) = \int_0^{\pi/2} \frac{dx}{\sqrt{1 - k^2 \sin^2 x}}. \quad (\text{A7})$$

The 3-D alignment disorder (mosaic spread) is described by a Gaussian distribution of the local membrane normal with respect to the average bilayer normal,

$p(\theta) = (1/\sigma \sqrt{2\pi}) \exp(-\theta'^2/2\sigma^2)$, where σ is the standard deviation about the mean of $\langle \theta' \rangle = 0$. For a random (spherical) distribution, the lineshape corresponds to the well-known Pake doublet:

$$|p(\xi_{\pm})| \propto \frac{1}{|\cos\theta|} \propto \frac{1}{\sqrt{1 \pm 2\xi_{\pm}}}. \quad (\text{A8})$$

The closed-form lineshape treatment for a semi-random, static uniaxial distribution, Eqs. (A5)–(A7), was programmed in Matlab. For the oriented samples, the simulation parameters included the C–C²H₃ bond orientation, the mosaic spread, the residual coupling $\langle\chi_Q\rangle$, and the intrinsic linebroadening. In the case of unoriented (powder-type) sample, the Pake formula, Eq. (A8), was used. To cross-validate the above procedure, an alternative Monte Carlo simulation of the lineshapes was implemented. The lineshape was accumulated numerically by randomly generating the angular variables according to their distribution functions, as in Eqs. (A1)–(A4)²⁶. Essentially identical results were obtained using the two methods.

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Abbreviations used

bR	bacteriorhodopsin
CD	circular dichroism
FWHM	full-width at half-maximum
GPCR	G protein-coupled receptor
HOOP	hydrogen-out-of-plane
meta I	metarhodopsin I
meta II	metarhodopsin II
PDB	Protein Data Bank
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
RMSD	root mean-square deviation

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Figure 1.

Powder-type ^2H NMR spectra for ^2H -labeled retinal in the dark state are indicative of rotating methyl groups with large order parameters. (a)–(c) Experimental ^2H NMR spectra for 11-Z-[9- C^2H_3]-retinylidene rhodopsin, *i.e.* having 11-*cis*-retinal deuterated at the C9 methyl group, in gel-phase POPC membranes (1:50 molar ratio) at pH 7 and $T = -30^\circ\text{C}$ (blue), -60°C (magenta), and -150°C (green), respectively. (d) Theoretical ^2H NMR spectrum for randomly oriented $\text{C}-\text{C}^2\text{H}_3$ groups undergoing fast three-fold rotation on the ^2H NMR time scale ($< (3\chi_Q/8)^{-1} \approx 10 \mu\text{s}$). Probability density $p(\xi_{\pm})$ is plotted against reduced frequency $\xi_{\pm}$ for the two $I=1$ spectral branches²⁴, which is expressed in units of $(3/4)\chi_Q^{\text{eff}} = 41.75 \text{ kHz}$ for rotating methyl groups. (e),(f) Representative ^2H NMR spectra for dark-state 11-Z-[5- C^2H_3]-retinylidene rhodopsin and 11-Z-[13- C^2H_3]-retinylidene rhodopsin, *i.e.* with 11-*cis*-retinal deuterated at the C5 methyl (yellow) or C13 methyl group (red), respectively, in POPC membranes (1:50) at pH 7 and $T = -100^\circ\text{C}$. Theoretical powder-type ^2H NMR spectra for $\text{C}-\text{C}^2\text{H}_3$ groups undergoing axial rotation (continuous color lines) are superimposed on the experimental spectra, with residuals below.

Figure 2.

Structure and orientation of retinal ligand of rhodopsin in membranes are investigated by solid-state ^2H NMR spectroscopy. Geometry of the tilt experiments is shown for aligned bilayers. (Top) 11-*cis*-retinylidene chromophore of rhodopsin in the dark state. For a given methyl group, θ_B is angle of $\text{C}-\text{C}^2\text{H}_3$ bond axis to the local membrane normal $\mathbf{n}$, with static rotational symmetry given by the azimuthal angle ϕ . Alignment disorder is described by angle θ' of $\mathbf{n}$ relative to the average membrane normal $\mathbf{n}_0$, and is likewise uniaxially distributed as characterized by ϕ' . Next, θ is the tilt angle from $\mathbf{n}_0$ to the main magnetic field $\mathbf{B}_0$, about which there is cylindrical symmetry. Lastly, θ'' and ϕ'' are the angles for overall transformation from $\mathbf{n}$ to $\mathbf{B}_0$. (Bottom left) Membrane-bound rhodopsin including N-retinylidene cofactor within its binding cavity. The van der Waals surface of rhodopsin is depicted in light grey, where the seven transmembrane helices are indicated by rods; the N-terminus is at top (extracellular side) and C-terminus at bottom (cytoplasmic side). (Bottom right) Schematic depiction of stack of aligned membranes containing rhodopsin within the radiofrequency coil of the NMR spectrometer, showing geometry relative to the magnetic field.

Figure 3.

Tilt series of ^2H NMR spectra for 11-*cis*-retinal in the dark state depends on methyl bond orientations and mosaic spread of aligned membranes. (a)–(c) ^2H NMR spectra for 11-*Z*-[5- C^2H_3]-retinylidene rhodopsin (blue), 11-*Z*-[9- C^2H_3]-retinylidene rhodopsin (magenta), and 11-*Z*-[13- C^2H_3]-retinylidene rhodopsin (green), respectively. Experimental ^2H NMR spectra are shown as a function of tilt angle θ of the average normal of membrane stack $\mathbf{n}_0$ to magnetic field $\mathbf{B}_0$ for rhodopsin/POPC bilayers (1:50) at pH 7 and $T = -150^\circ\text{C}$. Theoretical lineshapes (continuous color lines) assume a static uniaxial distribution of rhodopsin molecules, with rotating C- C^2H_3 groups of retinylidene cofactor.

Figure 4.

Angular sequence of ^2H NMR spectra for *trans*-retinal in meta I shows differences *versus* dark state. (a)–(c) Tilt series of ^2H NMR spectra for 11-*E*-[5- C^2H_3]-retinylidene rhodopsin, 11-*E*-[9- C^2H_3]-retinylidene rhodopsin, and 11-*E*-[13- C^2H_3]-retinylidene rhodopsin. ^2H NMR spectra are plotted at different values of tilt angle θ for meta I/POPC (1:50) membranes at pH 7 and $T = -100^\circ\text{C}$. Calculated lineshapes (continuous lines) assume a static uniaxial distribution of rhodopsin molecules with rotating methyl groups.

Figure 5.

Error surfaces for ^2H NMR spectra of 11-*cis*-retinal in the dark state give accurate methyl bond orientations. (a)–(c) Root mean square deviation (RMSD) of calculated *versus* experimental ^2H NMR spectra (normalized; *cf.* Figure 3) for rhodopsin with retinal deuterated at C5, C9, or C13 methyl groups, respectively. (d)–(f) Cross-sections through hypersurfaces at left showing well-defined minima in bond orientation and mosaic spread of aligned membranes. Fitting parameters at $T = -150^\circ\text{C}$ are: C5 ($\theta_B = 70 \pm 3^\circ$, $\sigma = 18 \pm 3^\circ$, $\Delta \nu_Q^{\text{powder}} = 39.0 \text{ kHz}$, FWHM = 3.8 kHz); C9 ($\theta_B = 52 \pm 3^\circ$, $\sigma = 21 \pm 3^\circ$, $\Delta \nu_Q^{\text{powder}} = 39.0 \text{ kHz}$, FWHM = 5.0 kHz); and C13 ($\theta_B = 68 \pm 2^\circ$, $\sigma = 20 \pm 2^\circ$, $\Delta \nu_Q^{\text{powder}} = 37.5\text{--}40.0 \text{ kHz}$, FWHM = 3.2 kHz). Global fitting parameters at $T = -60^\circ\text{C}$ (spectra not shown) are: C5 ($\theta_B = 70 \pm 5^\circ$, $\sigma = 18 \pm 4^\circ$, $\Delta \nu_Q^{\text{powder}} = 39.0 \text{ kHz}$, FWHM = 3.8 kHz); C9 ($\theta_B = 52 \pm 5^\circ$, $\sigma = 21 \pm 4^\circ$,

$\Delta \nu_Q^{\text{powder}} = 39.0 \text{ kHz}$, $\text{FWHM} = 5.0 \text{ kHz}$); and C13 ($\theta_B = 68 \pm 4^\circ$, $\sigma = 20 \pm 4^\circ$,
 $\Delta \nu_Q^{\text{powder}} = 37.5 - 40.0 \text{ kHz}$, $\text{FWHM} = 3.2 \text{ kHz}$).

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Figure 6.

Error analysis for ^2H NMR spectra of *trans*-retinal in meta I indicate differences in methyl bond orientations from dark state. (a)–(c) RMSD error hypersurfaces for meta I (*cf.* Figure 4) with retinal ^2H -labeled at C5, C9, or C13 methyl groups, respectively. (d)–(f) Cross-sections through error hypersurfaces displaying minima in bond orientation θ_B and mosaic spread σ . Fitting parameters for meta I at $T = -100^\circ\text{C}$ are: C5 ($\theta_B = 72 \pm 4^\circ$, $\sigma = 24 \pm 3^\circ$,

$\Delta \nu_Q^{\text{powder}} = 37.0\text{--}40.0\text{ kHz}$, FWHM = 3.2 kHz); C9 ($\theta_B = 53 \pm 3^\circ$, $\sigma = 25 \pm 3^\circ$,

$\Delta \nu_Q^{\text{powder}} = 38.0\text{ kHz}$, FWHM = 4.4 kHz); and C13 ($\theta_B = 59 \pm 3^\circ$, $\sigma = 22 \pm 3^\circ$,

$\Delta \nu_Q^{\text{powder}} = 35.0\text{--}40.0\text{ kHz}$, FWHM = 3.2 kHz).

Figure 7. Solid-state ^2H NMR yields structure and orientation of 11-*cis*-retinal in the dark state of rhodopsin. The retinal conformation is described by three planes of unsaturation (designated A, B, C). (a) Simple three-plane model with torsional twisting only about C6–C7 and C12–C13 bonds. (b) Extended three-plane model with additional pre-twisting about C11=C12 double bond. Extracellular side of rhodopsin is at top and the cytoplasmic side is at bottom (*cf.* text).

Figure 8.

Orientation and structure of *trans*-retinal in meta I are illuminated by solid-state ^2H NMR spectroscopy. Three planes of unsaturation (A, B, C) are depicted; the structures are mirror images related by a vertical reflection plane. Extracellular side of rhodopsin points up and the cytoplasmic side is down (*cf.* text).

Figure 9.

Solid-state NMR provides structure of retinal cofactor in the dark and pre-activated meta I states of rhodopsin. Vertical direction corresponds to membrane normal; the extracellular side is up and cytoplasmic side is down. (a) Comparison of NMR structure (green) of 11-*cis*-retinal in the dark state *versus* retinal structure (red) from X-ray crystallography (PDB accession code 1U19)¹⁷. Inset: NMR structure calculated with twisting of polyene about C12–C13 bond only (simple three-plane model; red) compared to structure with twisting about both C11=C12 and C12–C13 bonds (extended three-plane model; green); view angle is with horizontal rotation of β -ionone ring towards observer. (b) NMR structure for 11-*cis*-retinal in the dark state (green) compared to NMR structure of *trans*-retinal in the meta I state (red). (Figure produced with PyMOL⁸³.)

Cover.

Solid-state ^2H NMR structure of retinal ligand of G protein-coupled receptor rhodopsin in the dark (green) and pre-activated metarhodopsin I (red) states. [Four alternate cover figures are shown]

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Table 1

Retinal conformation in dark and meta I states of rhodopsin

dark state	bond orientation (θ_B/deg) ^d			torsional angle/deg		
	C5	C9	C13	C5=C6-C7=C8	C10-C11=C12-C13	C11=C12-C13=C14
² H NMR (this work; -150 °C) ^{b,c} 6- <i>s-cis</i> ,11- <i>cis</i> ,12- <i>s-trans</i>	70±3	52±3	68±2	-65±6 ^d	0 (simple) ^{d,e} -25±10 (extended)	147±4 (simple) ^{d,e} 176±6 (extended)
² H NMR ⁴¹ (-60 °C)	21±5	44±5	30±5	--	--	--
crystal ¹³ (1F88; -173 °C) ^f	74	65	55	-59	-1	171
crystal ¹⁴ (1H2X; -173 °C) ^f	71	54	70	-57	4	154
crystal ¹⁵ (1L9H; -173 °C) ^f	68	56	59	-77	0	171
crystal ¹⁶ (1GZM; -173 °C) ^f	79	61	56	-55	-14	173
crystal ¹⁷ (1U19; -183 °C) ^f	70	48	66	-32	-38	173
meta I state						
² H NMR (this work; -100 °C) ^{b,c} 6- <i>s-cis</i> ,11- <i>trans</i> ,12- <i>s-trans</i>	72±4	53±3	59±3	±32±7 ^d	0	±173±4 ^d

^a Bond orientations refer to angle between the retinylidene C-CH₃ axis and the normal to the local membrane surface.

^b Due to even parity of the quadrupolar interaction in ²H NMR θ_B cannot be distinguished from its supplement $\pi-\theta_B$.

^c Errors correspond to inverse curvature matrix of χ^2 hypersurface for nonlinear regression fits.

^d Torsion angle calculations include additional restraints from solid-state ¹³C NMR dipolar couplings and CD data where available. Random errors are propagated from Eq. (1) and assume a 10% error in the transition dipole orientation and a 4% distance error.

^e Simple three-plane model considers only the C6-C7 and C12-C13 dihedral angles; extended three-plane model also includes torsional twisting about C1=C12 double bond.

^f Atomic coordinates of PDB files were used to calculate the inertial tensor, which was diagonalized to obtain the principal axes. Methyl group