Deuterium Solid-State Nuclear Magnetic Resonance Studies of Methyl Group Dynamics in Bacteriorhodopsin and Retinal Model Compounds: Evidence for a 6-s-Trans Chromophore in the Protein†

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ABSTRACT: Solid-state deuterium NMR spectroscopy is used to examine the dynamic behavior of 18-CD_3 methyl groups in microcrystalline 6-*s-cis*-retinoic acid (triclinic) and 6-*s-trans*-retinoic acid (monoclinic) model compounds, as well as in the membrane protein bacteriorhodopsin (bR), regenerated with CD_3-labeled retinal. Temperature dependent quadrupolar echo line shapes and T_1 anisotropy measurements were used to characterize activation energies for 3-fold hopping motion of the methyl groups. These data provide supporting evidence that the conformation of the retinal chromophore in bR is 6-s-trans. The 6-s-cis conformer is characterized by strong eclipsing interactions between the 8-C proton and the 18-C methyl group protons; the 18-CD_3 group shows an activation energy barrier for methyl 3-fold hopping of 14.5 ± 1 kJ/mol. In contrast, the 18-CD_3 group in the 6-s-trans isomer shows a considerably lower activation energy barrier of 5 ± 1 kJ/mol. In bR, it is possible to obtain an approximate activation energy of 9 kJ/mol. This data is inconsistent with a 6-s-cis conformer but is consistent with the existence of a 6-*s*-trans-retinal Schiff base in bR with some interaction with the protein matrix. These results suggest that methyl rotor motions can be used to probe the van der Waals contact between a ligand and a protein binding pocket. The 6-s-trans conformer of the [16,17-$(CD_3)_2$]retinal in frozen hexane exhibits a major kinetic component with an activation energy barrier of 14 ± 2 kJ/mol. For the [16,17-$(CD_3)_2$]retinal in bR, line shapes indicate an activation energy of 13 ± 2 kJ/mol within error of that for the 6-s-trans model compound. Our results illustrate the use of deuterium NMR techniques to probe local group motions in a relatively large membrane protein like bR and they illustrate a novel solution to a structural problem by measuring molecular dynamics of pertinent functional groups.

Bacteriorhodopsin (bR),[1] the single protein in the purple membrane of *Halobacterium halobium*, contains a retinal chromophore attached via a protonated Schiff base linkage at lysine-216 (Wald, 1968; Stoeckenius *et al.*, 1979; Ottolenghi *et al.*, 1980). It is an integral membrane protein of molecular weight 26 000 daltons and functions as a photoreceptor. Light absorption initiates a photochemical cycle in which conformational change of the retinal chromophore drives proton translocation from the inside to the outside of the bacterial cell and produces a net pH gradient (Oesterhelt, 1976; Henderson, 1977; Lozier & Niederberger, 1977; Michel & Oesterhelt, 1976).

In spite of numerous studies on bR, questions remain as to the exact chemical structure of the protein, its conformational changes, and the mechanism of its proton pumping activity. It has been established that bR occurs in two forms, a dark-adapted state, containing a 2:1 mixture of 13-*cis*- and *all-trans*-retinal, and a light-adapted form, bR568, containing only *all-trans*-retinal (Oesterhelt & Stoeckenius, 1973; Pettei *et al.*, 1977).

The absorption maximum of bR568 is shifted by 5100 cm^{-1} relative to the protonated *all-trans*-retinal Schiff base model compound (λ_{max} = 440 nm), an effect which is termed the opsin shift. This shift is postulated to originate from special protein–chromophore interactions (Oesterhelt & Stoeckenius, 1973), such as cis/trans isomerization, the extent of conjugation between the ring and the polyene, electrostatic interactions between the retinal cyclohexene ring and an adjacent protein ion pair (Nakanishi *et al.*, 1980; Honig *et al.*, 1976; Derguini *et al.*, 1983; Kakitani *et al.*, 1983; Balogh-Nair et al., 1981; Lugtenburg *et al.*, 1986; Spudich *et al.*, 1986), hydrophobic stacking interactions which might occur with the protein Trp side chains (Mogi *et al.*, 1989; Henderson *et al.*, 1990), or electrostatic interaction of a counterion with the protonated Schiff base part of the chromophore (Oesterhelt & Stoeckenius, 1973).

Retinoid compounds with an all-trans polyene chain are known to assume two conformations, a 6-s-cis and a 6-s-trans

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[1] Abbreviations: bO, bacterioopsin; bR, bacteriorhodopsin; bR555, 13-cis, 15-syn component of dark-adapted bR; bR568, 13-trans, 15-anti component of dark-adapted bR; MAS, magic angle spinning; NMR, nuclear magnetic resonance; 18-CD_3-bR, [18,18,18-$^2H_3$]retinal labeled bR; 16,17-$(CD_3)_2$-bR, [16,16,16,17,17,17-$^2H_6$]retinal labeled bR; Trp, tryptophan.

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form. The relative energies of the two conformers depend on the extent of π electron conjugation, which is substantially broken in the 6-s-cis isomer and which extends into the ring for 6-*s-trans*-retinal. This occurs because in the 6-s-trans form there are eclipsing interactions between the 8-C hydrogen and the 16- and 17-methyl groups, forcing the ring out of the polyene plane (Honig *et al.*, 1971, 1975). Chemical shift analysis of the retinals in rhodopsin and bacteriorhodopsin implies that in bR the chromophore is 6-s-trans, while in rhodopsin it is 6-s-cis.

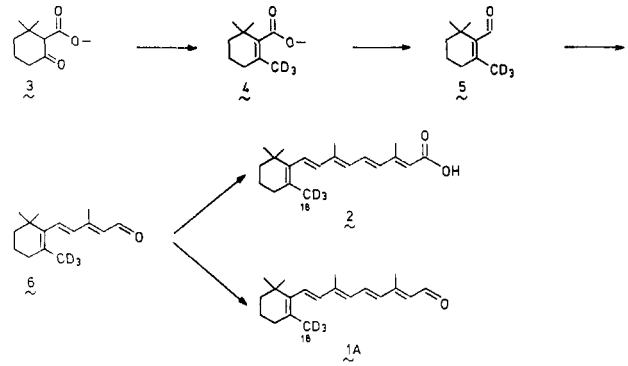
It has been implied that the conformation also affects function. The use of analog compounds has helped to delineate the critical features of the ring position and conformation. The extent of retinal binding to bR does not depend on the ring portion of the chromophore (Motto *et al.*, 1980; Umadevi *et al.*, 1983; Sheves *et al.*, 1984; Gartner *et al.*, 1984; Crouch *et al.*, 1986), although the kinetics of binding and the opsin shift do (van der Steen *et al.*, 1986; Gartner *et al.*, 1984). Retinals locked in either the 6-s-cis or the 6-s-trans form bind to bO, but the opsin shift and optimal proton pumping efficiency are both enhanced by the presence of a 6-s-trans conformation (van der Steen *et al.*, 1986). In contrast, the region near the Schiff base has distinct steric limitations and the length of the polyene side chain is also important for the light-induced photocycling and proton pumping of these pigments (Crouch *et al.*, 1986). It has been suggested that the ring conformation positions the chromophore among the protein α-helices so that the functional parts of the retinal are at the proper distance from the protein residues involved in proton transfer (Muradin-Szweykowska, 1984).

The purpose of this report is to clarify the question of the conformation about the 6-s bond and the relationship between conformation and the dynamics of the methyl groups on the cyclohexene ring. Harbison *et al.* (1985a) provided initial solid-state NMR evidence that the retinal chromophore in bR is in the 6-s-trans conformation and also has additional perturbations from its environment. Using ^{13}C MAS NMR, they measured the ^{13}C isotropic chemical shift and chemical shift tensor elements at the 5-C position of retinal in bR and compared these data with the data obtained for 5-C in 6-*s-cis*- and 6-*s-trans*-retinoic acid model compounds. For bR, and for the 6-s-trans compound, a downfield shift of the σ_{33} element was observed for the 5-C resonance relative to the 6-s-cis model compound.

In addition, Harbison *et al.* observed a long carbon T_1 for the 18-C methyl group (Harbison *et al.*, 1985a), which presumably reflects a short motional correlation time τ_c. Since T_1 is a double-valued function, it is also possible that the T_1 could correspond to a slow motion of the CH_3 group although it is the less precedented of the two possibilities. To resolve that point, preliminary solid-state deuterium NMR experiments on deuterated β-ionone derivatives were performed. We have also decided to pursue quantitative estimates of activation energies for the methyl group motion in direct 6-s-cis and 6-s-trans models and attempt to measure the barrier for the rotation in a retinal CD_3-labeled bR sample.

Solid-state deuterium NMR has been used extensively to study molecular dynamics in both small and large molecular systems (Seelig, 1977; Griffin, 1981; Davis, 1983; Spiess, 1985; Griffin *et al.*, 1987). Due to the size of the deuterium quadrupole coupling (e^2qQ/h = 1.67 × 10^5 Hz for a C–D bond), deuterium quadrupolar echo lines shapes provide a convenient method for studying molecular processes associated with τ_c in the range 10^{-3}–10^{-7} s (Beshah *et al.*, 1987; Beshah & Griffin, 1989), while measurement of the anisotropy of the deuterium spin lattice relaxation (T_1) extends the motional time scales being probed from 10^{-7} to 10^{-10} s (Torchia & Szabo, 1987).

Scheme 1: Synthetic Route for [18-CD_3]Retinal and [18-CD_3]Retinoic Acid



In this paper, we present deuterium solid-state NMR quadrupolar echo line shape and T_1 anisotropy data for 18-CD_3-bR, [18-CD_3]-6-*s-cis*- and -6-*s-trans* retinoic acid, 16,-17-$(CD_3)_2$-bR, and [16,17-$(CD_3)_2$]-*all-trans*-retinal in frozen hexane. From computer simulations of the line shapes, rate values are obtained as a function of temperature and activation energy values are derived. By comparing line shapes and energetic estimates for 18-CD_3-bR and [18-CD_3]-6-*s-trans*- and -6-*s-cis*-retinoic acid, we are able to determine unambiguously that the retinal chromophore is in the 6-s-trans conformation, and we demonstrate that the 18-CD_3 methyl motion is a very sensitive probe of chromophore structure. These results, together with recently measured 8–18 and 8–16 homonuclear ^{13}C–^{13}C through-space distances (Creuzet *et al.*, 1991; McDermott *et al.*, in press), confirm that retinal in bR is 6-s-trans.

EXPERIMENTAL METHODS

Synthesis of Deuterated [CD_3]-all-trans-Retinoic Acid and [16,17-$(CD_3)_2$]-all-trans-Retinal (Spijker-Assink et al., 1988). The synthesis of [18-CD_3]-*all-trans*-retinal (**1**) and [18-CD_3]-*all-trans*-retinoic acid (**2**) is summarized in Scheme 1. Reaction of the cyclic β-keto ester **3** with CD_3MgI, followed by dehydration using $POCl_3$, affords **4**. Dibal reduction of the ester function and subsequent oxidation by MnO_2 gives **5**. The aldehyde **5** is extended by a Horner–Emmons reaction with 5-C-phosphonate–nitrile followed by Dibal reduction to **6**. This two-step sequence is repeated once more and converts **6** into the [18-CD_3]-*all-trans*-retinal (**1**). A Horner–Emmons coupling of **6** with 5-C-phosphonate–ethyl ester gives ethyl retinoate. Saponification leads to the [CD_3]-*all-trans*-retinoic acid (**2**). The deuterium incorporation of **1**, determined from the mass spectrum, is 97.5% $^2H_3$. The retinoic acid (**2**) is expected to have the same enrichment.

[16,17-$(CD_3)_2$]Retinal was prepared according to the published procedure for the synthesis of [16,17-$^{13}C_2$]retinal (Gebhard et al., 1989) using acetone-d_6 instead of [1,3-$^{13}C_2$]-acetone. Mass spectral analysis gave a deuterium incorporation of 92%.

Crystallization of 6-s-cis- and 6-s-trans-Retinoic Acid. [18-CD_3]Retinoic acid was crystallized in the 6-s-cis and 6-s-trans conformations according to procedures described by Harbison et al. (1985b). The 6-s-cis (triclinic) modification of *all-trans*-retinoic acid was obtained by dissolving the material in an isooctane solution and heating the reaction mixture up to 90 °C for a few minutes. Subsequent cooling and evaporation of solvent produced 6-*s-cis*-retinoic acid

crystals. The 6-s-trans (monoclinic) form was prepared by dissolving retinoic acid in a minimum amount of hexane and bringing the reaction mixture to a temperature no higher than 45 °C. The reaction mixture was filtered hot and allowed to cool slowly. Monoclinic retinoic acid crystals appear within 1 or 2 days following dissolution in hexane. Both forms were checked by comparing the crystals microscopically with published morphologies (Stam, 1972; Stam & McGillivary, 1963). ^{13}C MAS NMR experiments were also performed to record 5-C isotropic chemical shift and shift tensor values to confirm that the samples were purely the 6-s-cis or the 6-s-trans isomers. Approximately 80 mg of [18-CD_3]-6-*s-cis*-retinoic acid and only 10–20 mg of the pure 6-s-trans isomer were used for NMR experiments.

Regeneration of Bacteriorhodopsin with Deuterated Retinals. Incorporation of deuterated retinal into bR was accomplished by bleaching native purple membrane with 0.5 M hydroxylamine hydrochloride (pH = 8.0) and incubating the protein in a warm water bath (T = 35 °C) overnight in the dark. The pH was raised to 8.2 during the last 2–3 hours of the bleaching process. The bleached membrane was subsequently washed several times with 50 mM HEPES buffer at pH 6.5 to neutralize the solution and remove residual NH_2-OH.

Purple membrane was regenerated with an equimolar ratio of [18-CD_3]- or [16,17-$(CD_3)_2$]-*all-trans*-retinal by additions of aliquots of retinal of ~1 mg/mL concentration in dry ethanol to a 1 mg/mL aqueous solution of the bleached bR. The process was followed spectroscopically by monitoring the increase in absorbance at 560 nm relative to that of retinal oxime at 360 nm (Oesterhelt & Schulmann, 1974). Excess retinal from the regeneration and retinal oxime were subsequently removed by washing the membrane ~20 times with a 2% solution of bovine serum albumin containing 1 mM NaN_3 (Sigma Chemical Co.). Finally, the protein samples were washed extensively with deuterium-depleted water, with the last wash containing 0.1 mM NaN_3. Solid-state deuterium NMR spectra of dark-adapted bR typically consisted of wet pellets of purple membrane containing ~80–100 mg of protein. The protein samples were packed in sapphire rotors, normally used for magic angle spinning experiments, because these holders have low natural abundance deuterium background signals.

NMR Experiments. The deuterium NMR spectra were recorded at 61.05 MHz on a homebuilt spectrometer, using a single resonance deuterium probe, with an evacuated cryostat as probe cover for variable temperature experiments (i.e., to reach temperatures as low as –185 °C). For all experiments, the deuterium pulse lengths and phases were carefully adjusted with a point sample of D_2O, and the quadrupole pulse sequence $90°_x$–τ–$90°_y$–τ–signal was employed for signal observation. Quadrature detection with a dwell time of 2 μs was used, and 90° ^2H pulse lengths were typically ~2.2 μs. Phase cycling through a set of eight quadrupole echoes during signal averaging was employed to eliminate artifacts from DC offsets and quadrature phase errors (Griffin, 1981). The repetition times used for data acquisition were at least five times longer than T_1, ranging from 3 s at room temperature to 30 s at T = –185 °C for retinal in frozen hexane. For T_1 measurements, a 180° pulse was applied for selective inversion prior to the quadrupole echo sequence as follows:

$$180°\text{–}\tau_1 \text{ delay (ms)–}90°_x\text{–}\tau\text{–}90°_y\text{–}\tau\text{–signal}$$

Quadrupole echo line shape spectra on 6-*s-cis*- and 6-*s-trans*-retinoic acid model compounds, as well as [16,17-$(CD_3)_2$]-retinal in hexane, could be acquired in 1 h or less with an excellent signal-to-noise ratio, whereas the 18-CD_3- or 16,-17-$(CD_3)_2$-bR samples required 4–8 h of signal averaging for acceptable spectra. Each T_1 inversion recovery experiment typically had 8–15 delay points and required 10–12 h of data acquisition, due to the loss of signal intensity at the T_1 zero crossing delay. For this reason, it proved difficult to perform T_1 anisotropy measurements at the zero crossing time on bR. The probe temperature was measured with a copper constantan thermocouple calibrated at ice and liquid nitrogen temperatures.

Computer Simulations of Deuterium Line Shapes. The details of the program and line shape calculations have been presented by Wittebort *et al.* (1987), with a recent modification to increase the computational speed. Corrections for finite pulse width effects (Bloom *et al.*, 1980) are included in the spectral calculations for ~2.0–2.4-μs 90° times. The rate of rotational jump is obtained by a visual comparison of the experimental and theoretical spectra. Errors were estimated and appropriately propagated assuming a 30% error in rate constant, a 2 °C error in the sample temperature, and 50% error in the theoretical kinetic prefactor in the cases for which this number was fixed. The error contribution due to the existence of minor kinetic components was neglected.

RESULTS

[18-CD_3]-6-s-cis-Retinoic Acid. Deuterium quadrupole echo spectra and corresponding simulated line shapes of polycrystalline [18-CD_3]-6-*s-cis*-retinoic acid are shown in Figure 1 for temperatures from T = 22 °C to T = –182 °C. At room temperature, the spectrum is an axially symmetric and motionally narrowed Pake pattern with a spectral width of 40 kHz. As the temperature is lowered from –145 °C to –175 °C, the signal intensity decreases, and the spectrum assumes the characteristic triplet line shape of a methyl group undergoing intermediate exchange. Around T = –165 °C, the signal intensity is severely diminished and small features on the edges of the center signal become apparent. These features are attributed to a small amount of a second crystal form of 6-*s-cis*-retinoic acid, although recrystallization of [18-CD_3]-6-*s-cis*-retinoic acid did not remove these additional spectral features. The amount of this amorphous material was estimated by comparing intensity losses in the intermediate exchange motional regime, indicating a value less than ~5% of the total sample weight.

The low-temperature line shapes are successfully reproduced by including one major kinetic component of deuterons undergoing three-site exchange and several minor components in the spectral calculations. The rates for the major kinetic component are presented in Table 1. This component, which we know to be the triclinic crystal form by ^{13}C studies of the same material, has the slowest motional rate, giving a nearly static spectrum at T = –182 °C, a triplet line shape at T = –165 °C and T = –175 °C, and a fast-limit spectrum at room temperature. The rates for this kinetic component are also plotted in Figure 7.

Anisotropic relaxation of the deuterium powder line shape in an inversion recovery experiment was used to provide information on both the rate and mechanism of molecular motion in the fast-limit line shape regime. The data and simulations for the 6-*s-cis*-retinoic acid at room temperature, shown in Figure 2, display pronounced anisotropy. The agreement between experimental and simulated spectra was very good and gave the rates indicated in Table 1 and plotted in Figure 7.

Table 1: Rates and Activation Energies for Methyl Three-Site Hop

T, K	18-C: 6-s-cis[a]	18-C: 6-s-trans[b]	18-C: bR[c]	16,17-C: 6-s-trans[d]	16,17-C: bR[e]
91	2.25×10^4				
95					8.65×10^5
98	5.06×10^4		$>5 \times 10^7$		
101				1.71×10^5	
108	3.84×10^5				
113	1.30×10^6				
116		2.1×10^{10}			
117				3.84×10^5	
118	1.94×10^6				
123			5×10^6		2.96×10^6
126				1.95×10^6	
128	4.38×10^6				
140		4.0×10^{10}			
139				2.96×10^6	
139				2.96×10^6	
158					$>5 \times 10^7$
179	2.0×10^8				
198			8×10^9		
228	2.6×10^9				
263	7.6×10^9				
297	1.5×10^{10}	1.2×10^{12}			
E(act), kJ/mol	14.5 ± 1	5 ± 1	9	14 ± 2	13 ± 2

[a] The sample was 18-CD_3-labeled retinoic acid in the triclinic form. Data below 130 K were from line shape analyses, and above that temperature data were from relaxation measurements. Only the predominant kinetic component is reported. [b] The sample was 18-CD_3-labeled retinoic acid in the monoclinic form. Data were from relaxation measurements. Only the predominant kinetic component is reported. [c] The sample was bR regenerated with 18-CD_3-labeled retinal. The lower limit on the rate was estimated from the line shape. [d] The sample was 16,17-$(CD_3)_2$-labeled retinal frozen in hexane, which is known to be in the 6-s-trans form as indicated in the text. All rates were estimated from line shapes. [e] The sample was bR regenerated with 16,17-$(CD_3)_2$-labeled retinal. All rates were estimated from line shapes. Only the predominant kinetic component is reported.

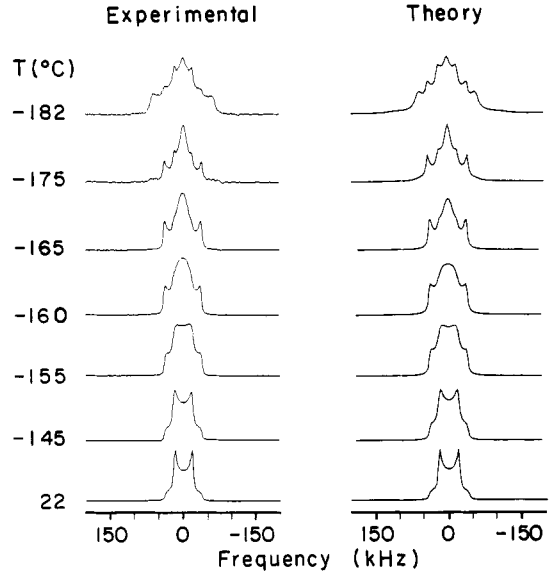


FIGURE 1: Experimental temperature dependent deuterium quadrupole echo spectra (left) and calculated line shapes (right) obtained for [18-CD_3]-6-*s-cis*-retinoic acid. Simulations involve a superposition of three-site jump line shapes with jump rates for the major component reported in Table 1.

[18-CD_3]-6-s-trans-Retinoic Acid. In contrast to the 6-s-cis isomer, the deuterium quadrupole echo line shapes shown in Figure 3 for [18-CD_3]-6-*s-trans*-retinoic acid are simple fast-limit Pake patterns for temperatures down to $T = -185$ °C. At $T = -185$ °C, the spectrum is still that of a 40-kHz-wide Pake pattern, indicating that $k > 10^7$ s^{-1}. The three-site

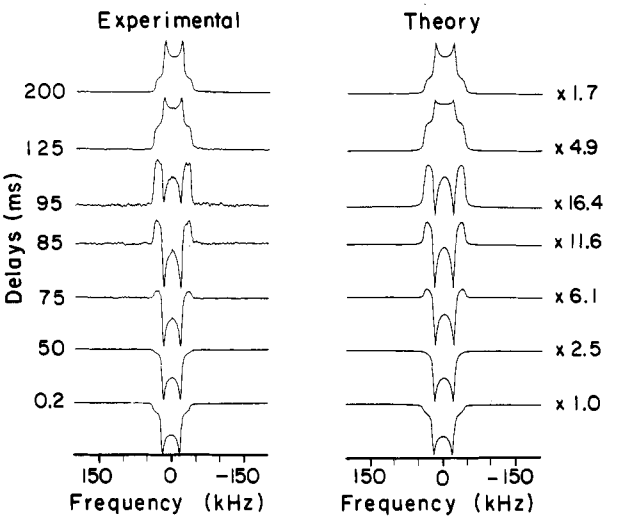


FIGURE 2: T_1 anisotropy experimental (left) and calculated (right) line shapes for [18-CD_3]-6-*s-cis*-retinoic acid at room temperature and with jump rate $k = 1.5 \times 10^{10}$ s^{-1}. The partially relaxed spectra are fitted using a discrete three-site hop which nicely fits the T_1 anisotropy anisotropy at the null near 85 ms. The intensities of both data and simulations have been expanded by the indicated ratios.

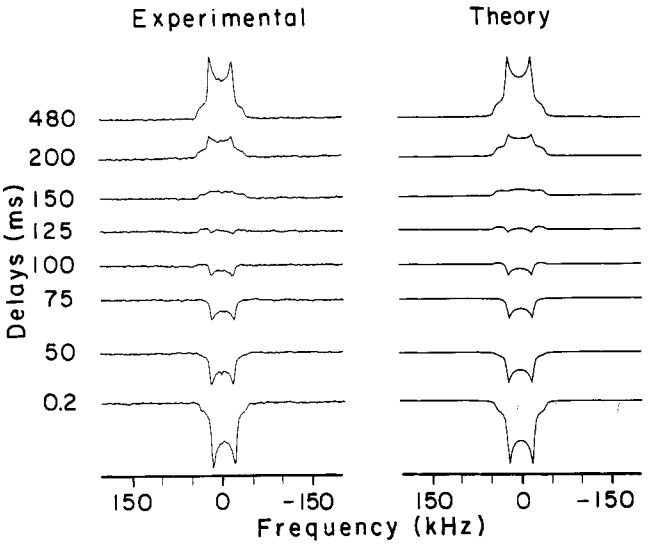


FIGURE 3: T_1 anisotropy experimental (left) and calculated (right) line shapes for the [18-CD_3]-6-*s-trans*-retinoic acid (monoclinic) at $T = 167$ °C using a discrete three-site hop rate of 2.1×10^{10} s^{-1}. At this temperature the zero crossing point occurs at a dealy of 125 ms.

hopping rates for [18-CD_3]-6-*s-trans*-retinoic acid were extracted more accurately from computer simulations of partially relaxed line shapes obtained by inversion recovery (T_1) experiments. Spectra collected at room temperature exhibit a very small T_1 anisotropy at the zero crossing for the trans isomer while the $T = -157$ °C spectra exhibit a distinct T_1 anisotropy relaxation behavior. The rates indicated by these data and simulations are given in Table 1 and Plotted in Figure 7.

18-CD_3-Labeled Bacteriorhodopsin. The quadrupolar echo spectra of 18-CD_3-bR at temperatures of –75 °C and –175 °C (Figure 4) exhibit a spectral breadth of 40 kHz. Like the low-temperature deuterium line shapes observed for [18-CD_3]-6-*s-trans*-retinoic acid, the bR spectra remain fast-limit to near liquid nitrogen temperatures; this is in distinct contrast to the 6-s-cis isomer, which shows static spectra at low temperatures due to eclipsing interactions. We interpret these results as confirmation that the retinal chromophore in bR is in the 6-s-trans conformation. From the line shape data, only

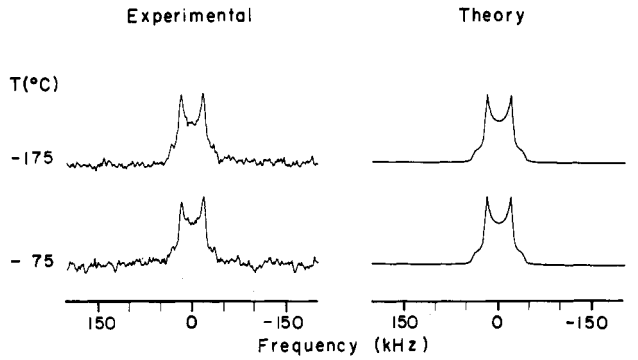


FIGURE 4: Experimental temperature dependent deuterium quadrupolar spectra (left) and calculated line shapes (right) for 18-CD_3-labeled bacteriorhodopsin (bR) for $T = -75$ and -175 °C. The low-temperature spectra of 18-CD_3-bR correspond to fast motionally averaged Pake patterns, indicating that, in bR, 18-CD_3 undergoes fast methyl rotation at the rate $k > 10^7$ s^{-1}. These quadrupolar echo line shapes are similar to the [18-CD_3]-6-*s-trans*-retinoic acid spectra (shown in Figure 2), an indication that, in bR, the retinal chromophore is 6-s-trans.

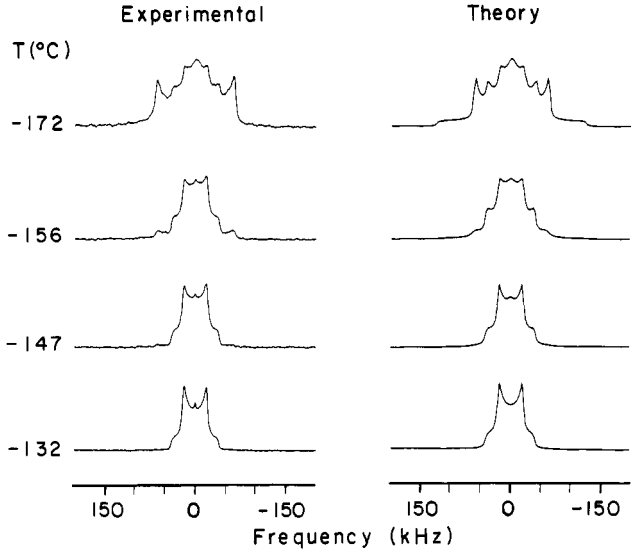


FIGURE 5: Experimental temperature dependent deuterium quadrupolar spectra (left) and calculated line shapes (right) obtained for [16,17-$(CD_3)_2$] retinal in a frozen hexane solution from a superposition of three-site hop line shapes with jump rates as recorded in Table 1.

a lower rate limit $k > 10^7$ s^{-1} could be obtained for every temperature; this constraint on the rate at the lowest temperature indicates an upper limit of activation energy of 9 kJ/mol (see Figure 7). The deuterium results on bR are of further interest since they illustrate the feasibility of investigating the dynamic behavior of a single CD_3 group in a membrane protein of molecular weight = 26 000. The quadrupolar echo line shapes in Figure 4 required ~8 h of data acquisition.

Inversion recovery experiments on 18-CD_3-bR require additional signal averaging and for this reason proved to be technically difficult. Nonetheless we obtained reliable T_1 crossing measurements at -151 and -75 °C of 65 and 35 ms, respectively, which together with the fast-limit line shapes constrained the rates to be 5×10^6 and 8×10^9 s^{-1}, respectively. From these two rates and an assumed prefactor we obtain an activation energy of 9 kJ/mol with error limits of approximately ± 2 kJ/mol.

[16,17-$(CD_3)_2$]- and [18-CD_3]Retinal in Frozen Hexane. Quadrupolar echo spectra were recorded for both [16,17-$(CD_3)_2$]- and [18-CD_3]retinal. Spectra of the [18-CD_3]retinal in hexane obtained at temperatures as low as $T = -184$ °C (data not shown) are analogous to the deuterium line shapes observed for [18-CD_3]-6-*s-trans*-retinoic acid and 18-CD_3-bR; i.e., they are fast-limit, motionally averaged Pake patterns with characteristic spectral breadth of 40 kHz. The 18-CD_3 data establish that, in liquid hexane, retinal is a mixture of 6-s-cis and 6-s-trans isomers, but as the temperature is lowered below freezing, the conformational equilibrium shifts dramatically toward the 6-s-trans isomer. This point was also confirmed by observation of the 5-C ^{13}C resonance by magic angle spinning as a function of temperature in hexane (data not shown).

Deuterium quadrupolar echo spectra of [16,17-$(CD_3)_2$]-retinal in hexane spectra were recorded as a function of

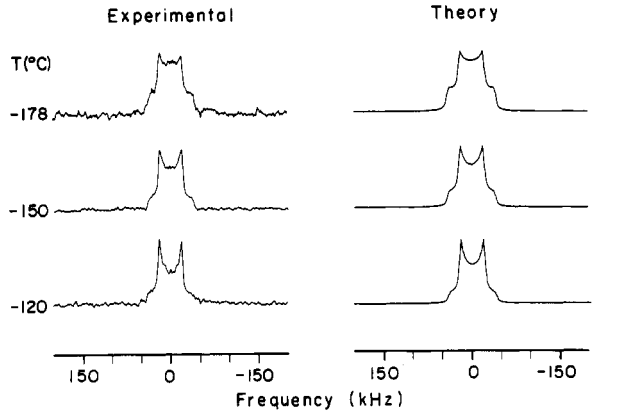


FIGURE 6: Experimental temperature dependent deuterium quadrupolar echo spectra (left) and calculated line shapes (right) for 16,17-methyl group motion in 16,17-$(CD_3)_2$-bR. The deuterium line shapes are only slightly affected by lowering the temperature to $T = -178$ °C, and they reflect the relatively high degree of mobility of the 16,17-methyl groups in bR for three-site hops. As discussed in the text, an estimate for the activation energy of this process is found to be 13 ± 2 kJ/mol.

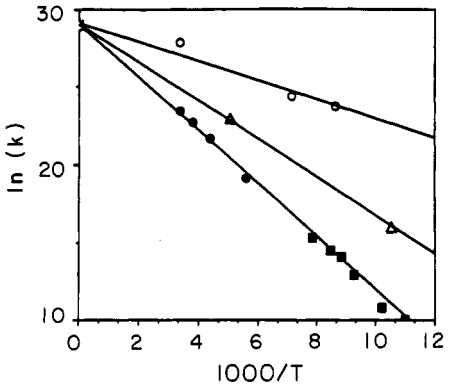


FIGURE 7: An Arrhenius plot of jump rate vs inverse temperature for [18-CD_3]-6-*s-cis*- and -6-*s-trans*-retinoic acid methyl motion and the 18-CD_3-labeled bR. The least-squares fit through all the 18-CD_3 6-s-cis data points includes jump rates obtained from T_1 anisotropy analysis (from $T = 22$ to -94 °C; lower curve, black circles) and from deuterium quadrupolar echo line shapes (from $T = -145$ to -182 °C; black squares). The slope of this line ([18-CD_3]-6-*s-cis*-retinoic acid) corresponds to $E_a = 14.5$ kJ/mol. The least-squares fit through the [18-CD_3]-6-*s-trans*-retinoic acid points (upper curve, open circles) corresponds to jump rates obtained only from T_1 analysis. From the slope of the line, the activation barrier is calculated to be 5 kJ/mol for 18-CD_3 motion in the 6-s-trans isomer. The lower limit for the jump rate for bR at low temperature based on the fast-limit line shapes constrains the activation barrier to be less than or equal to 9 kJ/mol. The T_1 measurements give rise to the data indicated by open triangles, which indicate an activation barrier of approximately 9 kJ/mol.

temperature to provide a data base of characteristic 16,17-$(CD_3)_2$ line shapes for a 6-s-trans conformer. These spectra are shown in Figure 5 with corresponding calculated line shapes. The $T = -132$ °C spectrum exhibits the characteristic motionally averaged deuterium line shape with the addition of a small, sharp resonance at the center of the Pake pattern due to an impurity. Figure 5 illustrates the complexity of the 16,17-$(CD_3)_2$ line shapes which can be simulated fairly accurately by assuming one major kinetic component (ca. 70%) and two minor components, all exercising three-site hopping motions. The fastest moving deuterons are a minor contribution and exhibit fast-limit motionally narrowed Pake pattern line shapes down to $T = -172$ °C. The slowest moving CD_3 deuterons have a marked effect on the appearance of the low-temperature data although they contribute only 10% of the total spectral intensity. We focused on the dominant component. Table 1 indicates the rates of this middle or dominant component, and an Arrhenius plot for these values gives an activation energy of 14 ± 2 kJ/mol. It is likely that the methyl motion of [16,17-$(CD_3)_2$]-6-*s-trans*-retinal would be influenced by interactions with the 8-C proton, and we speculate that the apparent barrier we measured reflects that intramolecular interaction. In that case this energy provides another fingerprint for the 6-s-trans conformation.

16,17-$(CD_3)_2$-Labeled bR. The quadrupolar echo spectra of 16,17-$(CD_3)_2$-bR are shown in Figure 6, from $T = -120$ °C to $T = -178$ °C. The slowing of the methyl rotations in bR is illustrated by the changes in line shape over temperature as shown in Figure 6. The line shape changes between $T = -150$ °C and $T = -178$ °C are not dramatic, making it difficult to calculate activation energies precisely. These deuterium line shapes show a greater degree of motional freedom than the slowest component of 16,17-$(CD_3)_2$ methyls in frozen hexane (Figure 5), but simulations show that the predominant component has kinetics comparable to those of the middle and predominant component of the frozen hexane solution. We concluded that the predominant component in the sample has an activation energy of 13 ± 2 kJ/mol. This value is within error of that measured for the 6-s-trans labeled retinal in hexane and therefore lends additional support to the 6-s-trans assignment in bR. Due to the dominant intramolecular barrier for methyl rotation at this site, it is probably difficult to detect any effect of the packing of the retinal into the protein.

DISCUSSION

In this work we obtained the temperature dependence of the rate of methyl group three-site hopping in a variety of labeled retinal compounds, both protein-bound and site specific model compounds. It is common to fit rate data such as these to an Arrhenius expression, since it is well established that the motion is an activated process at temperatures between 77 and 350 K. We have surveyed the extent of chemical variation in the prefactor (to be discussed in detail separately). In cases with extensive data on methyl group rotations, both from this study and from previously published data, the exponential prefactor did not vary appreciably from the theoretical value of $h/2\pi kT$, or approximately 10^{13} s^{-1} when the experimental temperatures were varied in the range 77–350 K. When analyzing methyl rotation data of poor signal-to-noise ratio, limited temperature range, or multiple kinetic components, we constrained the prefactor to be equal to the theoretical value. When decomposing into multiple kinetic components we constrained the amplitude of each component to be relatively temperature independent. The activation energies obtained from our data and their possible chemical interpretation are discussed below.

[18-CD_3]-6-s-cis- and -6-s-trans-Retinoic Acid and [18-CD_3]Retinal Labeled bR. For activation energy determinations, we employ three-site hopping rates evaluated at various temperatures for the predominant (slowest) component in the 6-*s-cis*-retinoic acid deuterium spectra, utilizing both line shape analysis and T_1 anisotropy analysis for the rates. The rates derived from line shape and relaxation analysis exhibit a common slope (Figure 7). The consistency of the data enables us to conclude that, over the range from room temperature to −180 °C, a single motional mechanism is operative for 18-CD_3 motion. The slope of the $\ln(k)$ vs $1/T$ plot gives an activation energy value of 15.0 ± 1.0 kJ/mol for the discrete three-site hopping motion of the 18-CD_3 methyl of 6-*s-cis*-retinoic acid.

For [18-CD_3]-6-*s-trans*-retinoic acid, the $\ln(k)$ vs $1/T$ plot (upper curve, Figure 7) indicates an activation energy value (6-s-trans) of 5 ± 1 kJ/mol for CD_3 methyl rotation. Interestingly, this is a much lower activation energy than the one for rotation of the corresponding 18-CD_3 methyl in 6-*s-cis*-retinoic acid. The measured barrier for the 6-s-cis conformer is presumably due to eclipsing interactions between the 18-C methyl group and the 8-C proton; from simple modeling one would assume that the protons attain distances much less than the sum of the two van der Waals radii at the transition state of the motion. For the 6-s-trans conformer we assume the barrier to be related to weak interactions with the neighboring molecules and therefore chemically less informative. *For both forms the exponential prefactor for the Arrhenius fit is quite close to the expected value of* $(h/2\pi kT)$.

The data collected on the 18-CD_3-labeled bR indicate a rate greater than 5×10^7 s^{-1} at every temperature. We conclude that the activation energy must be less than or equal to 9 kJ/mol, by utilizing the fast-limit rate at the lowest temperature together with the prefactor at infinite temperature. Using the T_1 values at two temperatures and a theoretical prefactor we can further say that the barrier is approximately 9 kJ/mol although the error in this is hard to estimate. We conclude therefore that the conformation must be 6-s-trans, since the barrier is substantially lower than that for the 6-s-cis compound with an eclipsing interaction. In addition, we interpret the higher barrier as compared with the 6-s-trans model compound as arising from a congested interaction with the protein matrix. It is conceivable that the interaction as evidenced by the methyl group is related to the congested protein environment and causes the retinal to be 6-s-trans in the protein; i.e., perhaps there is not enough room in the pocket for the 16- and 17-methyl groups at that site.

[16,17-$(CD_3)_2$]Retinal in Hexane and 16,17-$(CD_3)_2$-bR. Calculations of energies of activation for 16,17-$(CD_3)_2$ methyl motion in retinal in frozen hexane are complicated by the fact that the line shapes consist of multiple spectral components which cannot be unambiguously assigned to specific chemical entities. The middle component is dominant in terms of intensity and yields sufficient rate information at different temperatures to determine an activation energy value of 14 ± 2 kJ/mol *when the exponential prefactor is constrained to be equal to the theoretical value.* We attribute this kinetic component to 16,17-$(CD_3)_2$ methyl deuterons in 6-*s-trans*-retinal since both the mobility of the 18-methyl group and the 5-C chemical shift indicate that the overwhelmingly predominant species is 6-s-trans at this temperature. The barrier of 14 kJ/mol probably reflects eclipsing interactions between 16,17-$(CD_3)_2$ and the 8-C proton.

For 16,17-$(CD_3)_2$-bR the predominant kinetic component yields an activation energy barrier value of 13 ± 2 kJ/mol when analyzed with a constrained preexponential factor. It is of note that the barriers for rotation of the methyl groups in [16,17-$(CD_3)_2$]retinal in bR and the trans compound are within error, whereas the 16- and 17-methyl groups of the cis conformers would not have an intramolecular eclipsing interaction and might have a somewhat lower barrier. These additional data then support the conclusion of a 6-s-trans conformation of the retinal in bR.

CONCLUSIONS

By comparing deuterium line shapes and anisotropic relaxation with computer calculations for 18-CD_3-bR, [18-CD_3]-6-*s-cis*- and -6-*s-trans*-retinoic acid model compounds, and [16,17-$(CD_3)_2$ retinal in frozen hexane and bound to bR, we obtained activation energies for rotational motion of the deuterated methyl groups. The measured barriers for the 18 methyl group were 14.5 ± 1 kJ/mol in the 6-s-cis form (presumably reflecting interference between the methyl protons and the 8-C proton in the transition state complex), 5 ± 1 kJ/mol in the 6-s-trans form, and approximately 9 ± 2 kJ/mol in bR. For the 16- and 17-methyl groups we obtained rates of 14 ± 2 and 13 ± 2 kJ/mol for a 6-s-trans site model and for bR, respectively. These data offer support for a 6-s-trans conformation in bR. Furthermore they imply a tight packing in the protein around the 18-methyl group of the retinal.

Our deuterium results demonstrate that it is possible to probe the molecular dynamics of individual chemical moieties such as CD_3 in a relatively large membrane protein such as bR and illustrate a solution of structural problem by measurements of the molecular dynamics of pertinent functional groups.

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