

First Observation of Amino Acid Side Chain Dynamics in Membrane Proteins Using High Field Deuterium Nuclear Magnetic Resonance Spectroscopy*

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Robert A. Kinsey, Agustin Kintanar, Ming-Daw Tsai, Rebecca L. Smith, Nathan Janes, and Eric Oldfield†

From the School of Chemical Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

We have obtained the first deuterium NMR spectra of an individual membrane protein, bacteriorhodopsin in the purple membrane of *Halobacterium halobium* R1. Biosynthetic isotopic enrichment with [γ - $^2\text{H}_6$]valine and high field Fourier transform operation permitted rapid data acquisition on intact membranes, including measurement of relaxation times. At some temperatures high quality spectra could be obtained in < 1 s. [$\text{U-}^{14}\text{C}$]Valine tracer studies indicate that $\approx 2\%$ of valine added to the growth medium is broken down and incorporated into other membrane constituents. The NMR results indicate that the valine side chain is a rather rigid structure. Motion about $\text{C}^\alpha\text{-C}^\beta$ is slow ($< 10^5$ s $^{-1}$) at growth temperature, while motion about $\text{C}^\beta\text{-C}^\gamma$ is as expected fast ($> 10^5$ s $^{-1}$) at all accessible temperatures. The activation energy for methyl group rotation from spin-lattice relaxation data between -75 and 53°C is ~ 2.4 kcal/mol, in good agreement with previous ^1H NMR studies on solid alkanes. Preliminary data on [γ - $^2\text{H}_6$]valine-labeled *Acholeplasma laidlawii* B (PG9) cell membranes are also presented. Our results strongly suggest that it should now be possible to observe in great detail the motions of any type of amino acid side chain in membrane proteins, including the effects of lipid composition on protein dynamics.

work has been to determine, at a molecular level, how it is that sterols or other lipid molecules may affect membrane function, particularly the activity of membrane-bound enzymes (18, 19), and one of the more unexpected results of the studies to date is that membrane proteins have been shown to have relatively small effects on lipid order (16, 17, 20-22), in contrast to the very large ordering effects exerted by the sterol cholesterol (1, 12-14, 20, 21), due presumably to the mobile nature of the protein surfaces (16, 17, 20-24). Because of this unexpected result, it appeared to us that new approaches toward determining the role of lipids and sterols in membrane structure based on the direct observation of the effects of these species on protein (enzyme) static and dynamic structure should be developed. The first steps in implementing this strategy are clearly, first, to be able to observe signals from the membrane proteins themselves and to study the more global aspects of their dynamics, followed later by resolution, assignment, and determination of the static and dynamic structure(s) of specific groups in specific proteins.

In this communication we present the first observation of resonances from a specific group in a single type of amino acid in a defined membrane protein, the purple membrane protein, bacteriorhodopsin, from the extreme halophile *Halobacterium halobium* (R1). We show that with sufficiently sensitive NMR instrumentation it is now possible to study in some detail amino acid dynamics in this membrane protein. Such observations should allow comparison of motions in membrane proteins and protein crystals (23-27), where magnetic ordering techniques have already permitted residue resolution and assignment (25-27), and of course will facilitate direct observation of the effects of lipids and sterols on protein structure. We therefore present in this communication the first detailed study of the dynamics of an amino acid residue in a functional biological membrane, the purple membrane of *H. halobium*, and also show that these experiments may be extended to other membrane systems such as the cell membrane of *Acholeplasma laidlawii*. Our results also directly complement the static structural information currently being obtained on *H. halobium* using neutron beam methods (28).

MATERIALS AND METHODS

Nuclear Magnetic Resonance Spectroscopy—We used a "home-built" Fourier transform NMR spectrometer to record all spectra. It consists of an 8.5 Tesla 3.5-inch bore high resolution superconducting solenoid (Oxford Instruments, Osney Mead, Oxford, United Kingdom) together with a variety of digital and radiofrequency electronics, and a Nicolet 1180 computer interfaced to a Nicolet NIC-2090 50-ns transient recorder system (Nicolet Instrument Corporation, Madison, WI). Spectra were recorded using an 800- μl sample volume and a quadrupole echo (4) pulse sequence, using a 90° pulse width of about $3\ \mu\text{s}$. In almost all instances no phase corrections were required after Fourier transformation.

Spectral Simulations—Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corporation Cyber-175 system as described elsewhere (16).

Synthesis of Deuterium-labeled Amino Acid—We synthesized the labeled amino acid, [γ - $^2\text{H}_6$]valine, by reduction of [$^2\text{H}_6$]acetone (Merck, Sharpe and Dohme, Montreal, Canada) with NaBH_4 , followed by conversion of the [$1,3\text{-}^2\text{H}_6$]2-propanol produced to [$1,3\text{-}^2\text{H}_6$]2-bromopropane using concentrated HBr . The dried 2-bromopropane was then coupled with sodio-diethylacetamidomalonate (29), and the product hydrolyzed and decarboxylated to give [γ - $^2\text{H}_6$]valine. Purity was confirmed by thin layer chromatography on cellulose plates and by ^1H NMR spectroscopy at 90 MHz.

For almost 10 years now, there has been a tremendous interest in using deuterium nuclear magnetic resonance spectroscopy to study the structure of the lipid constituents of model (1-7) and "intact" biological membranes (8-11), particular emphasis having been placed on elucidating the nature of the interaction(s) between lipids and proteins and between lipids and sterols (1, 12-17). One of the goals of this type of

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Cell Growth—*H. halobium*, strain R1, were grown in a salt medium basically according to Onishi *et al.* (30), and purple membranes were isolated according to Becher and Cassim (31). *A. laidlawii* B (PG9), obtained from the United States National Institutes of Health Research Resource Reagent Center, were grown basically as described previously (32) except that avidin (25 units liter⁻¹) was added to prevent endogenous lipid synthesis (33), with possible ²H-label incorporation into lipid. *A. laidlawii* membranes, prepared by osmotic lysis as described (8), were collected by centrifugation at 100,000 × *g*. Both *H. halobium* purple membranes and *A. laidlawii* cell membranes were exchanged with ²H-depleted water (Aldrich Chemical Co., Milwaukee, WI) prior to NMR spectroscopy to remove most background HO²H. Samples which had not been so exchanged gave similar ²H NMR spectra, except the HO²H peak was much larger. To check for breakdown of deuterated valine, and its reincorporation into other amino acids or lipid, we grew both systems in the presence of [U-¹⁴C]valine (New England Nuclear) at a level of 50 μCi liter⁻¹. Samples were harvested in the normal manner, then were lipid-extracted (8) and the protein fractions hydrolyzed, followed by analysis using two-dimensional chromatography on cellulose plates, autoradiography, and direct counting after elution from the TLC plates.¹ It is estimated that less than 2–3% of total counts were scrambled away from the valine. Because of these very low levels, it was assumed that the ²H signals observed originated essentially entirely from labeled valyl side chains.

RESULTS AND DISCUSSION

The background necessary for interpretation of the ²H NMR spectra of membranes is discussed in some detail elsewhere (2, 14). The allowed transitions correspond to +1 ↔ 0 and 0 ↔ -1 and give rise to a quadrupole splitting of the absorption line ($\Delta\nu_Q$) with separation between peak maxima of

$$\Delta\nu_Q = \frac{3}{2} \frac{e^2 q Q}{h} \frac{3 \cos^2 \theta - 1}{2} \quad (1)$$

$e^2 q Q/h$ is the rigid lattice quadrupole coupling constant, about 168 kHz in aliphatic CD bonds (34, 35), and θ is the angle between the principal axis of the electric field gradient tensor (usually the CD bond axis) and the magnetic field, H_0 . In the case of molecular motion, it is necessary to take an average in time of $3 \cos^2 \theta - 1$ for motions faster than 168 kHz, in which case it is frequently convenient to express the results in terms of an order parameter for the C—D bond, S_{CD} , such that

$$\Delta\nu_Q = \frac{3}{4} \frac{e^2 q Q}{h} S_{CD} \quad (2)$$

We show in Fig. 1 the first requirement for the study of membrane proteins by NMR spectroscopy, the detection of signals in a reasonable period of time. The result of Fig. 1 was obtained in only 0.3 s of data acquisition. Such rapid data acquisition was possible since the valine residues incorporated were highly deuterated, there are some 21 valines (42 methyl groups, 126 deuterons) in bacteriorhodopsin (36, 37), we used a low temperature (−100 °C) to decrease the spin-lattice relaxation time (T_1), thereby permitting rapid pulsing, and the quadrupole splittings are relatively narrow. The result of Fig. 1 is nevertheless extraordinarily promising, indicating the feasibility of observing any type of group in this membrane protein, although data acquisition will still take numerous hours for more dilute labels, especially when relaxation times are long.

The quadrupole splitting of the sample of Fig. 1, from a spectral simulation, is 40 ± 1 kHz. For purposes of comparison we show in Fig. 2 ²H NMR spectra of [β -²H₁]valine and [γ -²H₆]valine, in the solid state at room temperature, together with their simulations. The spectrum of [β -²H₁]Val, Fig. 2A, is characteristic of a system totally immobile on the time scale

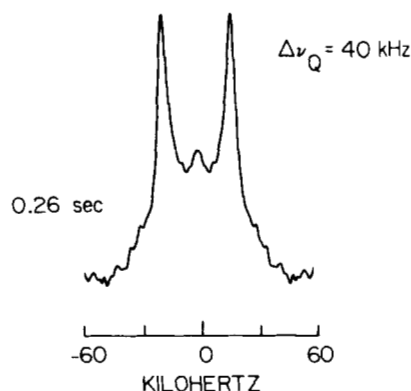


FIG. 1. Deuterium Fourier transform NMR spectrum of purple membrane. Deuterium Fourier transform NMR spectrum of bacteriorhodopsin containing biosynthetically incorporated [γ -²H₆]valine residues in the purple membranes of *H. halobium* R1 in excess deuterium-depleted water at ~ -100 °C, obtained using a quadrupole echo pulse sequence at 55.3 MHz, corresponding to a magnetic field strength of 8.5 Tesla. 10 scans, 26 ms recycle time (total time = 260 ms), $\tau_1 = \tau_2 = 50$ μs, 3.5 μs 90° pulse widths, 2 MHz digitization rate, 2048 data points, line broadening = 2000 Hz.

of the reciprocal of the anisotropy of the quadrupole coupling constant, that is to say on an approximately 1-μs time scale. The full rigid-lattice width of ~ 126 kHz, corresponding to a static quadrupole coupling constant $e^2 q Q/h$ of ~ 168 kHz (34, 35) is manifest. In Fig. 2B, however, we see the spectrum of the amino acid [γ -²H₆]valine, where fast methyl group rotation has averaged the static quadrupole coupling constant by a value of $\sim \frac{1}{2}(3 \cos^2 111.3^\circ - 1)$, i.e. by a factor of about 0.3 (15, 34) and a motionally averaged spectrum having a breadth of ~ 40 kHz is obtained. When we observe the ²H Fourier transform NMR spectra of membrane proteins, perhaps somewhat surprisingly, very similar results are obtained (Fig. 1). Observation of a motionally averaged quadrupole splitting of about 40 kHz (Figs. 1 and 2B) indicates unequivocally that there is very fast motion about only the C^β-C^γ bond in both free valine and in the labeled bacteriorhodopsin, motion about the C^α-C^β bond being very slow on our NMR time scale, such that no motional averaging of the quadrupole interaction occurs due to this motion. If motion about C^α-C^β were fast, then a methyl group splitting of about 13 kHz would be obtained, i.e. the quadrupole splitting would have to be reduced by another factor of ~ 3 (Fig. 2C). No such behavior is seen either in the purple membrane of *H. halobium* in the temperature range -100 °C to 53 °C (Fig. 2D),² or in the case of the *A. laidlawii* cell membrane between -29 °C and 37 °C, the temperature of growth (Fig. 2E).

In the case of bacteriorhodopsin in the purple membrane of *H. halobium*, the quadrupole splittings are remarkably temperature-independent (Fig. 2D), ranging from the rigid lattice valine $\Delta\nu_Q = 39 \pm 1$ kHz (due solely to fast Me rotation) below ~ -30 °C (found also in the model system [γ -²H₆]valine, Fig. 2B) to ~ 33 kHz at 60 °C. Please note however that above ~ 25 °C the spectra become considerably less "sharp" than those obtained at low temperature, due either to the onset of additional slow motions (38), or perhaps more likely to the occurrence of a broader distribution of $\Delta\nu_Q$ values, due to the basic heterogeneity of the purple membrane. In any case, as viewed from the biosynthetically incorporated [γ -²H₆]valine quadrupole splittings, the purple membrane remains a remarkably ordered structure over this wide range of temperature, the molecular order parameter (S_{mol}) being ~ 0.83 at growth temperature (2, 39). Results with the *A. laidlawii* membrane up to the temperature of growth exhibit similar ²H NMR spectra

¹ Rebecca L. Smith, Bernard Montez, T. Michael Rothgeb, and Eric Oldfield, unpublished results.

² Unpublished results.

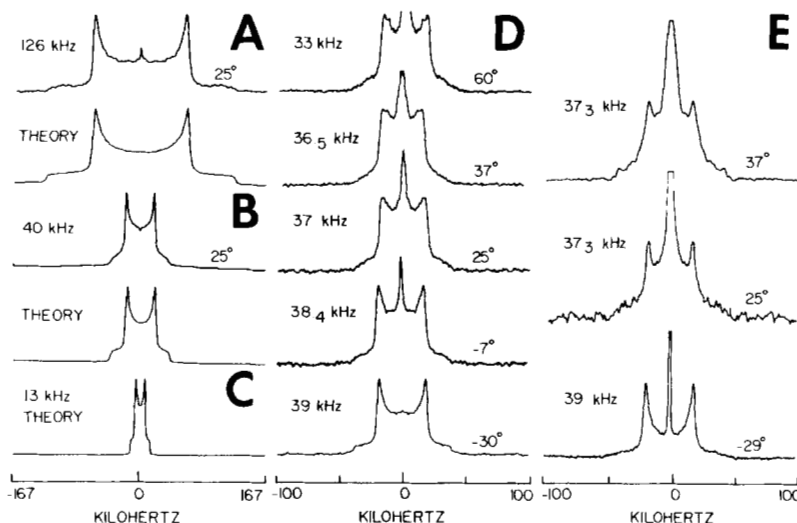


FIG. 2. Experimental and theoretical deuterium NMR spectra of deuterated valines and of deuterio-valine-labeled cell membranes. A, experimental and theoretical ^2H NMR spectra of $[\beta\text{-}^2\text{H}_1]\text{valine}$, 23 °C, 180 scans, recycle time = 31 s, $\tau_1 = \tau_2 = 50 \mu\text{s}$, 3 μs 90° pulse widths, 2 MHz digitization rate, 2048 data points, line broadening = 1500 Hz. The simulation used $\Delta\nu_Q = 126 \text{ kHz}$ and 2δ (W , the linewidth, Ref. 14) = 1000 Hz. B, experimental and theoretical ^2H NMR spectra of $[\gamma\text{-}^2\text{H}_6]\text{valine}$, 23°, 217 scans, recycle time = 590 ms, $\tau_1 = \tau_2 = 50 \mu\text{s}$, 3 μs 90° pulse widths, 2 MHz digitization rate, 2048 data points, line broadening = 400 Hz. The simulation used $\Delta\nu_Q = 40 \text{ kHz}$ and $2\delta = 100 \text{ Hz}$, corresponding to a C^2H bond angle of $\sim 110^\circ$.

(Fig. 2E), although spectral signal-to-noise ratios with this membrane system are less than those obtained with the *H. halobium* purple membranes, due to the lower protein-lipid ratio (40, 41).

The high signal-to-noise ratios of Figs. 1 and 2 also permit the direct study of amino acid side chain dynamics via determination of NMR relaxation rates and of their temperature dependencies. We present in Fig. 3A a series of partially relaxed Fourier transform ^2H NMR spectra of $[\gamma\text{-}^2\text{H}_6]\text{valine}$ -enriched *H. halobium* purple membranes, in excess deuterium-depleted water at the growth temperature of 37 °C obtained using a conventional inversion recovery pulse sequence modified for solids, *i.e.* ($180^\circ\text{-}\tau_3\text{-}90^\circ\text{-}\tau_1\text{-}90^\circ\text{-}\tau_2\text{-Echo-T}$) where T is the period of repetition of the pulse sequence ($>5T_1$), τ_1 and τ_2 are fixed delays of $\sim 70 \mu\text{s}$, and τ_3 is a variable delay between the inverting (180°) and sampling (90°) pulses (42). We show in Fig. 3B the results of three typical relaxation measurements where we have obtained T_1 values of 7.4 ms (-75°C), 35.7 ms (0°C), and 72.7 ms (53°C). The errors on these values are probably best estimated by the reader from the signal-to-noise ratios of Fig. 3A and the recovery curves of Fig. 3B, but should be in the region of $\pm 10\text{--}15\%$. Note that at low temperature the spin-lattice relaxation time is very short, only $\sim 7 \text{ ms}$ at -75°C , which facilitates rapid data acquisition as illustrated in Fig. 1. In Fig. 3C we plot the results of Fig. 3, A and B, together with additional unpublished results, in the form of an Arrhenius-type curve, which yields an activation energy (ΔE) for the relaxation process—methyl group rotation, of $\sim 2.4 \pm 0.2 \text{ kcal mol}^{-1}$. This is in excellent agreement with the value $\Delta E = 2.6 \pm 0.2 \text{ kcal mol}^{-1}$ obtained previously by Anderson and Slichter, who studied ^1H nuclear spin relaxation in solid *n*-alkanes (43). These workers also obtained a T_1 minimum (when $\omega_r\tau_c \sim 0.62$) at -125°C , essentially identical with that we have obtained at 34 MHz.³

³ Robert A. Kinsey, T. Michael Rothgeb, and Eric Oldfield, unpublished results.

C, computer simulation of valine side chain undergoing fast rotation about both $\text{C}^\alpha\text{-C}^\beta$ and $\text{C}^\beta\text{-C}^\gamma$, $\Delta\nu_Q = 13 \text{ kHz}$ and $2\delta = 1000 \text{ Hz}$, corresponding to $\theta_1 = 110^\circ$, $\theta_2 = 109.5^\circ$. D, temperature dependence of the ^2H NMR spectrum of bacteriorhodopsin in *H. halobium* purple membranes containing $[\gamma\text{-}^2\text{H}_6]\text{valine}$ residues. E, as D except for A. *laidlawii* B (PG9). The central narrow component in D and E “disappears” below $\sim -30^\circ\text{C}$ or upon sample lyophilization, and we believe they originate predominantly from residual HO^2H . We cannot absolutely rule out the possibility that highly mobile residues or membrane fragments contribute to these small signals, however.

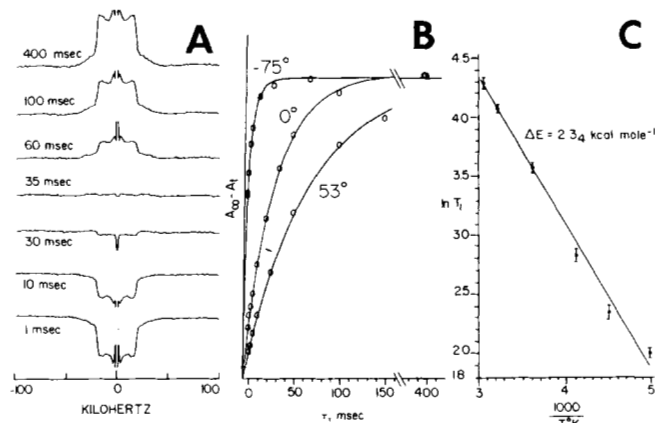


FIG. 3. 55 MHz deuterium NMR spin-lattice relaxation results for $[\gamma\text{-}^2\text{H}_6]\text{valine}$ -labeled bacteriorhodopsin in *H. halobium* purple membranes. A, partially relaxed Fourier transform NMR spectra obtained using a ($180^\circ\text{-}\tau_3\text{-}90^\circ\text{-}\tau_1\text{-}90^\circ\text{-}\tau_2\text{-Echo-T}$) sequence, at 37 °C, for membranes dispersed in excess deuterium-depleted water, 2000 scans, 500-ms recycle time, $\tau_1 = \tau_2 = 55 \mu\text{s}$, 4 μs 90° pulse widths, 2 MHz digitization rate, 2048 data points, line broadening = 400 Hz. The τ_3 values used are indicated on the figure. B, T_1 recovery curves, $(A - A_\infty)$ versus time, for $[\gamma\text{-}^2\text{H}_6]\text{bacteriorhodopsin}$ at -75°C , 0°C , and 53°C . The recycle time varied from 50 to 500 ms. C, Arrhenius plot of $[\gamma\text{-}^2\text{H}_6]\text{valine}$ -labeled purple membrane bacteriorhodopsin spin-lattice relaxation time as a function of temperature. The activation energy is $2.4 \pm 0.2 \text{ kcal mol}^{-1}$.

The picture of valine amino acid side chain dynamics that arises from the experiments we have discussed above is incomplete, and requires experiments with additional species, *e.g.* $[\alpha\text{-}^2\text{H}_1]$ - and $[\beta\text{-}^2\text{H}_1]\text{valine}$ -labeled membranes, together with relaxation experiments on different time scales (44) to more fully map out the spectrum of motions characteristic of this residue. The results do however show that the valine methyl groups undergo fast motion about the C3 axis ($\tau_c \approx 5 \times 10^{-11} \text{ s}$ at 37 °C, Ref. 45) with an activation energy of $\sim 2.4 \pm 0.2$

kcal mol⁻¹. Additional off-axis motions, which average the quadrupole splitting from its rigid lattice value of 40 kHz, are also important, and reduce the order parameter of the methyl group from $S_{\text{mol}} = 1.0$ at $\leq -30^\circ\text{C}$ to ~ 0.83 at growth temperature; development of simple motional models should permit interpretation of these reduced splittings in terms of atomic displacements (23, 24) on time scales $\leq 10^{-6}$ s. Our results suggest that by carrying out a large number of additional labeling and relaxation experiments it will soon be possible to obtain a detailed picture of the dynamics of all the amino acid side chains in many cell membrane systems, investigating for example, the effects of lipids, steroids, and various other ligands on protein motions. With the further development of techniques for electric, magnetic, or mechanical ordering of membrane samples or their microcrystalline aggregates (46), it should be possible to resolve and assign numerous sites in this and other ion or electron transport systems, offering new insights into the basis of biological energy transduction.

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