[41] Recent Advances in the Study of Bacteriorhodopsin Dynamic Structure Using High-Field Solid-State Nuclear Magnetic Resonance Spectroscopy

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There have been an impressive number of studies of the structure of proteins, both in the crystalline solid state[1] and in solution,[2,3] during the past decade. Direct information about protein structure most frequently

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[3] R. A. Dwek, "Nuclear Magnetic Resonance in Biochemistry; Applications to Enzyme Systems." Oxford Univ. Press (Clarendon), London and New York, 1973.

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comes from X-ray crystallography of single crystals, while solution structural information has been obtained most directly by nuclear magnetic resonance (NMR) spectroscopy. Of the two methods, X-ray diffraction gives direct three-dimensional or *spatial* structural information, whereas NMR spectroscopy is more suited to determination of the *dynamical* aspects of protein structure.[4,5] Unfortunately, membrane proteins have not yet been crystallized in forms suitable for X-ray diffraction studies and do not give rise to high-resolution NMR spectra in solution, so that the application of these two powerful conventional structure determination techniques has yielded little information about the systems of interest to readers of this volume: bacteriorhodopsin and rhodopsin.

Fortunately, however, solid-state NMR methods are, in principle, capable of giving information about the structures of condensed phases,[6,7] and in this chapter we present a summary of our results on the dynamics of single types of aliphatic and aromatic amino acids in the purple membrane protein, bacteriorhodopsin, from the extreme halophile *Halobacterium halobium* R_1. This system has the desirable NMR characteristics of only one protein, bacteriorhodopsin, in the purple membrane,[8] its sequence is known,[9-11] and its three-dimensional structure is becoming available.[12,13] The system may also be enriched biosynthetically with a number of deuterated amino acids[13,14] without undue label "scrambling." Moreover, the system has been oriented using electric[15] or magnetic[16] fields or by drying down onto glass or mica surfaces,[13] and preliminary results on formation of microcrystals have been reported,[17] opening up the possibility of obtaining oriented samples for NMR spectroscopy, which permits in favorable cases determination of residue orientations.[18,19]

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This Chapter demonstrates that with sufficiently sensitive NMR instrumentation it is now possible to study in some detail amino acid dynamics in this membrane protein. Such observations may eventually allow comparison of motions between proteins in membranes and in conventional three-dimensional crystals[20,21] and will, of course, facilitate *direct* observation of the effects of lipids and sterols on protein structure. Our results also directly complement the static structural information currently being obtained on *H. halobium* using neutron beam methods.[13]

Experimental Methods

Syntheses of ^{2}H-labeled amino acids. We have recently synthesized a wide range of ^{2}H-labeled amino acids, including the following species to be discussed: [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine, [ϵ_1, ϵ_2-$^2H_2$]tyrosine, [δ_1, ϵ_3, ζ_2, ζ_3, η_2-$^2H_5$]tryptophan, and [α-$^2H_1$]-, [β-$^2H_1$]-, and [γ-$^2H_6$]valine. The selectively labeled amino acids were synthesized using modifications of published procedures.[22,23] In addition, we obtained [α-$^2H_2$]glycine and [β-$^2H_3$]alanine from Merck, Sharpe and Dohme (Montreal). The positions of ^{2}H-label incorporation are thus as shown in Fig. 1.

Production of Labeled Membranes. Halobacterium halobium strain R_1 was the kind gift of Professor T. Ebrey and was grown in a salt medium basically according to Onishi *et al.*[24] with the addition of 2% malate,[25] except that ^{2}H-labeled amino acids were substituted, one by one for the normal nonlabeled amino acids. For the Trp-labeled membrane system we incorporated [$^2H_5$]Trp at a level of 5.0 g/10 liters, since the growth medium does not normally contain tryptophan. Purple membranes were isolated according to Becher and Cassim[26] and were then exchanged with ^{2}H-depleted water (Aldrich Chemical Company, Milwaukee, Wisconsin) to remove some background HO^{2}H. Samples were generally exchanged twice, then finally concentrated by ultracentrifugation for 10 hr at 100,000 *g* prior to NMR spectroscopy.

Radiotracer Experiments. To determine the level of deuterated amino acid breakdown, and reincorporation into other amino-acids, 1-liter batches of cells were grown and harvested basically as for the ^{2}H-labeled

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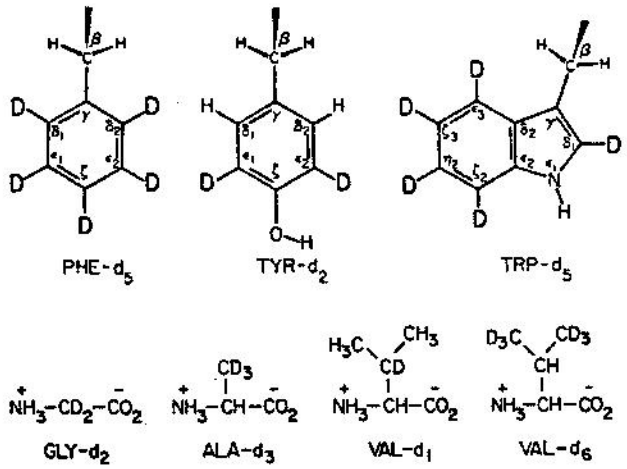

FIG. 1. Structures of the ^{2}H-labeled amino acids discussed in this publication, showing position of ^{2}H-label incorporation.

cells except that 50 μCi of either [^{14}C]Tyr, [^{14}C]Phe, [^{14}C]Trp, [^{14}C]Gly, [^{14}C]Ala, or [^{14}C]Val (New England Nuclear, Boston, Massachusetts) were added as radiotracers.

Spectroscopic Aspects. Nuclear magnetic resonance spectra were obtained using two "home-built" Fourier transform NMR spectrometers. The first one consists of an 8.5 Tesla 3.5-in. bore high-resolution superconducting solenoid (Oxford Instruments, Osney Mead, Oxford, U.K.), together with a variety of digital and radiofrequency electronics. We used a Nicolet 1180 computer, 293B pulse programmer, and Model NIC-2090 dual channel 50-nsec transient recorder (Nicolet Instrument Corporation, Madison, Wisconsin) for experiment control and rapid data acquisition, together with a dual Diablo Model 40 disk system for data storage (Diablo Systems, Inc., Haywood, California). In order to generate radio-frequency pulses of high enough power (~3 μsec 90° pulse widths) to cover the entire ^{2}H NMR spectral width, we used an Amplifier Reséarch (Amplifier Research, Souderton, Pennsylvania) Model 200L amplifier to drive a retuned Henry Radio (Henry Radio, Los Angeles) Model 2006 transmitter to a ~1000–1500 W output power level. The deuterium resonance frequency was 55.273 MHz. Deuterium NMR spectra were recorded on this instrument using an 800-μL sample volume and a quadrupole-echo[27,28] pulse sequence. The 90° pulse width varied between 2.0 and 3.5 μsec.

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The second instrument used was the medium-field spectrometer (5.2 Tesla) described previously,[29] except that it now uses a "homebuilt" 400 kHz data acquisition system based on a Digital Equipment Corporation LSI-11 microcomputer (Digital Equipment Corporation, Boston, Massachusetts) equipped with dual disks, together with a second Model 2006 transmitter. The 90° pulse widths (~2.0–3.5 μsec) and phase quadrature between the two radiofrequency pulses were established on both instruments by viewing quadrature free-induction decay signals of *S*-[*methyl*-$^2H_3$]methionine. Identical settings were used for ^2H-labeled membranes, and in essentially all cases no phase corrections were necessary after Fourier transformation. Sampling rates of 500 nsec per point were used at 8.5 Tesla, and 3 μsec per point at 5.2 Tesla. The zero frequencies of both instruments were established using a 1% D_2O reference, the zero frequency for the protein samples investigated being offset ~2 ppm downfield from this position for aromatic amino acids, or between 1 and 3 ppm upfield for aliphatics. Samples were run as solid high-speed pellets, probe temperature being regulated either by means of a liquid nitrogen boil-off system or by using a heated air flow.

Theoretical Background for NMR of Membrane Proteins. The allowed transitions for the spin I = 1 ^2H nucleus correspond to $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$ (Fig. 2A) and give rise to a "quadrupole splitting" ($\Delta\nu_Q$) of the absorption line, with separation between peak maxima of[29–31]

$$\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}\frac{3\cos^2\theta - 1}{2} \tag{1}$$

θ is the angle between the magnetic field H_0 and the principal axis of the electric field gradient tensor (frequently the C—D bond vector). All values of θ are possible for rigid polycrystalline solids and one therefore obtains a so-called powder pattern, Fig. 2B, having a peak separation corresponding to $\theta = 90°$, for which $\Delta\nu_Q = 3e^2qQ/4h$, and a shoulder separation corresponding to $\theta = 0°$, for which $\Delta\nu_Q = 3e^2qQ/2h$. A typical experimental example, [β-$^2H_1$]valine, is shown in Fig. 2C. For solid aliphatic compounds $\Delta\nu_Q$ values of 127 kHz ($\theta = 90°$) and 254 kHz ($\theta = 0°$), corresponding to an electric quadrupole coupling constant (e^2qQ/h) of about 168 kHz[32,33] are expected, and observed (Fig. 2B,C). These results assume that there are no fast ($> 10^5 sec^{-1}$) large-amplitude motions of the pertinent C-^2H vectors in the solid amino acid. In the presence of such

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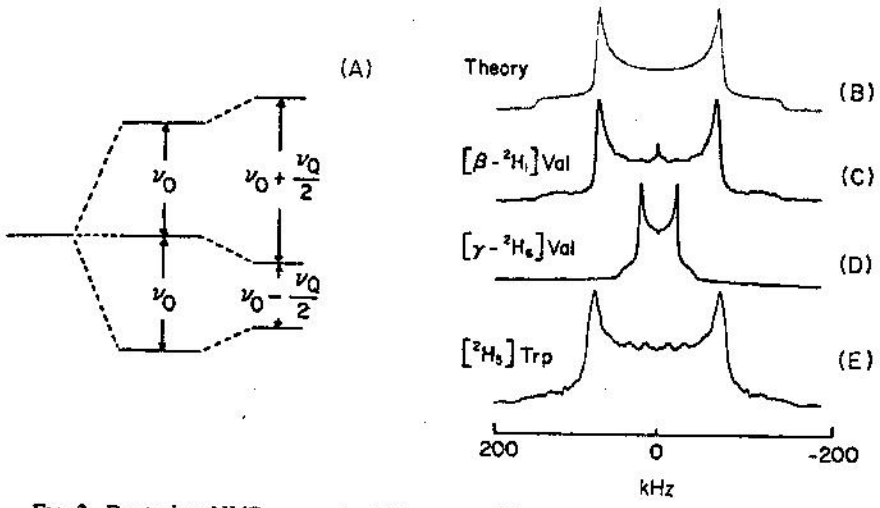

FIG. 2. Deuterium NMR energy level diagram and theoretical and experimental ^2H NMR spectra of polycrystalline amino acids. A, Energy level diagram showing Zeeman levels and presence of a first-order quadrupole perturbation. The transitions $-1 \leftrightarrow 0$ and $0 \leftrightarrow 1$ are shown, for an arbitrary crystal orientation. B, Theoretical ^2H NMR lineshape having $e^2qQ/h = 168$ kHz, $\eta = 0$, linewidth = 4000 Hz. C, Experimental spectrum of polycrystalline [β-$^2H_1$]valine, at 55.3 MHz, 23°. D, Experimental spectrum of polycrystalline [γ-$^2H_6$]valine, at 55.3 MHz, 23°. E, Experimental spectrum of polycrystalline [δ_1, ϵ_3, ζ_2, ζ_3, η_2-$^2H_5$]tryptophan, at 55.3 MHz, 23°.

fast large-amplitude motions it is necessary to take an average over the motions. Assuming that the asymmetry parameter (η) is zero, it can be shown that the motionally averaged splitting ($\Delta\nu$) is

$$\Delta\nu = \frac{3}{8}\frac{e^2qQ}{h}(3\cos^2\beta - 1)(3\cos^2\theta' - 1) \qquad (2)$$

where β is the angle between the principal axis of the electric field gradient tensor and the axis of motional averaging and θ' is the angle between the axis of motional averaging and H_0.

As an example of this motional averaging, consider the case of the C^2H_3 group in [γ-$^2H_6$]valine (Fig. 1, 2D). Methyl groups are expected to rotate rapidly about their C_3 axis at room temperature, in which case if we assume a tetrahedral geometry ($\beta = 109° 28'$), then a motionally averaged splitting ($\Delta\nu$) of about 42 kHz is predicted, in excellent agreement with the $\Delta\nu_Q \sim 39$ kHz observed experimentally in [γ-$^2H_6$]valine (Fig. 2D). Rotation about C^β—C^γ is thus very rapid at room temperature ($>> 10^5 sec^{-1}$). The unlikely possibility that fast rotation about C^α—C^β is the cause of the reduced splitting is eliminated by the observation of the

full rigid lattice breadth in the ^2H NMR spectrum of [β-$^2H_1$]valine (Fig. 2C). The results of Fig. 2B–D therefore give us a benchmark with which to compare ^2H NMR spectra of aliphatic amino acid labeled membranes.

In the case of aromatic residues quadrupole coupling constants are ~10% larger than for aliphatic species[34] and asymmetry parameters (η) for aromatic residues are nonzero; thus the full expression for the quadrupole splitting must be used[31]:

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}[3\cos^2\theta - 1 - \eta\sin^2\theta\cos 2\psi] \quad (3)$$

where θ and ψ define the orientation of the principal axis of the electric field gradient tensor (usually the C—D bond vector) with respect to the laboratory coordinates. The computed quadrupole coupling constant of about 183 kHz for [$^2H_5$]Trp (Fig. 2E) is therefore considerably in excess of those found in aliphatic C—^2H systems using NMR methods.[34,35] In addition, computer simulation of the lineshape of Fig. 2E indicates an asymmetry parameter $\eta = 0.05$. These results are consistent with the increased e^2qQ/h values found in a variety of other aromatic compounds,[34–36] the observed trends for the electric field gradient (EFG) values for C—D bonds being $sp > sp^2 > sp^3$,[37] the average value for naphthalene and anthracene, perhaps the most reasonable published models for [$^2H_5$]Trp, being ~184 kHz.[38–40] In addition, it is well known that C—D bonds in aromatic systems may have nonzero asymmetry parameters,[41] η values of 0.053 ± 0.015 being typical.[34]

Motions in Some Aliphatic Side Chains. Our first goal with NMR studies of membrane protein structure is to obtain a rather broad overview of the *rates* and *types* of motions of amino acid side chains, then to develop methods for the resolution and assignment of individual atomic sites and to investigate the effects of various membrane constituents, such as cholesterol and phospholipids, on protein structure. The results we have presented give the reader some idea of the shapes and widths expected for ^2H NMR spectra of solid, polycrystalline ^2H-labeled amino acids. What, then, do membrane protein spectra look like, if we can see them?

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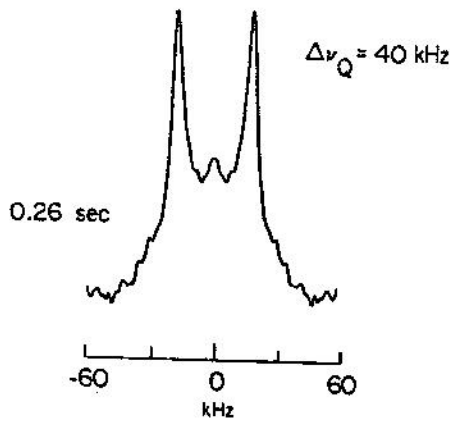

FIG. 3. 55.3 MHz ^{2}H NMR spectrum of [γ-$^2H_6$]valine labeled bacteriorhodopsin in the purple membrane of *H. halobium* R_1, $-100°$, 260 msec data acquisition time.

We show in Fig. 3 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. 3 was obtained in only 0.3 sec of data acquisition and is our fastest spectrum to date. Such rapid data acquisition was possible since the [γ-$^2H_6$]valine residues incorporated were highly deuterated; there are some 21 valines (42 methyl groups, 126 deuterons) in bacteriorhodopsin[9-11]; we used a low temperature ($-100°$) to decrease the spin-lattice relaxation time (T_1), thereby permitting rapid pulsing; and the quadrupole splittings are relatively narrow. The result shown in Fig. 3 is nevertheless extraordinarily exciting, indicating the feasibility of observing any type of group in this membrane protein, although data acquisition still takes numerous hours for more dilute labels, especially when relaxation times are long. For example, the ^{2}H spin-lattice relaxation time of the [γ-$^2H_6$]valine label is only a few milliseconds near the T_1 minimum ($\sim -150°$K, $\tau_c \approx \omega_0^{-1} \approx 10^{-9}$ sec), whereas T_1's of many seconds are obtained for rigidly bound residues ($\tau_c >> \omega_0^{-1}$), as noted later, greatly increasing the periods of time required for data acquisition.

Nevertheless, we have successfully incorporated and obtained spectra of the following ^{2}H labeled aliphatic amino acids in the purple membrane: [α-$^2H_2$]glycine, [β-$^2H_3$]alanine, [α-$^2H_1$]valine, [β-$^2H_1$]valine, and [γ-$^2H_6$]-valine, and additional studies are underway on the labeling of *Escherichia coli* and *Acholeplasma laidlawii* B (PG9) with these amino acids, and several deuterated leucines and isoleucines. Details of such experiments are being[14,35,41] or will be reported in detail elsewhere.

In Fig. 4 we present a selection of NMR results on ^{2}H-labeled aliphatic amino acids in the purple membrane of *H. halobium* R_1, at 37° in deute-

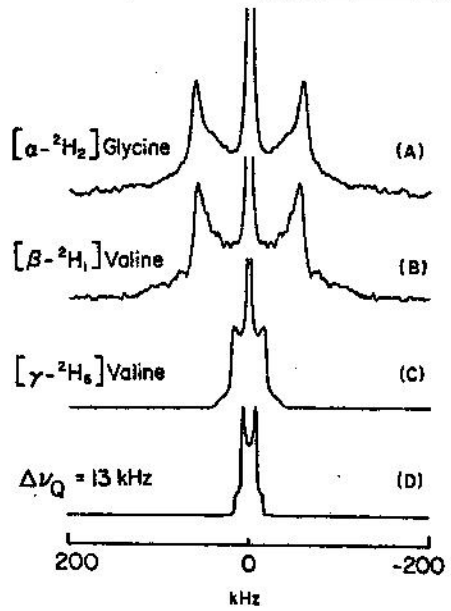

FIG. 4. 55.3 MHz ^{2}H NMR spectra of purple membranes of *H. halobium* R_1 at 37°, in ^{2}H-depleted water, together with a computer simulation of [γ-$^2H_6$]valine undergoing fast α–β and β–γ rotational diffusion. The labels were at the following sites: A, [α-$^2H_2$]glycine. B, [β-$^2H_1$]valine. C, [γ-$^2H_6$]valine. D, Computer simulation of [γ-$^2H_6$]valine undergoing fast rotational diffusion about C^α—C^β and C^β—C^γ. The quadrupole splitting $\Delta\nu_Q$ = 13 kHz.

rium-depleted water, which give some insight into the motions of these residues in bacteriorhodopsin. The spectrum of [α-$^2H_2$]glycine is essentially identical to that of the free amino acid[42] (and unpublished results of I. Baianu and E. O.), having e^2qQ/h = 168 kHz and $\eta = 0.05 \pm 0.02$. The nonzero η is not unexpected, because of the adjacent CO and NH groupings. The large value of the quadrupole splitting rules out all but small-amplitude torsional or vibrational motions of the CD_2 group, consistent with the observed magnitude and temperature dependence of the ^{2}H spin-lattice relaxation time (unpublished results of A. K., R. A. K. and E. O). Glycine is thus "rigid" in the purple membrane.

Results with [β-$^2H_3$]alanine are complicated by the "scrambling" of this amino acid into other species [e.g., phenylalanine (unpublished results)], nevertheless it seems that the major ^{2}H resonance is attributable to the methyl deuterons of [β-$^2H_3$]alanine, which as in Figs. 2 and 3 are undergoing fast internal rotation resulting in a quadrupole splitting $\Delta\nu_Q$ = 39 ± 2 kHz. Many of the alanine side chains in bacteriorhodopsin are thus "rigid," with methyl rotation being the only fast large-amplitude mo-

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tion. A similar statement may be made for the valine side chains (Fig. 4B,C). The ^2H NMR spectrum of [β-$^2H_1$]valine labeled membranes (Fig. 4B) has $\Delta\nu_Q \sim 116$kHz, about the same as that of the polycrystalline amino acid (Fig. 2C), and that of [γ-$^2H_6$]valine labeled purple membrane is also very similar, identical to that of the free amino acid (Fig. 4C, 2D).

Fast methyl group rotation averages the static quadrupole coupling constant by a value of $\sim\frac{1}{2}(3\cos^2 109.5° - 1)$, i.e., by a factor of about -0.3[14,32] and a motionally averaged spectrum having a breadth of ~ 40 kHz is obtained. Observation of a motionally averaged quadrupole splitting of about 40 kHz for [γ-$^2H_6$]valine labeled sites strongly suggests that there is very fast motion only about C^β—C^γ, motion about C^α—C^β being very slow on our NMR timescale, such that no motional averaging of the quadrupole interaction occurs because of 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. 4D), as would the splitting of the [β-$^2H_1$]species. No such behavior is seen in the purple membrane of *H. halobium* in the temperature range $-100°$–$53°$C.[14] The quadrupole splittings of [γ-$^2H_6$]valine-labeled bacteriorhodopsin in the purple membrane of *H. halobium* are in fact remarkably temperature independent,[14] ranging from the rigid lattice value $\Delta\nu_Q = 39 \pm 1$ kHz (due solely to fast Me rotation) below $\sim -30°$, found also in the model system [γ-$^2H_6$]valine (Fig. 2D), to ~ 33 kHz at 60°. Please note, however, that above $\sim 25°$ the spectra become considerably less "sharp" than those obtained at low temperature, either because of the onset of additional slow motions[43,44] or, perhaps more likely, because of the occurrence of a broader distribution of $\Delta\nu_Q$ values, resulting from the basic heterogeneity of the purple membrane. In any case, as viewed from the biosynthetically incorporated [γ-$^2H_6$]valine quadrupole splittings, the purple membrane remains a remarkably ordered structure over this wide range of temperature. We should point out that an "isotropic" component appears in all growth temperature spectra (Fig. 4A–C). We do not know the exact origin of this component, but attribute it in part to residual HO^2H. It disappears on sample freezing, or lyophilization, but could still arise from, e.g., mobile (surface) residues or from small membrane fragments.

The high signal-to-noise ratios obtained in Fig. 4 permit the direct study of amino acid side-chain dynamics via determination of NMR relaxation rates and of their temperature dependencies. For example, using a conventional inversion-recovery pulse sequence modified for solids, i.e. ($180°$-τ_3-$90°$-τ_1-$90°_{90°}$-τ_2-Echo-T), where T is the period of repetition of the

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pulse sequence ($>5\,T_1$), τ_1 and τ_2 are fixed delays of $\sim 70\,\mu$sec, and τ_3 is a variable delay between the inverting (180°) and sampling (90°) pulses, we obtain for [γ-$^2H_6$]valine labeled purple membranes T_1 values of 7.4 msec at −75°, 35.7 msec at 0°, 72.7 msec at 55°.[14] These, and the results of additional experiments, when plotted in the form of an Arrhenius-type curve, yield an activation energy (ΔE) for the relaxation process—methyl group rotation, of $\sim 2.4 + 0.2$ kcal mol^{-1}. This is in excellent agreement with the value $\Delta E = 2.6 \pm 0.2$ kcal mol^{-1} obtained previously by Anderson and Slichter,[45] who studied ^{1}H nuclear spin relaxation in solid n-alkanes. These workers also obtained a T_1 minimum (when $\omega_0\tau_c \sim 0.62$) at −125°, essentially identical to that we have obtained using our 5.2 Tesla instrument, at 34 MHz (unpublished results of E. Oldfield, A. Kintanar, M. Keniry, and B. Smith). These results suggest correlation times of $\approx 5 \times 10^{-11}$ sec for methyl group rotation, when analyzed using a simple relaxation model.[14]

Motions in Some Aromatic Side Chains. We have incorporated, and are incorporating, the following deuterated amino acids into bacteriorhodopsin in the purple membrane of *H. halobium* R_1, and a variety of other membrane systems: [α-$^2H_1$]phenylalanine, [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine, [ϵ_1, ϵ_2, ζ-$^2H_3$]phenylalanine, [ζ-$^2H_1$]phenylalanine, [α-$^2H_1$]tyrosine, [β-$^2H_2$]tyrosine, [ϵ_1, ϵ_2-$^2H_2$]tyrosine, [α-$^2H_1$]tryptophan and [δ_1, ϵ_3, ζ_2, ζ_3, η_2-$^2H_5$]tryptophan. Experiments with [ϵ-$^2H_1$]histidine are also under way with *E. coli*.

We show in Fig. 5 typical results obtained with [δ_1, ϵ_3, ζ_2, ζ_3, η_2-$^2H_5$]tryptophan, [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine and [ϵ_1, ϵ_2-$^2H_2$]tyrosine[35] at the growth temperature of the organism (37°). Note that the spectrum of [$^2H_5$]Trp is essentially identical to that of the free amino acid in the solid state (Fig. 2E), being simulated well by using $e^2qQ/h = 183.0$ kHz and $\eta = 0.05$.[35] Since the spectrum remains virtually the same between −85° and +85°, it seems reasonable to characterize the Trp residues as "rigid," only undergoing torsional or librational motions of $\approx 5 - 10°$ amplitude.[35]

By contrast, the ^{2}H FT NMR spectra of the Phe and Tyr labeled purple membranes have rather unusual lineshapes. It is therefore of some interest to examine the likely motions of a Phe or Tyr residue in the solid state, as shown in Fig. 6.

We show in Fig. 6A,B the ^{2}H NMR spectrum of a "rigid" [$^2H_5$]phenylalanine residue together with its simulation, characterized by a deuteron quadrupole coupling constant $e^2qQ/h \sim 180$ kHz and $\eta = 0.05$. These re-

[45] J. E. Anderson and W. P. Slichter, *J. Phys. Chem.* 69, 3099 (1965).

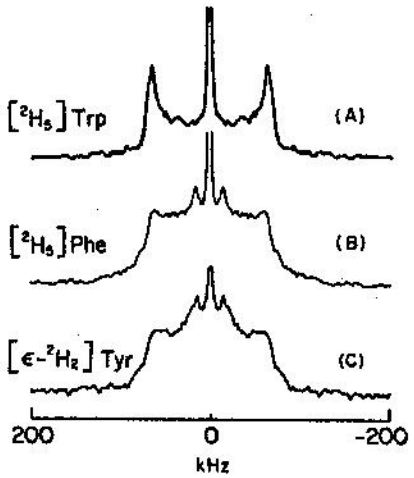

FIG. 5. Experimental ²H NMR spectra, at 55.3 MHz, of aromatic amino acid labeled purple membranes in ²H-depleted water, at 37°. The following labels were incorporated: *A*, [δ_1, ϵ_2, ζ_2, ζ_3, η_2-²H₅]tryptophan. *B*, [δ_1, δ_2, ϵ_1, ϵ_2, ζ-²H₅]-phenylalanine. *C*. [ϵ_1, ϵ_2-²H₂]tyrosine.

sults are in good agreement with the median values found for a series of monosubstituted benzenes of $e^2qQ/h \sim 181$ kHz and $\eta = 0.06$.[34,38,40,46] Let us now consider the effects of rapid ($>> 10^5$ sec^{-1}) motions on the observed ²H NMR spectrum. One possibility would be fast rotational diffusion about C^β—C^γ. In this case the phenyl ring would undergo rapid rotational diffusion about the C^γ—C^ζ axis, in which case the $C^{\delta1,\delta2,\epsilon1,\epsilon2}$-²H vectors would be at 60 ± 1° to the axis of motional averaging, and the C^ζ—²H vector would be at 0°. It is a simple matter to calculate the observed spectrum, and the result is shown in Fig. 6C. It is quite dissimilar to that observed for [²H₅]Phe in any native system investigated.

By contrast, a twofold "jump" model for phenylalanine motion, whereby the aromatic ring executes 180° reorientational "flips" about C^γ—C^ζ, and which has been detected previously in solution NMR studies of proteins by means of chemical shift data,[2,4,5,47] predicts a very different result (Fig. 6D). Assuming that motion is "fast" compared to the breadth of the rigid bond coupling, a motionally averaged tensor may be calculated by using the model of Soda and Chiba[48] for motional averaging of the deuterium quadrupole interaction by reorientation about a twofold axis.

[46] I. Y. Wei and B. M. Fung, *J. Chem. Phys.* **52**, 4917 (1970).
[47] C. M. Dobson, G. R. Moore, and R. J. P. Williams, *FEBS Lett.* **51**, 60 (1974).
[48] G. Soda and T. Chiba, *J. Chem. Phys.* **50**, 439 (1969).

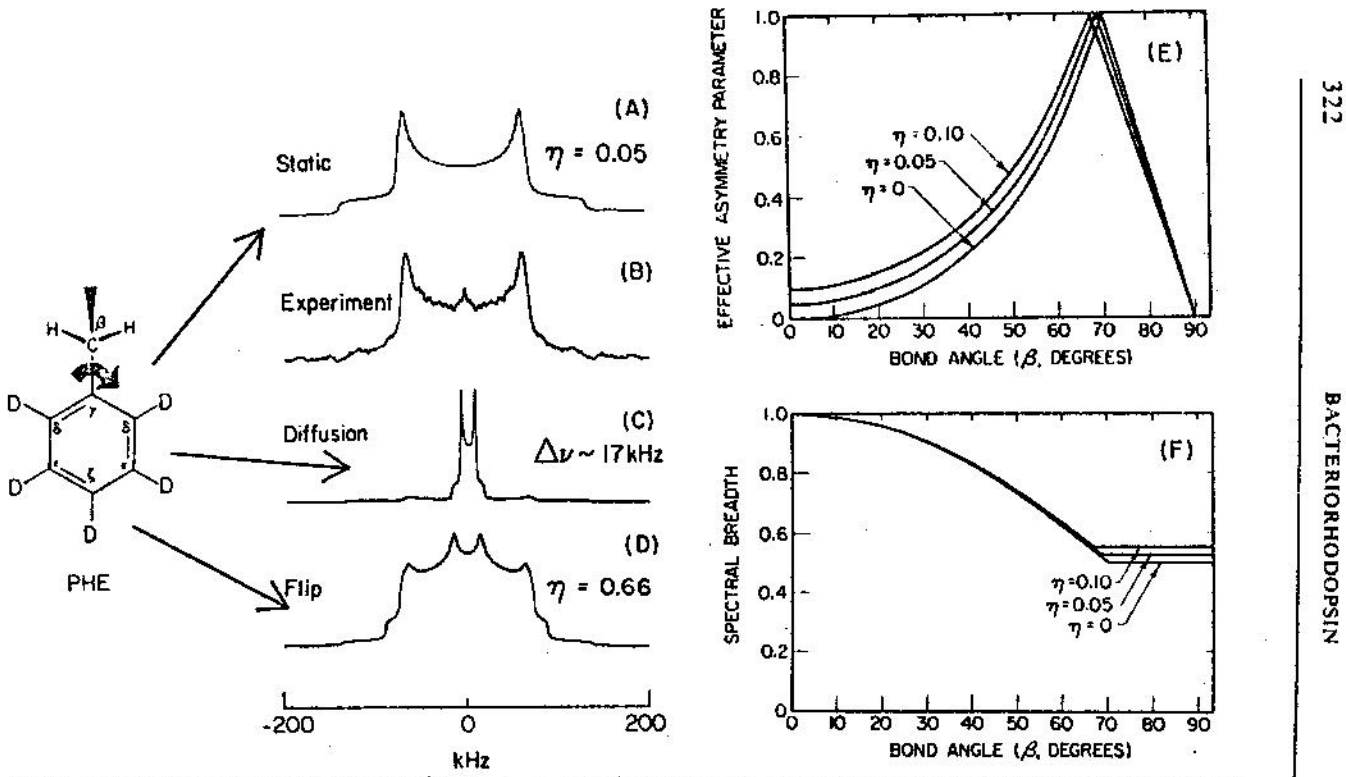

FIG. 6. ^{2}H HMR spectra and simulations of [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine, and theoretical plots of asymmetry parameter and spectral breadth as a function of flip-angle for a twofold flip. A, Computer simulation of rigid lattice lineshape using e^2qQ/h = 180 kHz, η = 0.05. B, Experimental 55.3 MHz ^{2}H NMR spectrum of [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine, at 23°C. C, Computer simulation of [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine undergoing fast (>>2 × 10^5 sec $^{-1}$) rotational diffusion about C^γ—C^ζ C_2 axis. D, As in C but motion is a twofold "flip." E, Dependence of the effective asymmetry parameter (η_{eff}) on the interbond angle β for the case of motional averaging by twofold flipping for several values of the rigid-bond asymmetry parameter (η). F, Dependence of the reduced spectral breadth on the interbond angle β for the case of motional averaging by twofold flipping for several values of the rigid bond asymmetry parameter (η).

The results for the averaged field gradient tensor components as a function of β, and the asymmetry parameter (η) of the static tensor are

$$V_{11} = -\tfrac{1}{2}q(1 + \eta)$$
$$V_{22} = +\tfrac{1}{4}q[(1 - 3\cos 2\beta) + \eta(1 + \cos 2\beta)] \qquad (4)$$
$$V_{33} = +\tfrac{1}{4}q[(1 + 3\cos 2\beta) + \eta(1 - \cos 2\beta)].$$

We show in Fig. 6E,F the new effective asymmetry parameters (η_{eff}) for the case of rapid 180° reorientations as a function of bond angle β together with the new maximum effective field gradients eq, i.e. the reduced spectral breadths $V_{33}-V_{11}$, from which it is a simple matter to calculate the new spectral lineshape for twofold flipping, as shown in Fig. 6D. The spectra now contain a new sharp narrow feature, corresponding to the separation between the singularities in the powder pattern, having $\Delta\nu_{Q1}$ -30 kHz (Fig. 6D). This dominant feature is easily detected in some intact membrane spectra, as discussed later. Unfortunately, however, the mere presence of such a predicted peak does not guarantee its origin in the spectra of intact membranes. For example, in a system as heterogeneous as a biological membrane, it seems quite likely that there could be a variety of environments in which Phe and Tyr residues might undergo a variety of different motions. For example, a "rocking" motion about C^{α}—C^{β} together with torsional motions about the peptide bonds or "rigid body" motions of whole segments of surface-exposed residues might easily occur and give rise to a second, reduced quadrupole splitting.

Two pieces of evidence indicate that these explanations do not apply in the case of Phe and Tyr-labeled bacteriorhodopsin. First, spectra of [β-$^2H_2$]tyrosine exhibit the full rigid-lattice splitting of $\sim$120 kHz and an asymmetry parameter $\eta = 0.00 \pm 0.02$, at the growth temperature of the organism. This rules out fast large-amplitude motions of the [β-$^2H_2$]tyrosine deuterons (unpublished results of A. Kintanar and E. Oldfield). Motional averaging of the tyrosine ring-deuteron spectrum must thus occur solely by motions about the C^{γ}—C^{ζ} axis. Similarly, ^2H NMR spectra of [ζ-$^2H_1$]phenylalanine, a *para*-substituted derivative, show essentially axially symmetric ($\Delta\nu_Q \sim 120$ kHz, $\eta = 0.05 \pm 0.02$) spectra at the temperature of growth, unlike the narrow, axially asymmetric spectra ($\eta \approx 0.65$) obtained for the [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine labeled samples (Fig. 5B and unpublished results of A. K. and E. O.). This arises from the fact that the *para*-deuteron's electric field gradient tensor principal axis lies directly along the axis of motional narrowing, i.e., $\beta = 0°$; consequently, there is essentially no averaging of the spectral linewidth, as shown in Fig. 6E, F.

Our results therefore indicate that tryptophan, phenylalanine, and

tyrosine residues are rigid at low temperatures ($< -30°$) while phenylalanine and tyrosine residues are each highly mobile at the temperature of growth of the *H. halobium* purple membrane (37°), undergoing fast ($> 10^5 - 10^6$ sec^{-1}) twofold jumps about C^β—C^γ. Tryptophan residues do not undergo this type of motion even at 85°, immediately prior to protein denaturation. On denaturation at ~90°, "narrow-line" spectra (having linewidths ~5–10 kHz) are obtained for all three aromatic amino acids (unpublished results of R. A. K., A. K. and E. O.), suggesting fast large-amplitude motions.

The Picture to Date. Our results may be summarized as follows. Backbone (C^α) labeled amino acids in the purple membrane of *H. halobium* exhibit "rigid-lattice spectra," except for a possible small population of surface residues. There is no evidence for fast motion about C^α—C^β for any aliphatic or aromatic amino acid investigated to date, except for fast methyl rotations in alanine and a possible small population (~5–10%) of surface residues. In the case of valine-labeled purple membranes, motion about C^β—C^γ is fast ($> 10^6$ sec^{-1}) at all temperatures investigated (down to 120°K). The increased bulk of the benzenoid rings in Tyr and Phe greatly impede motion of these side chains. When they do begin to move (at about the 37° growth temperature of the organism) rotation is not diffusive but occurs by a twofold flipping process, as has been detected previously in solution NMR studies of soluble proteins. The additional bulk of the indole ring in tryptophan prevents even this motion and only small-angle librations are allowed, even at 85°. These results are supported by spin-lattice relaxation data (unpublished results of R. A. K., A. K., Becky Smith, Max Keniry, Herbert Gutowsky and E. O.) that show that all systems (except for the methyl labels) have spin-lattice relaxation times that decrease with increasing temperature, since correlation times (τ_c) are all $>> \omega_0^{-1}$.

A generalized picture of our observations is presented in Fig. 7. This picture of the dynamics of most aliphatic and aromatic amino acids in the purple membrane is one of a rather rigid protein; in most instances the ^2H NMR spectra of the protein are rather similar to those of the solid amino acid, at the same temperature, except for the Phe and Tyr residues that undergo twofold "flipping." Since it appears that Phe residues at least are located toward the center of the protein,[13] where H^+ translocation may occur, it seems possible that these motions could be of importance in the energy transduction process. Interestingly, as discussed earlier, the Trp residues do not undergo any such fast motions.

Since we have now obtained ^2H spectra of [γ-$^2H_6$]valine labeled membranes in as short a time as 300 msec, it is hoped that future studies, perhaps using electrically or magnetically ordered materials[15,16,18,19] as reso-

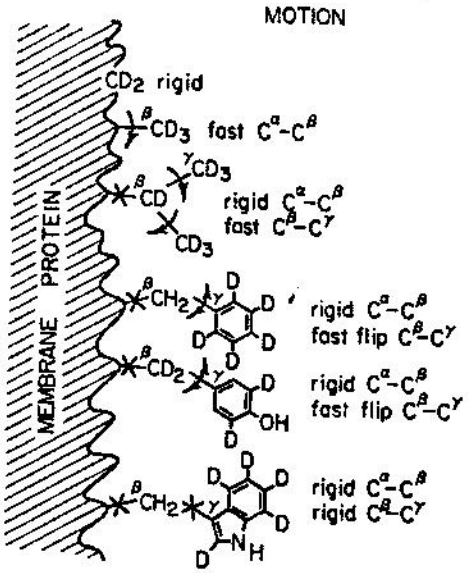

FIG. 7. Diagram showing the rates and types of motions of a variety of aliphatic and aromatic amino acid side chains of bacteriorhodopsin in the purple membrane of *H. halobium* R_1 at 37°, the temperature of growth.

lution-enhancing aids, may include time-resolved experiments aimed at determining the exact nature of conformational changes, both in the protein and in the retinal chromophore, during the photochemical cycle. At present, the major problem in carrying out such experiments is the availability of very high-field sperconducting magnets. Given fields of ~16–18 Tesla (~700–800 MHz ^{1}H frequency), within reach of current technology, time-resolved structural studies on this and other important energy-transduction systems should be quite feasible.

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