# Dynamics of Amino Acid Side Chains in Membrane Proteins by High Field Solid State Deuterium Nuclear Magnetic Resonance Spectroscopy

PHENYLALANINE, TYROSINE, AND TRYPTOPHAN\*

We have obtained the first deuterium NMR spectra

of individual types of aromatic amino acids in a defined

membrane protein, bacteriorhodopsin, in the photosyn-

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thetic purple membrane of Halobacterium halobium R1. Isotopic labeling and high field (8.5 Tesla) operation permitted relatively rapid data acquisition at a variety of temperatures. At the temperature of growth (37 °C), we find that all 7 tryptophan residues are rigid on the time scale of the NMR experiment ( $\sim 10^{-5}$  s), except for likely librational motions of  $\approx 10^{\circ}$  amplitude. By contrast, nearly all  $(9 \pm 2)$  of the 11 tyrosines and  $(13 \pm 2)$ 13 phenylalanines undergo rapid (>10<sup>5</sup> s<sup>-1</sup>) 2-fold rotational flips about  $C^{\gamma}$ - $C^{\beta}$ , causing formation of line shapes dominated by effectively axially asymmetric (asymmetry parameter  $\eta = 0.66$ ) deuteron electric field gradient tensors. On cooling the phenylalanine- and tyrosine-labeled samples to  $\sim -30$  °C, all such motions are frozen out, *i.e.* occur at rates  $<10^4$  s<sup>-1</sup>, and axially symmetric ( $\eta \approx 0.05$ ) line shapes are observed. At T > 0.0591 °C, phenylalanine-, tyrosine-, and tryptophan-labeled membrane spectra undergo dramatic narrowing to an isotropic line of ~4-9 kHz width. This transition is a reflection of the loss of tertiary structure in the membrane protein with resultant fast unrestricted motion of these aromatic side chains, and is only partly reversible. These results, in conjunction with those obtained using [y-2H6]valine-labeled bacteriorhodopsin (Kinsey, R. A., Kintanar, A., Tsai, M-D., Smith, R. L., Janes, N., and Oldfield, E. (1981) J. Biol. Chem., 256, 4146-4149) indicate the rather rigid nature of amino acid side chains in the H. halobium purple membrane, the principal fast large amplitude motions being methyl group rotation and discontinuous benzene ring "flip-

Over the past 10 years there has been considerable experimentation aimed at determining the nature of protein-lipid interactions in model and intact biological membranes. Some early studies (1, 2) emphasized the idea that proteins acted in a manner similar to that of the rigid tetracyclic sterol cholesterol (3, 4), causing an "ordering" of the lipid hydrocarbon chains, a view supported by several more recent theoretical studies (5-8), and experimental work using fluorescence and ESR techniques (9-12). More recently however, deuterium nuclear magnetic resonance spectroscopic techniques, in both model protein-lipid systems (13-16) and in biological membranes themselves (17-19), have given no indication of lipid ordering by protein molecules, which has thus led to a revision by some workers of previous protein-lipid interaction models. A popular view now is that lipids may be "immobilized" or perhaps trapped by proteins, but are not necessarily ordered (16, 20-22), but the crucial point about the protein-lipid interaction problem still remains unprobed, what does lipid "do" to protein? Since lipid phase behavior (23) and the presence of cholesterol (24) apparently have large effects on membrane enzyme activities, we have therefore begun a program aimed at elucidating the dynamic structures of proteins in membranes. As a "benchmark," we have already obtained NMR spectra of individual sites in crystals of some soluble proteins (25, 26) using a new magnetic ordering method which may eventually be applicable to membrane protein studies.

In this publication we report recent results obtained via  ${}^{2}\text{H}$ NMR spectroscopy at high field which indicate that the motions of individual types of amino acid residues in membrane proteins may now be studied in intact biological membranes. Our results show that protein dynamics in membranes may now be analyzed and compared with solution (27–29), micellar (30), and crystal data (31, 32) to provide a basis for more meaningful studies of protein-lipid interactions in biological membranes. Downloaded from www.jbc.org at University of Illinois - Urbana on November 30, 2007

As with studies of lipid dynamics in model or intact biological membranes (13, 15, 17) or in protein crystals (25, 26), we have chosen to use the <sup>2</sup>H nucleus as our structural probe. In this publication we concentrate on use of <sup>2</sup>H NMR powder pattern line shapes to deduce information about the rates and types of amino acid side chain motion (33, 34).

In this first detailed publication on amino acid dynamics in a "condensed phase" membrane protein, as opposed to the experimentally more accessible solubilized protein systems, we have chosen to investigate the "purple membrane" system of *Halobacterium halobium* R1. This system has the desirable NMR characteristics of only one protein, bacteriorhodopsin, in the purple membrane (35), its sequence is known (36–38), and its three-dimensional structure is becoming available (39, 40). The system may also be enriched biosynthetically with a number of deuterated amino acids (40, 41) without undue label "scrambling." Moreover, the system has been oriented using electric (42) or magnetic (43) fields, or by drying down onto glass or mica surfaces (40), and some preliminary results on formation of microcrystals have been obtained (44), opening up the possibility of obtaining oriented samples for NMR

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spectroscopy, which permits in favorable cases determination of residue orientations (25, 26). Finally, the enzyme has been shown to be susceptible to proteolytic cleavage and reassembly into an active proton-pumping system (37), which opens up the possibility of making specific NMR resonance assignments and studying the dynamics of individual residues.

## EXPERIMENTAL PROCEDURES

#### Syntheses of <sup>2</sup>H-labeled Amino Acids

We have synthesized the following <sup>2</sup>H-labeled amino acids: L-[ $\delta_1$ ,  $\delta_2$ ,  $\epsilon_1$ ,  $\epsilon_2$ ,  $\zeta^{-2}H_5$ ]phenylalanine, L-[ $\epsilon_1$ ,  $\epsilon_2^{-2}H_2$ ]tyrosine, and L-[ $\delta_1$ ,  $\epsilon_3$ ,  $\zeta_2$ ,  $\zeta_{3}$ ,  $\eta_2^{-2}H_5$ ]tryptophan, the positions of <sup>2</sup>H label incorporation being primarily as shown in Fig. 1. The selectively labeled amino acids were synthesized using modifications of published procedures, as follows.

 $L \cdot [\delta_1, \delta_2, \epsilon_1, \epsilon_2, \zeta^{-2}H_5]$  Phenylalanine  $-[{}^{2}H_{5}]$  Phe was prepared by platinum-catalyzed deuterium exchange using deuterium oxide as the <sup>2</sup>H source, by modification of the procedure of Norton and Bradbury (45). Finely divided platinum was prepared by the reductive procedure of Calf and Garnett (46), using NaBH<sub>4</sub>; 5 g of L-phenylalanine (Sigma) were added to 1 g of reduced Adams' catalyst in 100 ml of 99.8% <sup>2</sup>H<sub>2</sub>O (Merck Sharp & Dohme), followed by refluxing under N<sub>2</sub> gas for 24 h. The  ${}^{2}H_{2}O$  was removed by lyophilization and the  $[{}^{2}H_{5}]$ Phe was purified by recrystallization from EtOH/H2O (1:1, v/v). Adams catalyst and sodium borohydride were from Alfa Products (Danvers, MA). NMR spectroscopy in 3 M <sup>2</sup>HCl (<sup>1</sup>H at 360 MHz) indicated that ~80% of the aromatic hydrogens were exchanged; 20% <sup>1</sup>H intensity was retained at H<sup>81,82</sup>. H<sup>a</sup> and H<sup>#</sup> retained 100 ± 10% <sup>1</sup>H intensity.

 $L \cdot \epsilon_1, \epsilon_2 \cdot H_2 / Tyrosine - [^2H_2]$  Tyr was prepared using a modification of the acid-catalyzed exchange process of Matthews et al. (47). Ten g of L-Tyr (Sigma Chemical Co.) were dissolved in a solution consisting of 150 ml of <sup>2</sup>H<sub>2</sub>O and 30 ml of <sup>2</sup>H<sub>2</sub>SO<sub>4</sub> (Merck Sharpe & Dohme). The mixture was then refluxed under N<sub>2</sub> for 24 h, cooled, adjusted to pH 4.5 with 28% NH4OH, and then kept at 4 °C overnight. The precipitate collected by filtration was washed with 3 volumes of cold water to remove salts, followed by 1 volume of cold 95% ethanol. After drying overnight at 40 °C, 9 g of [<sup>2</sup>H<sub>2</sub>]Tyr crystals were recovered. 'H NMR spectroscopy at 90 MHz indicated that the product was 98  $\pm$  2% [ $\epsilon_1$ ,  $\epsilon_2$ -<sup>2</sup>H<sub>2</sub>]tyrosine.

 $L - [\delta_1, \epsilon_3, \zeta_2, \zeta_3, \eta_2 - {}^2H_5] Tryptophan = [{}^2H_5] Trp was prepared by acid$ catalyzed exchange in CF3CO2D, again using a modification of the procedure of Matthews et al. (47). Trifluoroacetic anhydride (67 ml) (99%, Aldrich) was added dropwise with stirring to 32 ml of cold 99.8% <sup>2</sup>H<sub>2</sub>O (Merck Sharpe & Dohme) in a 500-ml flask. L-Trp (15 g) (Sigma) was then dissolved in this solution, and the flask was sealed and left to stand at room temperature, in the dark, for 3 days. The solvent was removed by rotary evaporation to give a light brown tar. The exchange was repeated three more times, and the percent deuteration was assayed after each exchange using proton NMR spectroscopy at 220 MHz. After the fourth exchange, the solution was adjusted to pH 6 with 28% NH4OH, then kept overnight at 4 °C. The precipitate was filtered, redissolved in  $H_2O$ /ethanol (1:1, v/v), and decolorized using activated charcoal. The product was finally recrystallized from  $H_2O/$ ethanol (3:2, v/v), yielding 9 g of crystals. The product was  $92 \pm 3\%$ deuterated at positions  $\delta_1$ ,  $\epsilon_3$ ,  $\zeta_2$ ,  $\zeta_3$ , and  $\eta_2$  as determined by <sup>1</sup>H NMR spectroscopy at 220 MHz.

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# **Production of Labeled Membranes**

H. halobium strain R1 was the kind gift of Professor T. Ebrey, and was grown in a salt medium basically according to Onishi et al. (48) with the addition of 2% malate (49), except that either  $[^{2}H_{5}]$ Phe or <sup>2</sup>H<sub>2</sub>]Tyr were substituted for the normal nonlabeled amino acids. The growth medium thus typically contained the following amino acids (in g/10 liters): L-alanine, 2.15; L-arginine hydrochloride, 2.0; Lcysteine, 0.5; L-glutamic acid, 13.0; glycine, 0.6; L-isoleucine, 2.2; Lleucine, 8.0; L-lysine hydrochloride, 8.5; L-methionine, 1.85; L-phenylalanine, 1.3; L-proline, 0.5; L-serine, 3.05; L-threonine, 2.5; L-tyrosine, 2.0; and L-valine, 5.0. Note that the growth medium does not normally contain tryptophan, thus, for the Trp-labeled membrane system we incorporated [<sup>2</sup>H<sub>5</sub>]Trp at a level of 5.0 g/10 liters into the medium. Purple membranes were isolated according to Becher and Cassim (50) and were then exchanged with <sup>2</sup>H-depleted water (Aldrich) to remove some background HO<sup>2</sup>H. Samples were generally exchanged twice, then finally concentrated by ultracentrifugation for 12 h at  $100,000 \times g$ , prior to NMR spectroscopy.

#### **Radiotracer** Experiments

To determine the level of deuterated amino acid breakdown and reincorporation into lipid and other amino acids, 1-liter batches of cells were grown and harvested basically as for the <sup>2</sup>H-labeled cells, except that 50  $\mu$ Ci of either [<sup>14</sup>C]Tyr, [<sup>14</sup>C]Phe, or [<sup>14</sup>C]Trp (New England Nuclear) were added as radiotracers. Purple membranes were lyophilized, lipid-extracted (3 times with CHCl<sub>3</sub>/MeOH, 1:2, v/ v), and then hydrolyzed in 6 m HCl (24 h at 110 °C, under vacuum). The hydrolysates were chromatographed using two-dimensional thin layer chromatography on 160-µm Eastman Kodak cellulose Chromagram plates (Rochester, NY) in 2-propanol, 1 M HCl, 2-butanone (60: 25:15, v/v/v; first dimension), then 2-methyl-2-propranol, 2-butanone, acetone, H2O, 28% NH4OH, MeOH (40:20:20:14:5:1, v/v/v/v/v; second dimension). Individual amino acids were detected using ninhydrin spray, after which the "spots" were removed from the TLC plates and counted on a Tracor Analytic Model 6892 scintillation counter (Elk Grove, IL), using Aquasol-2 (New England Nuclear). Our results indicate that  $\leq 5\%$  of [<sup>14</sup>C]Phe and  $\sim 0\%$  [<sup>14</sup>C]Tyr counts 'scrambled" into other amino acids, strongly suggesting a similar low level of incorporation into lipid. Similarly, with [<sup>14</sup>C]Trp, even though there is amino acid breakdown during hydrolysis, comparison of the relative number of counts in the region of the Trp hydrolysis product with those on the rest of the chromatogram indicated little, if any, incorporation of <sup>2</sup>H label into any other amino acid. Our results with Phe incorporation are consistent with those of Engleman and Zaccai (40) who, using  $[{}^{3}H]$ Phe, determined that less than 1%  ${}^{3}H$  was incorporated into other amino acids and whose neutron diffraction data indicated only small <sup>2</sup>H label incorporation into lipid upon [<sup>2</sup>H] Phe supplementation.

#### Spectroscopic Aspects

Nuclear magnetic resonance spectra were obtained on a "homebuilt" Fourier transform NMR spectrometer which 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. We used a Nicolet 1180 computer, 293B pulse programmer, and Model NIC-2090 dual channel 50 ns transient recorder (Nicolet Instrument Corp., Madison,



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WI) for experiment control and rapid data acquisition, together with a dual Diablo model 40 disc system for data storage (Diablo Systems, Inc., Haywood, CA). In order to generate high enough power radiofrequency pulses (~3  $\mu$ s 90° pulse widths) to cover the entire <sup>2</sup>H NMR spectral width, we used an Amplifier Research (Amplifier Research, Souderton, PA) model 200L amplifier to drive a retuned Henry Radio (Henry Radio, Los Angeles, CA) model 2006 transmitter to a ~1000-1500-watt 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 (51, 52) pulse sequence. The 90° pulse width varied between 2.0 and 3.5  $\mu$ s; 90° pulse widths and phase quadrature between the two radiofrequency pulses was established by viewing quadrature free induction decay signals of S-[methyl-2H3]methionine. The same settings were used for data acquisition on <sup>2</sup>H-labeled membranes. In essentially all cases, no phase corrections were necessary after Fourier transformation. Typically, data were collected using sampling rates of 500 ns/point. The zero frequencies of both instruments were established using a 1% D<sub>2</sub>O reference, the zero frequency for the protein samples investigated being offset ~2 ppm downfield from this position. Samples were run as solid high speed pellets (100,000  $\times g$ for 12 h). Sample temperature was regulated either by means of a liquid nitrogen boil off system or by using a heated air flow. The temperatures reported were measured using a calibrated Doric Trendicator (San Diego, CA) with a copper-constantan thermocouple and are the gas flow temperatures measured next to the sample. Separate experiments indicate that this temperature is accurate to  $\pm 1-2$  °C over the entire sample volume. All samples had normal optical absorption spectra after NMR spectroscopy, except after heating to >90 °C when "loss" of chromophore 560 nm absorbance occurred.

Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corporation Cyber-175 system, which is interfaced to a Tektronix 4006 graphics terminal and interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory, as described previously (15).

# RESULTS AND DISCUSSION

We shall discuss first the basic background theory to the study of the <sup>2</sup>H NMR of amino acid dynamics in membrane proteins, concentrating on a residue that we intuitively expect to be one of the most rigid, or irrotationally bound: tryptophan. We show in Fig. 2 theoretical and experimental results for <sup>2</sup>H NMR of  $[\delta_1, \epsilon_3, \zeta_2, \zeta_3, \eta_2$ -<sup>2</sup>H<sub>5</sub>]tryptophan, the free amino acid in the solid state, at 25 °C. As has been discussed in detail elsewhere (34, 53–55), for <sup>2</sup>H NMR the allowed transitions correspond to +1  $\leftrightarrow$  0 and 0  $\leftrightarrow$  -1 and give rise to a "quadrupole splitting" of the absorption line with separation  $\Delta \nu \rho^1$  between peak maxima of

$$\Delta \nu_Q = \frac{3}{4} \frac{e^2 q Q}{h} \left[ 3 \cos^2 \theta - 1 - \eta \sin^2 \theta \cos 2\Psi \right] \tag{1}$$

where  $\theta$  and  $\Psi$  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, eq is the field gradient  $V_{zz}$ , and eQ is the deuteron quadrupole moment. Assuming axial symmetry (asymmetry parameter  $\eta = 0$ ) to eliminate  $\Psi$  dependence, as is certainly appropriate for aliphatic C—<sup>2</sup>H bonds (56), then Equation 1 may be rewritten in a simpler form

$$\Delta \nu = \frac{3}{4} \frac{e^2 q Q}{h} \left( 3 \cos^2 \theta - 1 \right). \tag{2}$$

For rigid polycrystalline solids all values of  $\theta$  are possible and one obtains a so-called "powder pattern," Fig. 2A, having a singularity separation  $(\Delta \nu_{Q_1})$  corresponding to  $\theta = 90^\circ$  and an edge separation  $(\Delta \nu_{Q_3})$  corresponding to  $\theta = 0^\circ$ . For solid aliphatic compounds,  $\Delta \nu$  values of 127 kHz ( $\theta = 90^\circ$ ) and 254

kHz ( $\theta = 0^{\circ}$ ), corresponding to an electric quadrupole coupling constant  $(e^2 q Q/h)$  of 168 kHz (56-58), are expected. Spectral simulation of the results of Fig. 2B (solid [2H5]Trp, 25 °C), using the line shape equations of Bloembergen and Rowland (59) and Cohen and Reif (55) show, however, that the best fit to the experimental spectrum (Fig. 2B) is obtained using  $e^2 q Q/h = 183 \pm 3$  kHz and an asymmetry parameter  $\eta = 0.05 \pm 0.02$  (Fig. 2A and Table I). The observed quadrupole coupling constant for  $[^{2}H_{5}]$ Trp (Fig. 2B), is therefore considerably in excess of the  $\sim 168$  kHz found in aliphatic C-<sup>2</sup>H systems using NMR methods (56). This result is nevertheless consistent with the increased  $e^2 q Q/h$  values found in a variety of other aromatic compounds (34, 56), the observed trends for the electric field gradient values for C—D bonds being  $s_{D} >$  $sp^2 > sp^3$  (60). The average value for naphthalene and anthracene, perhaps the most reasonable published models for  $[{}^{2}H_{5}]$ Trp. is ~184 kHz (61-63). Also, in addition to having substantially larger coupling constants, it is well known that C--D bonds in aromatic systems may have non-zero asymmetry parameters,  $\eta$  (64). In those aromatic systems where asymmetry parameters have been investigated,  $\eta$  values = 0.053  $\pm$ 0.015 have been determined (56).

Our results therefore suggest that there is essentially no large amplitude motion of the Trp molecule on the time scale of the <sup>2</sup>H NMR experiment ( $\sim 10^{-5}$  s) at 25 °C, since the quadrupole splitting is that expected for a rigid, crystalline, aromatic species.

What motions then, if any, do the Trp residues of bacteriorhodopsin in *H. halobium* purple membranes undergo? We show in Fig. 3 deuterium NMR spectra, obtained by the quadrupole echo Fourier transform method at 55.3 MHz (corresponding to a magnetic field strength of 8.5 Tesla) of  $[^{2}H_{5}]$ -



FIG. 2. Deuterium quadrupole-echo Fourier transform NMR spectrum at 55.3 MHz and 25 °C of  $[^{2}H_{b}]$ tryptophan as a crystalline powder, together with its computer simulation. Spectral conditions: 5 scans, 300-s recycle time,  $\tau_{1} = \tau_{2} = 50 \ \mu\text{s}$ , 2.7- $\mu\text{s}$  90° pulse widths, 1-MHz digitization rate, 1,024 real data points, line broadening = 2,000 Hz. The simulation used a deuteron quadrupole coupling constant (DQCC) = 183 kHz,  $\eta = 0.05$ , and  $2\delta = 4,000$  Hz.

Experimental deuterium quadrupole coupling constants and asymmetry parameters for tryptophan, phenylalanine, and tyrosine

	Quadrupole splitting <sup>a</sup>	Asymmetry parameter*
	kHz	η
L- $[\delta_1, \epsilon_3, \zeta_2, \zeta_3, \eta_2 - {}^2H_5]$ Tryptophan <sup>e</sup>	183.0	0.05
L- $[\delta_1, \delta_2, \epsilon_1, \epsilon_2, \zeta^{-2}H_5]$ Phenylalanine <sup>c</sup>	180.0	0.05
L- $[\epsilon_1, \epsilon_2^{-2}H_2]$ Tyrosine <sup>c</sup>	181.3	0.05

" Obtained from a spectral simulation; error is  $\pm 3$  kHz.

<sup>b</sup> Obtained from a spectral simulation; error is  $\pm 0.02$ .

° Solid amino acid at  $25 \pm 2$  °C.

<sup>&</sup>lt;sup>1</sup> The abbreviations used are;  $[{}^{2}H_{5}]$ Phe, L- $[\delta_{1}, \delta_{2}, \epsilon_{1}, \epsilon_{2}, \zeta^{-}H_{5}]$ phenylalanine;  $[{}^{2}H_{2}]$ Tyr, L- $[\epsilon_{1}, \epsilon_{2} - {}^{2}H_{2}]$ tyrosine;  $[{}^{2}H_{5}]$ Trp, L- $[\delta_{1}, \epsilon_{3}, \zeta_{2}, \zeta_{3}, \eta_{2} - {}^{2}H_{5}]$ tryptophan;  $\Delta\nu_{Q}$ , quadrupole splitting;  $\eta$ , asymmetry parameter; DQCC, deuteron quadrupole coupling constant.

tion and resuspension, and the observation that this component is much larger before exchange with <sup>2</sup>H-depleted water. There are almost certainly no isotropically reorienting Trp residues in the purple membrane below ~90 °C, since at least a 14% intensity (1 Trp in 7) would be expected for 1 mobile Trp. The results of Fig. 3 therefore indicate that Trp residues of bacteriorhodopsin in the purple membrane of H. halobium undergo no large amplitude motions on a time scale  $\leq 10^{-5}$  s below 90 °C. The actual upper limits on estimation of the amplitudes of motion of the Trp ring depend, of course, on our ideas as to what these motions may be, *i.e.* what models we may use to describe them. Linear extrapolation of the quadrupole coupling constant of [2H5]Trp-labeled purple membranes to  $0 \text{ K}^2$  indicates that there can be no more than a ~5-10-kHz decrease in  $\Delta \nu_{\varphi}$  at ~37 °C from the absolute zero temperature value, due to (presumed) torsional or librational motions of the Trp residue. Assuming a Gaussian distribution of such motions, where in plane and out of plane motions are equally weighted, we may estimate a maximum C-<sup>2</sup>H vector motion of  $\approx \pm 5-10^{\circ}$  (53). If we assumed a 2-fold jump model, the resulting jump angle would be  $\approx \pm 10-20^{\circ}$  (33). We believe that the first model (53) is the more plausible. Tryptophan residues in the purple membrane are therefore properly throught of as being rigid at the temperature of growth (37 °Č).

Upon heating the  $[{}^{2}H_{5}]$ Trp-labeled purple membranes above ~90 °C, there is a dramatic change in the  ${}^{2}H$  NMR spectrum, as shown in Fig. 3. The large quadrupole splitting collapses and a relatively narrow "isotropic" line, of ~4-kHz width, is obtained. Assuming isotropic rotational motion, a quadrupole coupling constant  $e^{2}qQ/h = 183$  kHz and  $\eta = 0.05$ (Fig. 2), this line width corresponds to a rotational correlation time  $\tau_{R}$  of ~100 ns (65) and is presumably due to protein denaturation. Upon cooling to 37 °C, there is a reappearance of some ~140-kHz component, although this does not necessarily imply any protein renaturation.

The results obtained with tryptophan were not unexpected and are supported by the observation of essentially irrotationally bound Trp residues in a variety of proteins in solution (27-29, 65-67). With this body of solution NMR data in mind, it is therefore worth asking if the types of motions observed for other aromatic residues, such as phenylalanine and tyrosine (27, 29), carry over into the solid state. In particular, we would like to know if these residues may "flip," as has been shown in a number of <sup>1</sup>H solution NMR studies (27, 29, 67, 68).

What, therefore, are the manifestations of such large amplitude residue motions in the <sup>2</sup>H NMR spectra of amino acids in membrane proteins in the solid state? We show in Fig. 4 a spectrum of  $[{}^{2}H_{5}]$ Phe in the crystalline solid state, together with simulated <sup>2</sup>H NMR spectra, for a variety of possible amino acid motions. For the case of no motion we obtain a rigid lattice powder spectrum (Fig. 4A), which as expected is in good agreement with the experimental result obtained with  $[{}^{2}H_{5}]$ Phe (Fig. 4B). The quadrupole coupling constant  $(e^2 q Q/h)$  is 180 ± 3 kHz and the asymmetry parameter is  $\eta = 0.05 \pm 0.02$ . These results are in good agreement with the median values found for a series of monosubstituted benzenes of  $e^2 q Q/h \sim 181$  kHz and  $\eta = 0.06$  (56, 61, 63, 69). Let us now consider the effects of rapid ( $\gg 10^5 \text{ s}^{-1}$ ) motions on the observed <sup>2</sup>H NMR spectrum. One possibility would be fast rotational diffusion about  $C^{\beta}$ - $C^{\gamma}$ . In this case the phenyl ring would undergo rapid rotational diffusion about the  $C^{\gamma}$ - $C^{\zeta}$  axis, in which case the  $C^{\delta_1,\delta_2,\epsilon_1,\epsilon_2}$ <sup>2</sup>H vectors would be at 60 ± 1° to the axis of motional averaging (70), and it is a simple matter to calculate the observed spectrum. Ignoring for simplicity the small non-zero asymmetry parameter, then we find

FIG. 3. Deuterium quadrupole-echo Fourier transform NMR spectra at 55.3 MHz of [<sup>2</sup>H<sub>5</sub>]tryptophan and [<sup>2</sup>H<sub>5</sub>]tryptophanlabeled bacteriorhodopsin in the purple membrane of H. hal**obium R1.** All experimental spectra were collected with  $\tau_1 = \tau_2 = 50$  $\mu$ s, 2.7- $\mu$ s 90° pulse widths, 1-MHz digitization rate, 1,024 real data points, and line broadening = 2,000 Hz. A, crystalline powdered [<sup>2</sup>H<sub>5</sub>]tryptophan at 25 °C, 5 scans, 300-s recycle time. Simulation used DQCC = 183 kHz,  $\eta = 0.05$ ,  $2\delta = 4,000$  Hz. *B*, temperature dependence of the <sup>2</sup>H NMR spectrum of bacteriorhodopsin in H. halobium R1 purple membranes containing biosynthetically incorporated  $[{}^{2}H_{5}]$ tryptophan residues. The temperatures are indicated in  $^{\circ}C. -85 ^{\circ}C:$ 1,080 scans, 25-s recycle time, simulation used DQCC = 186.7 kHz,  $\eta = 0.05, 2\delta = 4,000$  Hz. -30 °C: 9,900 scans, 4-s recycle time, simulation used DQCC = 184 kHz,  $\eta = 0.05$ ,  $2\delta = 4,000$  Hz. 37 °C: 15,000 scans, 1-s recycle time, simulation used DQCC = 183 kHz,  $\eta = 0.06$ ,  $2\delta = 4,000$  Hz. 63 °C: 13,000 scans, 1-s recycle time, simulation used DQCC = 180 kHz,  $\eta = 0.06$ ,  $2\delta = 4,000$  Hz. 75 °C: 12,000 scans, 1-s recycle time, simulation used DQCC = 175 kHz,  $\eta = 0.06$ ,  $2\delta = 4,000$ Hz. 95°C: 16,000 scans, 1-s recycle time, simulation used an isotropic Lorentzian with  $2\delta = 4,000$  Hz.

Trp-labeled membranes, as a function of temperature, together with, for comparison, the <sup>2</sup>H NMR spectrum of  $[^{2}H_{5}]$ -Trp at 25 °C. The results of Fig. 3 indicate that there is essentially no change in the spectrum of  $[^{2}H_{5}]$ Trp-labeled purple membranes between -85 and 75 °C, and in addition, the quadrupole splitting and asymmetry parameter of the  $[^{2}H_{5}]$ Trp-labeled purple membrane are extremely close to those of the solid amino acid (Figs. 2B and 3A). The slight differences in the case of the purple membrane do not necessarily imply differing motions, since the rigid lattice quadrupole coupling constant may have been changed slightly due to peptide bond formation, or there may be slight differences due to "lattice effects," e.g. hydrogen bonding interactions in the Trp crystal. Please note that the sharp central component in Fig. 3B (37°, 63°, and 75°) arises mainly from HO<sup>2</sup>H, as determined by additional experiments<sup>2</sup> involving lyophiliza-

<sup>2</sup> R. Kinsey and E. Oldfield, unpublished results.



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FIG. 4. Theoretical and experimental deuterium NMR spectra of [<sup>2</sup>H<sub>5</sub>]phenylalanine. A, computer simulation of the deuterium NMR spectra of  $[{}^{2}H_{5}]$  phenylalanine as a rigid polycrystalline solid; DQCC = 180 kHz,  $\eta$  = 0.05,  $2\delta = 5,000$  Hz. B, deuterium quadrupole echo Fourier transform NMR spectrum at 55.3 MHz and 25 °C of [<sup>2</sup>H<sub>5</sub>] phenylalanine as a crystalline powder; 15 scans, 40-s recycle time,  $\tau_1 = \tau_2 = 50 \ \mu s$ , 2.7-µs 90° pulse widths, 1-MHz digitization rate, 1,024 real data points, line broadening = 2,000 Hz. C, computer-simulated line shape in which the phenylalanine ring is assumed to undergo rapid rotational diffusion about  $C^{\gamma}$ - $C^{\zeta}$  C<sub>2</sub>-axis. D, computer-simulated line shape in which the phenylalanine ring is assumed to undergo a rapid 2-fold flipping motion about the  $C^{\gamma}$ - $C^{\tilde{\chi}}$  axis.

$$\Delta \nu = \frac{3}{8} \frac{e^2 q Q}{h} \left( 3 \cos^2 \beta - 1 \right) (3 \cos^2 \theta' - 1)$$
(3)

where  $\beta$  is the angle between the principal axis of the electric field gradient tensor and the axis of motional averaging and  $\theta'$  is the angle between the axis of motional averaging and  $H_0$ . For the case  $\theta' = 90^\circ$ , a quadrupole splitting  $\Delta \nu \sim 17.0$  kHz is predicted, having an intensity corresponding to four deuterons. In addition, a 20% intensity contribution from the  $\zeta^{-2}$ H is predicted, having the same breadth as the rigid lattice spectrum, since the  $C^{\xi}$ -<sup>2</sup>H vector is along the axis of motional averaging, *i.e.*  $\beta = 0$ . The composite spectrum (corrected for the nonuniform labeling of our sample) is shown in Fig. 4*C*. It is quite dissimilar to that observed for  $[^{2}H_{5}]$ Phe in any native system investigated.

By contrast, a 2-fold "jump" model for phenylalanine motion, whereby the aromatic ring executes  $180^{\circ}$  reorientational flips about C<sup>7</sup>-C<sup>5</sup>, and which has been detected previously in solution NMR studies of proteins by means of chemical shift data, predicts a very different result (Fig. 4*D*).

Assuming that motion is "fast" compared to the breadth of the rigid bond coupling ( $\gg 2 \times 10^5 \text{ s}^{-1}$ ), a motionally averaged tensor may be calculated by using the model of Soda and Chiba (33) for motional averaging of the deuterium quadrupole interaction by reorientation about a 2-fold axis. The results for the averaged field gradient tensor components as a function of  $\beta$  and the asymmetry parameter ( $\eta$ ) of the static tensor are

Shown in Fig. 5 are the new effective asymmetry parameters 
$$(\eta_{eff})$$
 for the case of rapid 180° reorientations as a function of bond angle,  $\beta$ , together with the new maximum effective field gradients,  $eq$ , *i.e.* the reduced spectral breadths  $V_{33}$ - $V_{11}$ . As expected,  $\eta_{eff}$  is only slightly dependent on  $\eta$  for the very small  $\eta$ 's of interest. Using  $\beta = 60^{\circ}$ ,  $\eta = 0.05$ , and  $e^2 q Q/h = 180$  kHz (the best fit spectral simulation parameters from Fig. 4, A and B), we calculate for the case of flipping PheC <sup>$\delta_1, \delta_2, \epsilon_1, \epsilon_2$</sup> -<sup>2</sup>H vectors a new effective asymmetry parameter  $\eta_{eff} = 0.66$  and a reduced maximum spectral breadth (corresponding to the outer edges of the absorption line shape) of 179 kHz. The spectra now contain a new sharp narrow feature, corresponding to the separation between the singularities in the powder pattern, having  $\Delta v_{Q1} \sim 30.3$  kHz (Fig. 4D). This dominant feature is easily detected in some intact membrane spectra, as discussed below. Note that we have not yet considered the  $\zeta^{-2}$ H. This lies on the axis of motional averaging and its spectrum will therefore be essentially unaffected by the motion ( $\beta = 0^{\circ}$ , Fig. 5), except for a second order effect due to an initial non-zero  $\eta$ . The composite spectrum of Fig. 4D therefore contains two components: 75% intensity from  ${}^{2}H^{\delta_1,\delta_2,\epsilon_1,\epsilon_2}$  having  $\eta = 0.66$ ,  $\Delta \nu_{Q1} = 30$  kHz, and a total breadth of ~210 kHz (Fig. 5). It is perhaps worth noting that in general for a  ${}^{2}H$  powder spectrum, the splittings of the singularity, step, and edge as a function of breadth ( $\nu_Q$ ) and asymmetry parameter ( $\eta$  or  $\eta_{eff}$ )

$$V_{11} = -\frac{1}{2}eq(1 + \eta) \qquad \Delta \nu_{Q1} = \frac{1}{2}\nu_Q(1 - \eta)$$

$$V_{22} = +\frac{1}{4}eq[(1 - 3\cos 2\beta) + \eta(1 + \cos 2\beta)] \qquad (4) \qquad \Delta \nu_{Q2} = \frac{1}{2}\nu_Q(1 + \eta)$$

$$V_{33} = +\frac{1}{4}eq[(1 + 3\cos 2\beta) + \eta(1 - \cos 2\beta)]. \qquad \Delta \nu_{Q3} = \nu_Q$$

(5)

bc



FIG. 5. Dependence of the effective asymmetry parameter  $(\eta_{\text{eff}})$  and reduced spectral breadth on the interbond angle,  $\beta$ , for the case of motional averaging by 2-fold flipping for several values of the rigid bond asymmetry parameter  $(\eta)$ .

such that  $\nu_Q$  and  $\eta$  are fully determined by measurement of any two of the frequency parameters,  $\Delta \nu_{Qi}$ . In practice, however, especially in systems as complex as cell membranes, where different residues of the same chemical type may undergo different motions at any given temperature, and where adventitious <sup>2</sup>H label incorporation may have occurred due to label scrambling, either in synthesis or biosynthesis, such analyses of spectral line shapes will require considerably higher spectral signal-to-noise ratios than those obtained so far.

The above results have concentrated exclusively on the deuterium NMR spectra of flipping phenylalanine rings: such line shape changes as discussed above will also, of course, be manifest in <sup>13</sup>C spectra, and similar calculations have been presented by Spiess (71) for the chemical shift tensor,  $\sigma$ .

In Fig. 6 we present the first <sup>2</sup>H NMR results on [<sup>2</sup>H<sub>5</sub>]Phelabeled bacteriorhodopsin in the purple membranes of H. halobium, together with comparison spectra of  $[^{2}H_{5}]$ Phe in the solid state (Fig. 6A). The deuteron spectra of all "native" membranes (Fig. 6, B-F) are extremely broad and have characteristic line shapes (Fig. 4, A, B, and D). At low temperatures (-85 °C, -30 °C, Fig. 6, B and C), the spectra of  $[^{2}H_{5}]$ Phe-labeled purple membranes are virtually superimposable on that of the solid amino acid at room temperature (Fig. 6A). The quadrupole splittings of the membrane spectra at low temperatures are, as with the [2H5]Trp-labeled membranes (Fig. 2), only very slightly temperature-dependent between  $\sim 0$  and -85 °C, but in contrast to the Trp sample, upon warming above  $\sim -30$  °C there is a continuous change in spectral line shape up to  $\sim 55$  °C, characterized by a loss of the ~130-kHz component and a growth of a ~30-kHz component (Fig. 6, E-G). As will by now be clear, this narrow spectral component ( $\Delta v_{Q1} \sim 31$  kHz,  $\eta_{eff} = 0.66$ ) most likely originates from [2H5]Phe rings undergoing 2-fold flips. At the temperature of growth (Fig. 6F), all  $(13 \pm 2)$  of the 13 Phe residues appear to be undergoing rapid 2-fold jumps, and as expected at >50 °C, all Phe residues again appear to be undergoing such motions, although our spectral simulation at 55 °C is less satisfying than those obtained at lower temperatures, since adequate agreement between experiment and computer simulation is only achieved using  $\eta_{eff} = 0.60$  (rather than  $\eta_{\rm eff} = 0.66$ ), suggesting that additional motions to those discussed above have begun to occur. Interestingly, the spectra of Fig. 6, D-F, show little evidence of broad line shapes due to flipping at "intermediate exchange" frequencies. One likely explanation for this result is that the <sup>2</sup>H NMR spectra of such intermediate exchange residues will be characterized by rather rapid decays of the quadrupole echo intensity in the  $90^{\circ} - \tau - 90^{\circ}_{90^{\circ}}$  experiment (72) and we have indeed observed rather rapid and anisotropic quadrupole echo decay rates at 3° (in addition to large differential spin-lattice relaxation behavior) in Fig. 6D. Accurate determinations of flipping to nonflipping ratios at intermediate temperatures will thus require considerably higher signal-to-noise ratio spectra than we have obtained so far, in addition to  $\tau$ -dependence studies, the use of slow motional models (73, 74) to accurately simulate the spectra of residues undergoing intermediate rate motions  $(\sim 10^4 - 10^5 \text{ s}^{-1})$ , computations of echo decay rates in such intermediate exchange situations, together with development of instrumentation having dead times shorter than those currently available. Nevertheless, at ~3 °C our results are quite well simulated using a simple superposition of two individual states and suggest that ~45% of the spectrum of Fig. 6D may be attributed to deuterons having  $\Delta v_{Q1} = 31.0$ 



FIG. 6. Deuterium quadrupole-echo Fourier transform NMR spectra at 55.3 MHz of [<sup>2</sup>H<sub>5</sub>]phenylalanine and [<sup>2</sup>H<sub>5</sub>]phenylalanine-labeled bacteriorhodopsin in the purple membrane of *H. halobium* R1, together with their spectral simulations. All experimental spectra were collected with  $\tau_1 = \tau_2 = 50 \ \mu s$ , 2.7- $\mu s$  90° pulse widths, 1-MHz digitization rate, 1,024 real data points and line broadening = 2,000 Hz. *A*, crystalline powder [<sup>2</sup>H<sub>5</sub>]phenylalanine at 25 °C, 15 scans, 40-s recycle time, simulation used DQCC = 180 kHz,  $\eta = 0.05$ ,  $2\delta = 5,000$  Hz. *B*, temperature dependence and spectral simulations of the <sup>2</sup>H NMR spectrum of [<sup>2</sup>H<sub>5</sub>]phenylalanine-labeled bacteriorhodopsin in *H. halobium* R1 purple membranes. Important features of the spectral simulation results are discussed in the text. The number of scans and recycle times were in the range 3,000-7,000 and 3-10 s, respectively, for spectra between 90 °C and -30 °C, and 552 scans at 80 s for the -85 °C spectrum.

kHz (the singularity separation of a flipping ring) and  $\eta \sim 0.66$ . Taking into account the relative deuterations of  $H^{\delta_1,\delta_2,\epsilon_1,\epsilon_2,\zeta}$ , this could correspond to 9 (±2) of the 13 Phe rings in bacteriorhodopsin undergoing fast  $C^{\gamma}-C^{\zeta}$  flips at ~3 °C. Residues which might be expected to be mobile at low temperatures include the chymotrypsin-accessible surface residue Phe-72 and perhaps Phe-229, near the carboxyl terminus.

A second problem that arises in interpretation of the results of Fig. 6 is that we have so far only speculated that the  $\Delta \nu_Q \sim$ 30-kHz component originates from the *ortho* and *meta* deuterons undergoing flipping. An alternative possibility is that this signal arises from a second population of  $[{}^{2}H_{5}]$ Phe residues undergoing some alternative specialized motion, such as a "rocking" motion about C<sup>a</sup>-C<sup> $\beta$ </sup>. Fortunately, we have eliminated this possibility by synthesis of  $[\zeta - {}^{2}H_{1}]$ Phe together with <sup>2</sup>H NMR of  $[\zeta - {}^{2}H_{1}]$ Phe-labeled membranes, in which axially symmetric spectra are obtained at 37 °C.<sup>3</sup> Our results thus suggest that at growth temperature almost all Phe residues are undergoing fast (>10<sup>5</sup> s<sup>-1</sup>) 2-fold flipping motions about C<sup> $\gamma$ </sup>-C<sup> $\zeta$ </sup>, while at  $\lesssim - 25$  °C all residues are undergoing slow motions (<10<sup>4</sup> s<sup>-1</sup>).

Upon heating the sample of Fig. 6F to  $\gtrsim 90$  °C, there is a dramatic change in the <sup>2</sup>H NMR spectrum from a state characterized by having  $\Delta v_{Q1} \sim 31$  kHz and  $\eta = 0.6$  (Fig. 6F) to a relatively sharp "isotropic" line shape having a width of ~9 kHz (Fig. 6G). There seem to be two main possible explanations for this result. First, the protein could simply be denatured, resulting in an isotropic line shape or series of overlapping Lorentzian lines, due to motion of the Phe ring with a correlation time  $\sim$ 200 ns (65). The second possibility is that the Phe rings begin to undergo fast continuous rotational diffusion about  $C^{\beta}$ - $C^{\gamma}$  at >90 °C, resulting in a line having  $\Delta \nu_{\varphi} \sim 17$  kHz (Fig. 4C), the splitting being in addition further reduced by some additional off-axis motions, together with a contribution from HO<sup>2</sup>H helping to obscure the quadrupole splitting. However, it seems unlikely that the HO<sup>2</sup>H component's total integrated intensity is sufficiently large to effect this obfuscation. The spectrum of Fig. 6G may be quite well simulated using a ~10% flipping component ( $\Delta v_{Q1} \sim 31$  kHz) and a ~90% isotropic component ( $W \sim 9$  kHz). We thus favor the notion that the bacteriorhodopsin has become at least partially unfolded by heating above ~90 °C. Upon cooling the sample of Fig. 6G to 37 °C, a broad splitting is again obtained, superimposed on an isotropic line. Such a spectrum is not observed in the initial heating runs, suggesting that there is a partial refolding of the protein after cooling. Although this spectrum (Fig. 6G) is rather difficult to simulate, we estimate that  $\sim 40\%$  of the Phe residues remain in the mobile, denatured state. Optical absorption spectra indicate loss of the 560 nm chromophore absorption and presumably a non-native protein structure is obtained after such high temperature excursions. It may be worth noting that the collapse of the native membrane type spectrum occurs over a rather narrow range of temperature, between 85 and 90 °C.

The results of Fig. 6 indicate that phenylalanine side chains in bacteriorhodopsin of the purple membrane of *H. halobium* undergo only a limited variety of motions at their growth temperature. In particular, it seems that the vast majority undergo 2-fold flipping motions at 37 °C about  $C^{\beta}$ -C<sup> $\gamma$ </sup>, and only between 0 and 2 are rigid. If therefore seems reasonable to ask whether such motions are also seen with the aromatic residue tyrosine, since 2-fold flips have previously been noted in <sup>1</sup>H NMR spectra of several proteins in solution (29, 68).

We show therefore in Fig. 7 <sup>2</sup>H NMR spectra of  $[^{2}H_{2}]$ Tyr in the crystalline solid state (Fig. 7A), together with spectra of

the membrane, as a function of temperature. At room temperature the amino acid spectrum may best be simulated (Table I, Fig. 7A) using  $e^2 q Q/h = 181.3 \pm 3$  kHz and  $\eta =$  $0.05 \pm 0.02$ . An essentially identical spectrum is obtained for the  $[^{2}H_{2}]$ Tyr-labeled purple membrane at ~-90 °C (Fig. 7B), where spectral simulation reveals an observed splitting of ~124 kHz and asymmetry parameter  $\eta = 0.05$ , corresponding to a coupling constant  $e^2 q Q/h = 180$  kHz. As with the case of the [2H5]Phe-labeled membranes, this slight reduction in coupling in the membrane may be due to peptide bond formation, or to lattice effects, e.g. hydrogen-bonding differences between amino acid crystal and lipoprotein membrane, or more likely due to experimental error. As with the  $[{}^{2}H_{5}]$ Phe results (Fig. 6), increasing temperature again results in a decrease of the broad spectral component and an increase in the narrower component having  $\Delta \nu_{Q1} \sim 27-29$  kHz (Fig. 7). At the temperature of growth (37 °C), the spectrum of the [<sup>2</sup>H<sub>2</sub>]Tyr-labeled purple membrane (Fig. 7) is qualitatively quite similar to that of the  $[{}^{2}H_{5}]$ Phe membrane at the same temperature (Fig. 6D). We find that good agreement with the experimental result of Fig. 7 (membranes at 37 °C) is obtained using an 82% spectral contribution (due to flipping) have  $\Delta v_{Q1} = 30.0$  kHz,  $\eta = 0.66$ , and an 18% contribution having  $\Delta v_{01} = 118$  kHz and  $\eta = 0.05$ . Note that all ring deuterons in  $[^{2}H_{2}]Tyr$  generate axially asymmetric ( $\eta \sim 0.66$ ) powder pattern line shapes in the case of fast 2-fold flipping motion, since there is no  ${}^{2}H^{\zeta}$  nucleus present. The spectral simulation results therefore indicate that  $9 \pm 2$  of the 11 Tyr residues in the purple membrane are undergoing fast  $(>10^5 \text{ s}^{-1})$  flipping motions at the temperature of growth, *i.e.* as with the case of  $[{}^{2}H_{5}]$  Phe-labeled membranes, essentially all benzene rings are flipping at growth temperature.



FIG. 7. Deuterium quadrupole echo Fourier transform NMR spectra at 55.3 MHz of  $[{}^{2}H_{2}]$ tyrosine and  $[{}^{2}H_{2}]$ tyrosine-labeled bacteriorhodopsin in the purple membrane of *H. halobium* R1, and their spectral simulations. All experimental spectra were collected with  $\tau_{1} = \tau_{2} = 50 \ \mu s$ , 2.7- $\mu s$  90° pulse widths, 1-MHz digitization rate, 1,024 real data points and line broadening = 2,000 Hz. *A*, deuterium NMR spectrum of random crystalline powder  $[{}^{2}H_{2}]$ tyrosine, 20 scans, 200-s recycle time, simulation used DQCC = 181.3 kHz,  $\eta = 0.05$ ,  $2\delta = 4,000$  Hz. *B*, temperature dependence of the  ${}^{2}H$  NMR spectra of  $[{}^{2}H_{2}]$ tyrosine-labeled bacteriorhodopsin in *H. halobium* R1 purple membranes. Data acquisition conditions were as follows: -90 °C, 1,967 scans, 25-s recycle time; -25 °C, 2,734 scans, 2-s recycle time; 37 °C, 5,500 scans, 1-s recycle time; 54 °C, 8,000 scans, 1-s recycle time; 95 °C, 28,000 scans, 400-ms recycle time. Important features of the spectral simulations are discussed in the text.

The Journal of Biological Chemistry

<sup>&</sup>lt;sup>3</sup> A. Kintanar and E. Oldfield, unpublished results.

Upon heating the sample of Fig. 7 to ~85 °C, there is relatively little change in the <sup>2</sup>H NMR spectrum, but between ~86 and 92 °C the protein again apparently unfolds and a narrow line spectrum is obtained at 95 °C, as seen previously with [<sup>2</sup>H<sub>5</sub>]Phe. Upon cooling to 37 °C, the majority of the narrow component remains, indicating little return to the native protein structure.

The results we have presented in this publication represent the first attempt at detailing the motions of aromatic amino acids in a functional biological membrane protein, bacteriorhodopsin in the purple membrane of *H. halobium* R1. Our results indicate that tryptophan, phenylalanine, and tyrosine residues are rigid at low temperatures (<-30 °C), but phenylalanine and tyrosine residues are both highly mobile at the temperature of growth of the *H. halobium* purple membrane (37 °C), undergoing fast ( $>10^5-10^6 \text{ s}^{-1}$ ) 2-fold jumps about C<sup>β</sup>-C<sup>γ</sup>. Tryptophan residues do not undergo this type of motion even at 85 °C, immediately prior to protein denaturation. Upon denaturation at ~90 °C "narrow line" spectra (having line widths ~5-10 kHz) are obtained for all three aromatic amino acids, suggesting fast large amplitude motions.

The above results are to be compared with those reported previously (41) for the aliphatic system  $[\gamma^{-2}H_6]$  value-labeled bacteriorhodopsin. In all instances, there is no evidence for fast motion about  $C^{\alpha}$ - $C^{\beta}$  at any temperature investigated. In the case of valine-labeled purple membranes, motion about  $C^{\beta}$ -C<sup> $\gamma$ </sup> is fast (>10<sup>6</sup> s<sup>-1</sup>) 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 growth temperature of the organism), rotation is not diffusive but occurs by a 2-fold flipping process, as has been detected previously in solution NMR studies of soluble proteins (27, 29, 67, 68). The additional bulk of the Trp-indole ring prevents even this motion and only small angle librations are allowed. These results are supported by spin-lattice relaxation data to be reported elsewhere,<sup>2</sup> which show that all systems except for the  $[\gamma^{-2}H_6]$ valine membranes have spin-lattice relaxation times which decrease with increasing temperature since correlation times  $(\tau_c)$  are all  $\gg \omega_0^{-1}$ .

Basically similar results with <sup>2</sup>H-labeled aromatic amino acids in this and other systems have recently been reported in abstracts by three other independent groups (75–77), indicating that the types of motions we have observed in the solid state may be of general occurrence.

We believe that the way is now open to examining a whole new area in membrane molecular biology by focusing on the active species, the membrane enzymes, rather than solely observing the membrane lipids, which for technical reasons have been the most attractive species to study during the last 10 years. Clearly, spectral sensitivity is now sufficient to permit extremely detailed investigation of the rates and types of motion of amino acid residues in membrane proteins, including the effects of, *e.g.* cholesterol and membrane lipid "fluidity" on protein structure.<sup>4</sup>

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# 9036

The Journal of Biological Chemistry

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