Fluorotyrosine M13 Coat Protein: Fluorine-19 Nuclear Magnetic Resonance Study of the Motional Properties of an Integral Membrane Protein in Phospholipid Vesicles†

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ABSTRACT: We have prepared in vivo a fluorotyrosyl derivative of M13 coat protein and have incorporated it at high levels in small phospholipid vesicles, using a urea–cholate dialysis procedure. ^{19}F nuclear magnetic resonance experiments at 254 MHz with this system indicate a T_1 of 0.32 s and line width of 300 Hz. The observed line width increases dramatically below the gel to liquid-crystalline transition temperature for the lipid, indicating that the probe is sensitive to the phase state of the bilayer. Nuclear Overhauser enhancement and field dependence of line width were used to establish the relative contributions of dipolar interactions and chemical-shift anisotropy to the observed T_1 and line width. From this relaxation data, we have constructed a model for the motional properties of the protein in the lipid bilayer. This model is characterized by correlation times for rotation about the $\alpha\beta$ and $\beta\gamma$ bonds of the two tyrosyl residues of 2×10^{-8} and 5×10^{-9} s, respectively. Rapid intermolecular dipole–dipole interactions are required to account for the estimated dipolar contribution to T_1. A reasonable model for these interactions is that lipid methylene protons are involved in relaxation of the fluorine probes (which reside in the hydrophobic region of this integral membrane protein). We estimate a minimum translational diffusion coefficient for such lipids of $D \geq 3 \times 10^{-9}$ cm^2/s.

The motional characteristics of proteins in phospholipid bilayers have been examined to the extent that lateral diffusion has been estimated for a few restricted eukaryotic systems by methods such as measurement of the rate of intermixing of surface antigens (Frye and Ediden, 1970), rate of movement of fluorescent patches (Ediden and Fambrough, 1973), and fluorescence recovery after photobleaching (Poo and Cone, 1974). Although these techniques have provided useful information, they are not easily generalized to the study of membrane protein motion in a reconstituted vesicle system and do not provide details of the various internal molecular motions involved. While nuclear magnetic resonance spectroscopy has been applied extensively to the characterization of the motional properties of lipids (for reviews, see Wennerström and Lindblom, 1977; Lee et al., 1974; Smith et al., 1977), it has not been utilized to examine the same properties of membrane proteins. The reason NMR[1] has not been used to study membrane proteins is that the intrinsic ^1H and ^{13}C NMR spectra cannot be seen due to a combination of low amounts of protein present, slow motions resulting in large line widths, and the dominating resonances from the lipids. The utility of ^{19}F NMR as a probe of protein structure and dynamics has been elaborated previously (Sykes et al., 1974; Hull and Sykes 1974, 1975a,b; Lu et al., 1976). In the present context, ^{19}F NMR of fluoro-labeled proteins has the additional advantage that the only resonances to be seen come from the protein studied, with no interference from the lipids.

The major coat protein (gene 8) of the nonlytic coliphage M13 behaves as an integral membrane protein during certain stages of the viral infection (Smilowitz et al., 1972; Smilowitz, 1974) and as such offers an attractive model for study of lipid–protein interactions. It is a 5240-dalton polypeptide comprised of a basic carboxyl terminus, a very hydrophobic central region, and an acidic amino terminus (Figure 1; Asbeck et al., 1969; Nakashima and Konigsberg, 1974) and is one of the best characterized membrane proteins (Knippers and Hoffman-Berling, 1966; Wickner, 1976; Zwizinski and Wickner, 1977). It offers a further advantage in that it may be easily purified in lipid-free form from intact phage.

We have prepared in vivo a fluorotyrosyl derivative of M13 coat protein and have developed a methodology for its incorporation at high levels into synthetic phospholipid vesicles suitable for NMR studies. We report here the results of ^{19}F NMR investigations into the motional properties of the protein in synthetic phospholipid vesicles.

Experimental Procedure

Materials. M13 phage was the gift of Dr. William Paranchych, Department of Biochemistry, University of Alberta. *Escherichia coli* AT2471 (CGSC 4510, Hfr, *thi, rel, tyrA*$_4$, λ^-) was obtained from Dr. B. Bachmann at the *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn. Trizma base and phospholipids were from Sigma (St. Louis, Mo.), while *m*-fluoro-DL-tyrosine was from Aldrich Chemical Co. (Milwaukee, Wis.). Na^{125}I (542 mCi/mL, 17 Ci/mg) was obtained from New England Nuclear, Canada (Lachine, Quebec). Reagent-grade urea and antifoam B were from Baker (Phillipsburg, N.J.), and Sepharose 4B was obtained from Pharmacia (Piscataway, N.J.). D_2O was from Bio-Rad Laboratories (Richmond, Calif.).

Growth of the Fluorotyrosyl M13 Phage. For innocula, *E. coli* AT2471 was grown to stationary phase at 37 °C on M63 medium (Miller, 1972) supplemented with 1% glycerol, 10 μg/mL thiamine, and 8 μg/mL tyrosine (a growth-limiting tyrosine level). For phage preparation, 20 L of M63 medium

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[1] Abbreviations used: NMR, nuclear magnetic resonance; DMPC, dimyristoylphosphatidylcholine; NOE, nuclear Overhauser enhancement; DSS, sodium 4,4-dimethyl-4-silapentane-5-sulfonate; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hyrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

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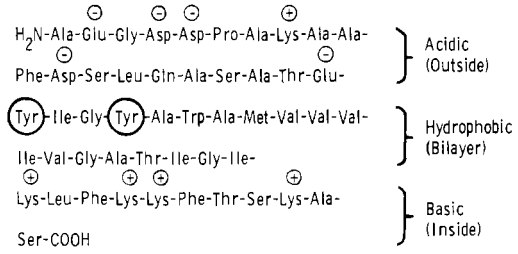

FIGURE 1: The sequence of M13 coat protein (Nakashima and Konigsberg, 1974). The two tyrosine residues, which were replaced by fluorotyrosine, reside in the hydrophobic central region which spans the cytoplasmic membrane during viral infection.

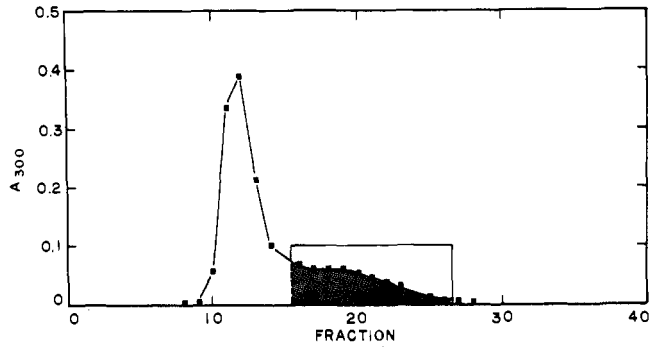

FIGURE 2: Elution profile from Sepharose 4B chromatography of vesicles. The shaded fractions were combined and concentrated with an ultrafiltration apparatus.

supplemented with 1% glycerol, 10 μg/mL thiamine, and 3 μg/mL tyrosine was innoculated to an A_{400} of 0.05 with tyrosine-starved AT2471. Fifteen minutes after innoculation, 5 drops of antifoam B was added. When the culture reached an A_{400} of 0.10, M13 phage was added at a multiplicity of infection of twenty. Eight minutes later, 1.20 g of *m*-fluoro-DL-tyrosine (final concentration of 60 μg/mL) was added slowly as a pH 9.5 solution in 120 mL of water. Additional tyrosine to sustain growth was added as follows: 3 μg/mL at 6 h after phage addition, 2 μg/mL at 10 h, and 2 μg/mL at 15 h. Total growth time was 18 h. Cells were removed by continuous-flow centrifugation at 10 000*g* in a Cepa refrigerated centrifuge. Isolation of phage was carried out as described by Wickner (1975), except that the final preparation was lyophilized and stored dry at 4 °C. Yield of fluorotyrosyl M13 phage from 20 L was 750 mg dry weight.

Isolation of Fluorotyrosyl Coat Protein. Protein was extracted from fluorotyrosyl M13 phage as described by Knippers and Hoffman-Berling (1966) and stored dry at 4 °C. Protein was determined by dry weight with typical yields of 90%. Based on the 20-fold preference of the tyrosyl-tRNA synthetase for tyrosine over *m*-fluoro-DL-tyrosine (Calender and Berg, 1966), we estimate 50% replacement of tyrosine by fluorotyrosine with our procedures.

Preparation of ^{125}I-Labeled Coat Protein. Coat protein was prepared as described above from unlabeled M13 phage grown by the Wickner (1975) procedure. Protein was iodinated by the Syvanen et al. (1973) procedure using 0.5 mCi of $Na^{125}I$ in a 10-μL volume and 100 μg of protein (100 μL of a 1 mg/mL solution). The cold KI dilution step was omitted. The specific activity of the purified preparation was 220 000 cpm/μg, as determined by liquid scintillation counting of β emission in toluene–Triton counting fluid (Weiner and Heppel, 1971) in a Beckman Model LS-230 scintillation counter.

Preparation of Vesicles Containing Fluorotyrosyl Coat Protein. Forty-eight milligrams of dipalmitoylphosphatidic acid (1,2-dihexadecanoyl-3-*sn*-phosphatidic acid) and 385 mg of dimyristoylphosphatidylcholine (1,2-ditetradecanoyl-3-*sn*-phosphatidylcholine) were weighed into a 50-mL round-bottom flask. Forty-eight milligrams of beef heart cardiolipin was added as an ethanol solution (3.4 mg/mL). This mixture was dried on a rotary evaporator until it had a consistency of a thick cream, whereupon 6.0 mL of benzene and 6 drops of H_2O were added. The resulting clear solution was thoroughly dried on a rotary evaporator and redried after the addition of 3.0 mL of ether to remove residual solvent. The dried lipids were then suspended in 5.5 mL of 8.0 M urea, 5.0 mM Tris-HCl (pH 8.0), 2.0% sodium cholate, 0.1 mM EDTA, and 20 mM $(NH_4)_2SO_4$ in a 15-mL polystyrene conical tube and sonicated with a titanium microprobe on a Bronwill Biosonik III sonicator at maximum power for a total of 10 min (2-min bursts with cooling periods). Twenty-seven milligrams of fluorotyrosyl coat protein and 150 000 cpm of ^{125}I-labeled coat protein (28 000 cpm/μg) were added and solubilized by incubation in a 55 °C water bath and vortexing. The solution was then dialyzed against 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, and 10% methanol for a total of 4 days, with buffer changes at 20 and 40 h. The sample was divided into two portions, and each portion was layered over 5.0 mL of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA and centrifuged for 4.5 h at 15 °C in a Beckman Ti50 rotor at 150 000*g*. Each pellet was resuspended in 3.5 mL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM KCl, and 0.02% NaN_3 (TEKA buffer) with a combination of vortexing and sonication at maximum power setting with a titanium microprobe (3 min total). The two fractions were combined and sonicated for a total of 20 min (microprobe, maximum power setting, 2-min bursts separated by cooling periods).

Sizing of Fluorotyrosyl Coat Protein-Containing Vesicles. A 60-mL (2.5 × 50 cm) Sepharose 4B column was equilibrated with TEKA buffer and saturated with phospholipid by passage of a dimyristoylphosphatidylcholine suspension (150 mg of DMPC in 4.0 mL of TEKA buffer, sonicated at maximum power with the microprobe for 20 min total) at a flow rate of 4.9 mL/(cm² h). The vesicle sample was then chromatographed in three equal portions, under the same elution conditions. For each column, 50 fractions of 1.7 mL each were collected and assayed for phospholipid by their A_{300} (Huang, 1969). The fractions corresponding to unilamellar vesicles (Huang, 1969) were pooled (Figure 2) and concentrated in a 10-mL Amicon ultrafiltration apparatus (YM-10 membrane) from an initial volume of 63 mL to a final volume of 3 mL. Three milliliters of TEKA buffer prepared with D_2O (in place of H_2O) was added to provide a lock for NMR experiments, and the sample was reconcentrated to 2.2 mL. One hundred microliters of this sample was placed in 5.0 mL of toluene–Triton counting fluid and counted for determination of protein incorporated.

Electron Microscopy of Sample. A 1:64 dilution of the NMR sample in TEKA buffer was applied for 30 s to carbon-coated parlodian 150 mesh copper grids which had been treated with a glow discharge apparatus. The grids were then washed once with TEKA buffer and stained for 10 to 15 s with 1% sodium phosphotungstenate (pH 7). After drying, grids were examined under a Philips 300 electron microscope at an accelerating voltage of 80 kV and an objective aperture of 50 μm.

NMR Methods. All ^{19}F measurements were made at 254 MHz on a Bruker HXS-270 NMR spectrometer, except for one line-width measurement at 84.67 MHz made on a Bruker HX-90 NMR spectrometer. Both spectrometers were operated

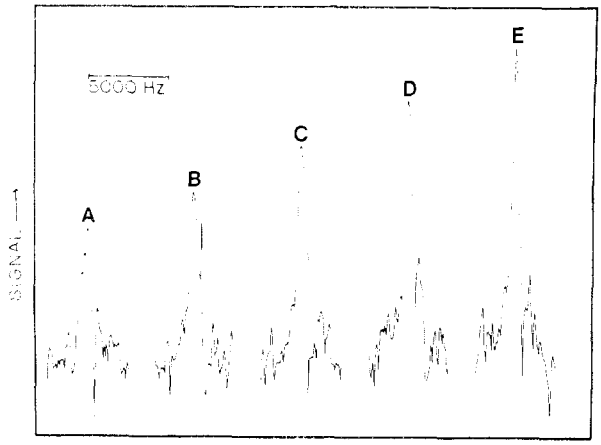

FIGURE 3: 254-MHz ^{19}F spin–lattice relaxation time (T_1) measurement using the progressive-saturation method on the fluorotyrosyl M13 coat protein in synthetic vesicles. The delay times between spectra were (A) 0.1, (B) 0.2, (C) 0.3, (D) 0.4, and (E) 0.5 s. Spectra were acquired with a sweep width of ±6000 Hz, a pulse width of 11 μs (90°), and an acquisition time of 0.085 s. The line broadening was 100 Hz and all spectra represent 25 000 transients. The glitch on the upfield side of the peak is an instrumental artifact.

in the Fourier transform mode. Specific acquisition parameters are given for various spectra in the figure legends. ^1H decoupling for nuclear Overhauser enhancement measurements was accomplished in a pseudo-pulsed homonuclear decoupling mode (Jesson et al., 1973); that is, the ^1H decoupler was pulsed off during the acquisition of the individual points in the ^{19}F free-induction decay and pulsed on between points. ^{19}F spin–lattice relaxation times were measured by the Fourier transform progressive saturation experiment (Freeman and Hill, 1971). Variable-temperature measurements were accomplished using the Bruker variable-temperature controller calibrated against ice water. All chemical shifts are measured relative to 10 mM sodium trifluoroacetate in D_2O, pH 7. Negative shifts are upfield. All line widths are reported as the observed line width minus the line broadening applied by the computer as an exponential weighting function to improve the signal to noise ratio in the spectrum.

Results and Discussion

Characteristics of Vesicles Containing Fluorotyrosyl Coat Protein. The application of nuclear magnetic resonance spectroscopy to the study of this integral membrane protein required development of a system for incorporation of high levels of protein into small, rapidly tumbling vesicles which were stable for the time course of NMR experiments. There were several problems associated with this, however. M13 coat protein has a very strong tendency to self-aggregate, presumably due to electrostatic and hydrophobic interactions, and our numerous initial attempts at vesicle preparation in standard cholate systems (Racker et al., 1975; Racker and Kandrach, 1972; Wickner, 1976) were unsuccessful. Inclusion of 8 M urea in the detergent buffer used for vesicle preparation solubilized the protein sufficiently, and subsequent dialysis to remove urea and cholate yielded vesicles containing the desired high levels of protein. The other major problem related to the tendency of coat protein containing vesicles to gradually aggregate during NMR experiments. Incorporation of 20% acidic phospholipid as described under Experimental Procedure resolved this problem and also stabilized the formation of smaller species of vesicles. Chromatography on Sepharose 4B resulted in a preparation of small vesicles of defined size range and desired characteristics.

The mean diameter of coat protein containing vesicles was 285 Å with a standard deviation of 115 Å, as determined by measurement of 129 vesicles from electron micrographs of the NMR sample. As larger vesicles contain more protein and thus contribute more to the signal obtained, an average diameter d_{av} weighted for surface area was computed according to the relation

$$d_{av} = \left(\frac{\sum_{i=1}^{N} d_i^2}{N} \right)^{1/2}$$

where d_i is an individual diameter measurement and N is the number of measurements. The value obtained was 310 Å. This was used where applicable in analysis of NMR results. For determination of the lipid to protein ratio, 100-μL aliquots of the NMR sample were counted to quantitate the ^{125}I-labeled tracer protein, and 200-μL aliquots were subjected to careful dry weight determinations. Assuming that [^{125}I]protein distributes itself in the same manner as the fluoro analogue during vesicle preparation, we arrive at a lipid to protein mole ratio of 180:1. Thus, the typical vesicle seen in NMR experiments may be described as 310 Å in diameter and containing 24 molecules of fluorotyrosyl coat protein for its 4400 phospholipid molecules. Such a vesicle has a surface area of 1.4×10^5 Å^2 on the inner face of the bilayer, which provides a region 5600 Å^2 in area (75 × 75 Å) for each protein molecule. It is thus unlikely that steric hindrance of lateral diffusion is a factor to be considered in interpretation of results.

NMR Results. When the fluorotyrosyl coat protein is incorporated into vesicles as described above, the ^{19}F spectrum appears as a single broad resonance ($\Delta\nu$ = 300 Hz at 254 MHz for T = 301 K) at −61.09 ppm. The line width of this sample is decreased to 60 Hz when measured under the same conditions at 84.67 MHz. The spin–lattice relaxation time T_1 of this resonance was measured using the progressive-saturation method at 254 MHz. These spectra are presented in Figure 3. The T_1 obtained from a nonlinear least-squares analysis of the data is 0.32 ± 0.02 s. All of the above spectra were recorded in the absence of proton decoupling. The validity of T_1 measurements under these conditions has been discussed previously (Hull and Sykes, 1975a).

The nuclear Overhauser enhancements obtained in the presence of irradiation of the protons provide a measure of fluorine proton dipolar interactions and are presented in Figure 4. The NOE measured when the proton irradiation was centered at 7.0-ppm downfield from DSS in the ^1H NMR spectrum, near the tyrosyl aromatic protons, was −0.55. The NOE measured when the irradiation was centered at 1.55 ppm from DSS, near the lipid aliphatic protons, was −0.3.

The line width and intensity of the protein ^{19}F resonance are a strong function of temperature as seen in Figures 5 and 6. These measurements were made in a relatively random order, so the line-width variation with temperature is reversible.

Analysis of NMR Results. To analyze the above measurements we propose a physical model for the mobility of the fluorotyrosine residues in the protein and the mobility of the protein in the membrane. We then attempt to determine the range of numbers which can account for the various relaxation measurements observed. (In all of the analyses to be presented below, it must be emphasized that we are attempting only to identify the general range of numbers characterizing our physical model and consistent with the experimental results and do not claim a high precision.) We must first estimate the overall correlation time τ_c for vesicle tumbling, which can be

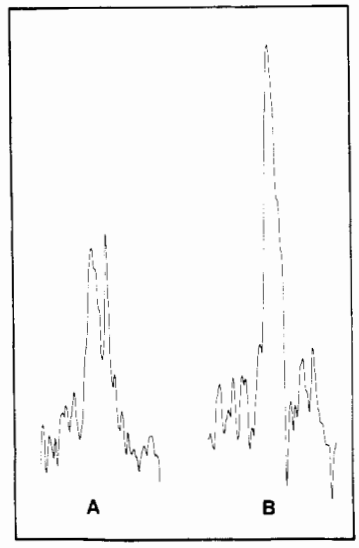

FIGURE 4: 254-MHz ^{19}F NMR spectra of the fluorotyrosyl M13 coat protein in synthetic vesicles showing effect of irradiation in the ^1H NMR spectrum of the protein. (A) Irradiation centered at 7.0 ppm downfield from DSS in the 270-MHz ^1H NMR spectrum. (B) Irradiation far off resonance. Irradiation was performed in a pseudo-pulsed homonuclear mode (see Experimental Procedure for details) to reduce interference between the two channels. Spectra were acquired with a sweep width of ±6000 Hz, pulse width of 10 μs, and an acquisition time of 0.34 s. Line broadening applied was 100 Hz. The glitch on the upfield side of the peak is an artifact. Number of acquisitions was 50 000.

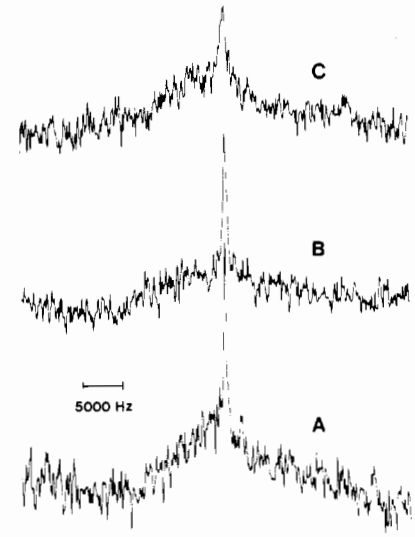

FIGURE 5: 254-MHz ^{19}F NMR spectra of the fluorotyrosyl M13 coat protein in phospholipid vesicles as a function of temperature. The resonance is centered at −61.09 ppm from 10 mM CF_3CO_2Na in D_2O. These spectra have been scaled vertically to represent an approximately equal number of transients: (A) T = 309 K, number of transients 42 000, scaling X2; (B) T = 300 K, number of transients 83 714, no scaling; (C) T = 288 K, number of transients 81 564, no scaling. These spectra were acquired with a sweep width of ±25 000 Hz, a pulse width of 7 μs (~65°), and an acquisition time of 0.082 s with a delay between transients of 0.34 s. Line broadening used was 100 Hz.

obtained from the Stokes-Einstein equation:

$$\tau_c = \frac{4}{3}\frac{\pi r^3 \eta}{kT}$$

and is equal to 3.6×10^{-6} s for a vesicle of diameter 310 Å, T = 301 K, $\eta = 10^{-2}$ P (the viscosity for water).

We start the analysis with the NOE measurement which can be written as:

$$\text{NOE} = \left(\frac{\gamma^H}{\gamma^F}\right)\frac{\sigma^{FH}}{\rho^{FH} + \rho^{CSA}}$$

in the nomenclature of Hull and Sykes (1975a), where γ^H and γ^F are the gyromagnetic ratios for protons and fluorine, respectively, ρ^{FH} is $1/T_{1,FH}$ (the contribution of fluorine-proton dipolar interactions to the spin-lattice relaxation rate), ρ^{CSA} is $1/T_{1,CSA}$ (the contribution of the fluorine chemical-shift anisotropy to the spin-lattice relaxation rate), and σ^{FH} is the proton fluorine cross relaxation rate which can have a magnitude $0.5\rho^{FH} > \sigma^{FH} > -\rho^{FH}$. In the absence of any internal motion for the fluorotyrosine, σ^{FH} is expected to be equal to $-\rho^{FH}$ for $\tau_c = 3.6 \times 10^{-6}$ s. If ρ^{CSA} were equal to zero, this would predict an NOE of approximately −1 (as γ^H/γ^F = 1.06). The observed NOE of −0.55 therefore indicates that $\rho^{CSA}/\rho^{FH} \simeq 0.9$ under the assumption that the correlation time of 3.6×10^{-6} s is appropriate, as can be seen in Figure 7. If we remove this assumption (allow a shorter τ_c), we conclude that ρ^{CSA}/ρ^{FH} is less than 0.9. This can be seen in Figure 7 by choosing a shorter τ_c and then observing that a smaller relative contribution from ρ^{CSA} is consistent with the observed NOE. We would also conclude that 0.9 was an upper limit for ρ^{CSA}/ρ^{FH} if the experimentally used level of irradiation were insufficient to completely saturate the proton resonance, that is, if the NOE was less than −0.55. Combining this with the observed spin-lattice relaxation time ($T_{1,obsd}$) of 0.32 s

$$\rho^{FH} + \rho^{CSA} = \frac{1}{T_{1,FH}} + \frac{1}{T_{1,CSA}} = \frac{1}{T_{1,obsd}} = 3.1\ s^{-1}$$

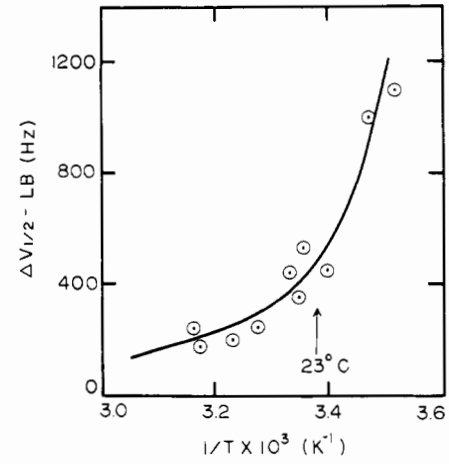

FIGURE 6: ^{19}F NMR line widths for the fluorotyrosyl M13 coat protein in phospholipid vesicles plotted as a function of reciprocal temperature. The line widths are the experimentally observed line widths minus line broadening applied during Fourier transformation to improve signal to noise.

we obtain

$$\frac{1}{T_{1,FH}} \geq 1.6\ s^{-1}; \frac{1}{T_{1,CSA}} \leq 1.5\ s^{-1}$$

The line width can also be separated into contributions from fluorine-proton dipolar interactions ($\Delta\nu_{FH}$) and from fluorine chemical-shift anisotropy ($\Delta\nu_{CSA}$) because the latter contribution is dependent on the square of the resonance frequency of the measurement whereas the former is frequency independent (Hull and Sykes, 1975b). Again this is strictly true for τ_c in the range expected for this system. For intermediate-sized molecules, there could be some decrease in the linewidth contribution from either source with increasing field strength. Our line widths at 84.67 and 254 MHz clearly increase with frequency as expected and when plotted vs. fre-

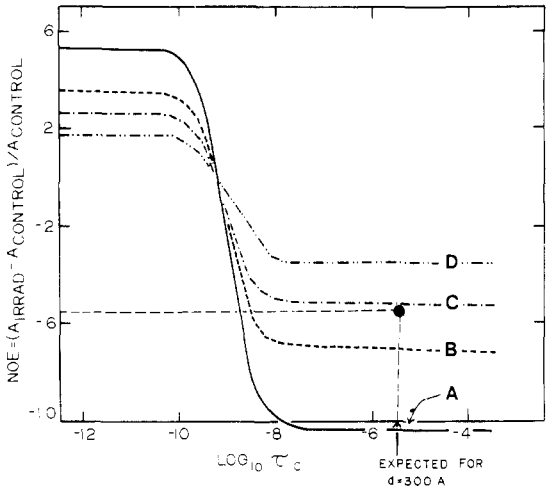

FIGURE 7: Calcuated $^{19}F-\{^1H\}$ nuclear Overhauser enhancement for ^{19}F at 254 MHz as a function of isotropic rotational correlation time (τ_c) and as a function of the relative contribution of dipolar interactions and chemical-shift anisotropy to the relaxation of the ^{19}F nucleus. Curve A is for $1/T_{1,CSA} = 0$; curve B is for $1/T_{1,HF} = 2/T_{1,CSA}$; curve C is for $1/T_{1,HF} = 1/T_{1,CSA}$; and curve D is for $1/T_{1,HF} = 0.5/T_{1,CSA}$.

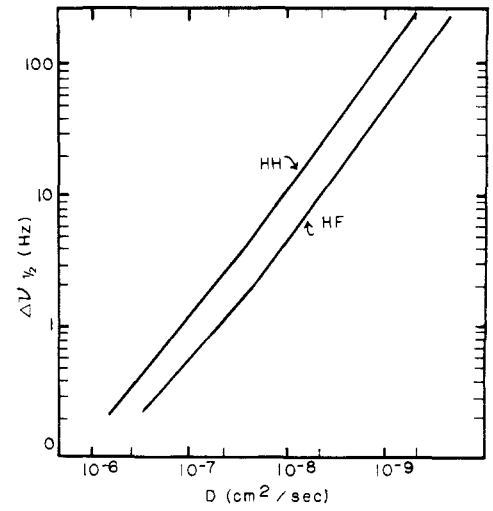

FIGURE 8: The calculated line broadening of a fluorine nucleus buried in a lipid bilayer as a function of the lateral diffusion coefficient of the lipid. This figure is adapted from Figure 2 of Lee et al. (1973) using their eq 12-14 for unlike spins.

quency squared we can obtain the contributions to the line width at 254 MHz of $\Delta\nu_{FH} = 30$ Hz and $\Delta\nu_{CSA} = 270$ Hz.

The contribution of fluorine chemical-shift anisotropy to the line width and spin-lattice relaxation rate can now be used to characterize the motion of the fluorotyrosines in the protein. Using formulas 11-17 and the chemical-shift tensor parameters from Table 2 of Hull and Sykes (1975b), we can calculate the predicted $\Delta\nu_{CSA}$ and $1/T_{1,CSA}$ as a function of internal motion around the $\beta\gamma$ bond of tyrosine using $\tau_c = 3.6 \times 10^{-6}$ s as the overall rotational correlation time. This is certainly the first motion which would be expected. The predicted values of $\Delta\nu_{CSA}$ are, however, greater than the observed line width for all values of τ_{INT} ($\beta\gamma$), the correlation time for this internal motion. This suggests that further internal motions are possible such as about the $\alpha\beta$ bond of tyrosine. Since no formalism is available to analytically treat this situation of an overall correlation time and two links of internal motion, one about $\alpha\beta$ and one about $\beta\gamma$, we have approximated the effects of rotation about $\alpha\beta$ by reducing τ_c in the calculation. Using $\tau_c = 1 \times 10^{-6}$ s, we obtain $\Delta\nu_{CSA}^{calcd} = 325$ Hz and $1/T_{1,CSA}^{calcd} = 0.8$ s^{-1} for τ_{INT} ($\beta\gamma$) $= 2 \times 10^{-9}$ s, chosen to bring $\Delta\nu_{CSA}^{calcd}$ down toward 300 Hz and simultaneously to maximize $1/T_{1,CSA}^{calcd}$.

We now turn to analyzing the contribution of intramolecular proton-fluorine dipolar interactions to $\Delta\nu_{FH}$ and $1/T_{1,FH}$. The only significant proton on the aromatic ring is the proton ortho to the fluorine at an internuclear distance of 2.6 Å. Internal rotation about the $\beta\gamma$ bond of tyrosine does not affect this interaction, since the rotation axis and internuclear vector are parallel. Consequently, the problem can be parameterized in terms of internal motion about the $\alpha\beta$ bond, τ_{INT} ($\alpha\beta$), using eq 3-9 from Hull and Sykes (1974) and with $\tau_c = 3.6 \times 10^{-6}$ s. The results obtained are $\Delta\nu_{FH}^{calcd} = 45$ Hz and $1/T_{1,FH}^{calcd} = 0.6$ s^{-1} for τ_{INT} ($\alpha\beta$) $= 5 \times 10^{-8}$ s, chosen to reduce $\Delta\nu_{FH}^{calcd}$ toward 30 Hz and to maximize $1/T_{1,FH}^{calcd}$.

The numbers in the above calculations can be manipulated to make the agreement between the calculated and observed line widths exact. However, the main point we wish to make here is that a very reasonable model for the internal mobility of tyrosyl residues of a protein in a vesicle [$\tau_c = 3.6 \times 10^{-6}$ s, τ_{INT} ($\alpha\beta$) $= 5 \times 10^{-8}$ s, τ_{INT} ($\beta\gamma$) $= 2 \times 10^{-9}$ s], involving increasing mobility of the tyrosyl residue away from backbone of the protein, can account for all of the features of the observed relaxation [$\Delta\nu_{FH} \simeq 30$ Hz, $\Delta\nu_{CSA} \simeq 270$ Hz, and $1/T_{1,CSA} < 1.5$ s], except the proton-fluorine dipolar contribution to $1/T_{1,FH}$ which must be ≥ 1.6 s^{-1}.

Increasing the calculated value of $1/T_{1,HF}$ requires interaction of the fluorine with other protons, with the restriction that the time scale of the interactions be reasonably rapid so that $1/T_{1,HF}$ is increased without also increasing $\Delta\nu_{FH}$ significantly. One possibility, if the tyrosyl residues are in the bilayer, is that the fluorine is interacting with protons from the lipid molecules which diffuse by. Similar conclusions about the rates of the motions required were drawn by Lee et al. (1973) in their discussion of the relaxation of lipid protons by intermolecular interactions among the lipids. We have modified their Figure 2 according to their eq 12-14 to correspond to the contribution to the ^{19}F line width from lipid protons as a function of the translational diffusion coefficient of the lipids (Figure 8). The condition that the motion not significantly contribute to the calculated line width (say <10 Hz) places a lower limit on the lipid translational diffusion coefficient of $D \geq 3 \times 10^{-9}$ cm^2/s. That the fluorines interact with the lipid protons is supported by the existence of a negative NOE when the lipid aliphatic protons are irradiated. This could, however, be mediated via other protons on the protein (Sykes et al., 1978) and further experiments are presently in progress to settle this issue. Other possible sources for the necessary additional contribution to the fluorine relaxation are other protons on the protein or solvent protons. Regarding the possibility of relaxation by other protons on the protein, it seems unlikely that two parts of the protein would move relative to one another as fast as is required, although relaxation via methyl groups is possible. Solvent protons would, however, diffuse by on the fast time scale required.

We come now to the meaning of the strong dependence of the observed ^{19}F line width for the protein tyrosyl residues on temperature (Figure 6). The line width reflects the expected lipid phase transition temperature of about 23 °C, the resonance broadening dramatically and appearing to lose intensity below this temperature. The observed line width, as we have seen above, is dominated by $\Delta\nu_{CSA}$. This in turn reflects the overall rotational correlation time for the vesicle and the correlation times for the various modes of internal motion. The overall rotational correlation time is not expected to be a strong

function of temperature nor to reflect any phase transition in the lipids. Therefore, the line broadening must be the result of an influence on the protein from the change in state of the lipids which results in a more restricted mobility of the tyrosyl residues. Some line broadening could also occur if the translational diffusion of the lipids was reduced to the point where interactions with the lipid protons contributed to the observed line widths.

Conclusions

The nuclear magnetic relaxation parameters which we have investigated, namely, line width and spin–lattice relaxation time, are a function of the strength of the various interactions and of the time scale on which they occur. The interactions involved are between fluorine and proton nuclear spins (both intra- and intermolecular) and anisotropy in the fluorine chemical shift and are modulated by the overall tumbling time of the protein–vesicle complex as well as internal motions of tyrosines in the protein and motions of the protein in the membrane.

We have used nuclear Overhauser enhancement measurements and the field dependence of line width to separate out the relative contributions of dipolar interactions and fluorine chemical-shift anisotropy to the observed T_1 and line width, respectively. As contributions by fluorine chemical-shift anistropy to the line width and spin–lattice relaxation time are a function only of the motional characteristics of the CF bond and not HF interactions, we subsequently utilized these to characterize the motional properties of the fluorotyrosyl residues. In analyzing the dipolar contributions to relaxation, however, we found that simple interaction of fluorine with the adjacent ring proton was insufficient to account for the observed $T_{1,HF}$, and had to invoke rapid interactions of the fluorine with other protons. If these protons are lipid methylene protons, we arrive at a minimum translational diffusion coefficient of $D \geq 3 \times 10^{-9}$ cm^2/s at 28 °C, in good agreement with values obtained from ESR (see Scandella et al., 1972) and ^{1}H nuclear magnetic resonance (Lee et al., 1973) studies.

From the results presented in this communication, we conclude that it is possible to construct a reasonable model for the motional properties of a membrane protein in a phospholipid model bilayer in terms of principles derived from solution protein studies. The model we have presented is characterized by a vesicle tumbling correlation time τ_c of 3.6×10^{-6} s, correlation times for internal tyrosine bond rotation of 5×10^{-8} and 2×10^{-9} s for the $\alpha\beta$ and $\beta\gamma$ linkages, respectively, and rapid intermolecular FH interactions.

Examination of Figure 6 shows that motions within the protein are dramatically influenced by the phase state of the bilayer. For the lipid mixture we have employed (80% DMPC), we expect a broad gel to liquid-crystalline transition centered around 23 °C and find that above this temperature the observed line width sharpens much more than can be accounted for by the decrease in vesicle tumbling correlation time. The broad line width seen below the lipid phase-transition temperature reflects reduced mobility of the tyrosyl residues and possibly increased significance of intermolecular dipole–dipole interactions (with retarded translational diffusion of the lipid). These effects could, however, be indirectly mediated by the protein if the fluorotyrosyl residues are, in fact, exposed to the solvent.

Throughout our treatment of the spectra obtained for protein in vesicles, we have assumed the two fluorotyrosyl residues separated by only two amino acids to be in essentially identical environments. We have been unable to detect any gross peak heterogeneity, and more detailed investigations are underway to resolve this question and the possibility of any preferential orientation of the protein in the vesicle.

We have demonstrated in this communication that nuclear magnetic resonance spectroscopy can be applied successfully to analysis of the motional properties of a membrane protein. We have utilized a low-molecular-weight viral coat protein, which behaves as an integral membrane protein during infection, for our investigation and are presently attempting to generalize these techniques to more complex membrane-associated proteins.

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Relation between Structure and Specificity of Antibodies: Nuclear Magnetic Resonance Study of Binding Fluorine-19 Labeled Nitrophenyl Haptens to Myeloma Immunoglobulins M315, M460, and X25†

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ABSTRACT: The relation between structure and specificity of antibodies has been explored by ^{19}F NMR studies of the binding of trifluoromethyl analogues of nitrophenyl haptens to the three mouse myeloma immunoglobulins M315, M460, and X25. We have used haptens with trifluoromethyl groups located at the ortho or para positions of the phenyl ring or attached to the side chain, two atoms removed from the ring (i.e., $-NHCH_2CF_3$). The changes in chemical shift between hapten free in solution and bound to antibody are sensitive to microenvironment and range from 1.7-ppm downfield to 1-ppm upfield. The shifts of *p*-trifluoromethylnitrophenyl haptens bound to M315 and M460 are both large downfield shifts, which are likely caused by van der Waals interaction and ring-current effects, particularly from tyrosine-34 (L); these haptens do not show similar shifts when bound to X25 which has a deletion of tyrosine-34 (L). Other differences in the binding of the aromatic rings of haptens by M315, M460, and X25 are observed and their origins considered. The importance of hydrogen bonding in the thermodynamic affinity of antibody for hapten has been estimated by comparisons of binding affinities for haptens with trifluoromethyl groups in place of nitro groups.

In their recognition of foreign substances, antibodies display both a high degree of specificity and a wide diversity (Richards and Konigsberg, 1973) whose molecular origins have been probed by a variety of physical techniques (Nissonoff et al., 1975). Complementarity between hapten and binding site plays an important role in the antibody–antigen interaction (Kabat, 1976), though we do not as yet have a sophisticated understanding of the "strange cross reaction" of apparently dissimilar haptens for the same antibody (Michaelides and Eisen, 1974). A molecular understanding of the interaction between antibody and antigen (or hapten) should help to resolve some of these questions and might also serve as a preliminary basis for subsequent elucidation of the way in which binding of antigen to certain classes of antibodies triggers the various effector functions (Spiegelberg, 1974) such as the complement cascade (Müller-Eberhard, 1968) and lymphocyte transformation (Vitetta and Uhr, 1975).

Homogeneous proteins (Potter, 1972) secreted by myeloma tumors provide an ideal subject for molecular studies of antibody–antigen interactions. Many of these proteins have been screened by precipitin assay against a variety of antigens (Potter, 1971), and groups of proteins have been identified which exhibit specificities for substances related to phosphorylcholine (Leon and Young, 1970), dinitrophenol (Schubert et al., 1968), and various oligosaccharides (Vicari et al., 1970).

The three plasmacytomas studied grow in BALB/c mice and produce IgA type antibodies secreted primarily as oligomers ($[H_2L_2]_n$; n = 2–5 or more). M315 has a λ_2 light chain (Dugan et al., 1973), whereas M460 and X25 have a κ light chain (Jaffe et al., 1971; Sharon and Givol, 1976). M315 has a high affinity for 2,4-dinitrophenyl (DNP)[1] and 2,4,6-trinitrophenyl (TNP) haptens ($K_{assoc} \approx 10^6$ to 10^7 M^{-1}) (Eisen et al., 1968) and also binds menadione (vitamin K_3) with an affinity of 10^5 M^{-1}, a "cross reaction" of the type mentioned earlier. The binding specificity of M315 with a variety of other haptens has also been studied (Haimovich and Eisen, 1971). M460 binds nitrophenyl haptens with moderate affinity ($K_{assoc} \approx 10^5$ M^{-1}), exhibits high affinity for 2,4-dinitronaphthol ($K_{assoc} \approx 10^7$ M^{-1}) (Haimovich et al., 1972), and binds menadione with an affinity comparable to that for nitrophenyl haptens (Johnson et al., 1974). X25 displays a moderate af-

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[1] Abbreviations used are: BGG, bovine γ-globulin; DNP, 2,4-dinitrophenyl; EDTA, ethylenediaminetetraacetate; NMR, nuclear magnetic resonance; ppm, parts per million; $NaDodSO_4$, sodium dodecyl sulfate; TNP, 2,4,6-trinitrophenyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; GABA, γ-aminobutyric acid.

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