^{19}F NUCLEAR MAGNETIC RESONANCE STUDIES OF THE COAT PROTEIN OF BACTERIOPHAGE M13 IN SYNTHETIC PHOSPHOLIPID VESICLES AND DEOXYCHOLATE MICELLES

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ABSTRACT The nonlytic, filamentous coliphage M13 offers an excellent model system for the study of membrane-protein interactions. We prepare derivatives of the protein containing fluorine-labeled amino acids and use ^{19}F nuclear magnetic resonance (NMR) to study the protein in both deoxycholate micelles and phospholipid vesicles. We have previously described the in vivo preparation of an m-fluorotyrosyl derivative of M13 coat protein and also a method for incorporation of high levels of this protein into small, uniformly sized phospholipid vesicles of defined composition. Herein we describe the in vivo preparation and the characterization of an m-fluorophenylalanine derivative. We simultaneously compare the environment and mobility of the tyrosine and phenylalanine residues (the former in the hydrophobic region of the protein and the latter in the hydrophilic regions) as influenced by bile salt detergent or lipid interactions.

INTRODUCTION

The process of phage M13 assembly takes place entirely within the inner membrane of the *Escherichia coli* host, and the major coat protein (Gene 8) is stored during infection as a cytoplasmic membrane-spanning protein (10, 11, 14). The coat protein contains 50 amino acids. The amino acid and messenger RNA sequences are known (see Fig. 1), and its physical properties have been extensively characterized (6). The coat protein can be prepared in large quantities from intact phage recovered from the growth medium.

By combining an m-fluorotyrosyl derivative of M13 coat protein and an m-fluorophenylalanine derivative in either micelles or vesicles, we can simultaneously compare the environment and mobility of the tyrosine and phenylalanine residues. The tyrosines are in the hydrophobic region of the protein, whereas two of the phenylalanines are in the basic C-terminal region and one is in the acidic N-terminal region. ^{19}F NMR allows us to characterize the environment, exposure, and mobility of the individual labeled amino acids, especially as these parameters are influenced by bile salt detergent or lipid-protein interactions. We show the results of preliminary experiments where fluorine-labeled fatty acids are also present in the vesicle samples, permitting us simultaneously to monitor the fluidity of the vesicle lipids.

Our previous studies (3–5) have shown that the fluorotyrosines were inaccessible to solvent when reconstituted in phospholipid vesicles, and that the fluorotyrosine sidechain mobility was influenced by the gel-liquid crystalline state of the lipids. These results are consistent with the hydrophobic region of the protein being buried in the lipid bilayer. In contrast the phenylalanyl residues are in the hydrophilic terminal regions of the protein, and *a priori* we might expect them to be much more exposed and more mobile. However, we find that the fluorophenylalanyl residues are not exposed to proteolytic digestion and are not much more mobile than the fluorotyrosyl residues, while the side chain mobility of the fluorophenylalanyl residues is not influenced by the phase transition of the lipids.

METHODS

E. coli KA197 (CGSC 5243, Hfr, Thi, PheA97, RelA1, λ-) and AT2471 (CGSC 4510, Hfr, Thi, Rel, TyrA4, λ-) were obtained from Dr. Barbara Bachmann, Yale University School of Medicine. m-fluoro-D, L-phenylalanine, L-phenylalanine and L-tyrosine were bought from Sigma Chemical Co. (St. Louis, MO). α-Chymotrypsin (65.4 units/mg) was obtained from Worthington Biochemical Corporation (Freehold, NJ). All other materials were as described previously (3, 4).

The fluorotyrosine labeled M13 was grown in 2–10 liter fermentors as described previously (3), except that 0.2% glucose was used as the carbon source instead of 1% glycerol and the culture was supplemented with 2 μg/ml of L-tyrosine. The culture was made 40 μg/ml in m-F-tyrosine

Abbreviations used: NMR, nuclear magnetic resonance; m-FPHE, m-fluoro-D,L-phenylalanine; m-FTYR, m-fluoro-D,L-tyrosine; DOC, deoxycholate; EDTA, (ethylenedinitrilo) tetra-acetic acid.

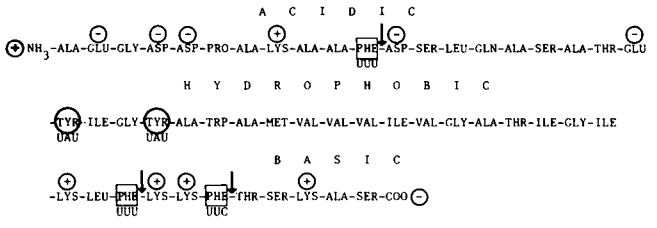

FIGURE 1 The amino acid sequence of M13 coat protein (1, 9) and the mRNA sequence of the closely related phage, fd for the amino acids of interest to this work (12). The arrows indicate the chymotryptic cleavage sites of deoxycholate micelle-bound protein. Fluorine labels have been incorporated at the circled and boxed amino acids.

after addition of phage at a multiplicity of infection of 20. Additional L-tyrosine was not necessary for maintenance of cell growth and phage production. Phage were harvested from the growth medium as described previously (3) and after lyophilization, 628 mg of m-F-tyrosine-labeled phage was obtained.

E. coli KA197 was used to grow m-F-phenylalanine labeled M13. M13 was grown as above except that the growth medium was supplemented with 80 μg/ml m-F-phenylalanine. Addition of L-phenylalanine was not necessary. 368 mg of lyophilized m-F-phenylalanine labeled phage were obtained from 2–10 liter cultures.

Preparation of Deoxycholate Micelles

The method of preparation of micelles containing both m-F-phenylalanine and m-F-tyrosine labeled coat protein (m-FPHE/m-FTYR micelles) was as outlined previously (4) except that 0.1 M NH_4HCO_3, pH 9.0, rather than 0.1 M $NaHCO_3$, pH 9.0, was used. The samples were concentrated using an Amicon ultrafiltration apparatus with a YM10 membrane (Amicon Corp. Scientific Syst. Div., Lexington, MA).

Preparation of Vesicles

The coat protein was first extracted into deoxycholate micelles (4). The bile salt detergent was removed by extensive dialysis (48 h) against 3 liter 0.01 M NH_4HCO_3, pH 9.0 (two changes), followed by 3 liter H_2O (two changes) and then lyophilized. Vesicles were made using our previously described procedure (3), with the following modifications: 400 mg dimyristoylphosphatidylcholine, 50 mg dipalmitoylphosphatidic acid, and 50 mg beef heart cardiolipin were used, with 15 mg each of m-F-phenylalanine- and m-F-tyrosine-labeled coat protein. The vesicles were dialyzed for 24 h against 3 liter of 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA and 10% methanol (five changes).

Chymotryptic Digestion of Micelles and Purification of Fragments

Micelles were prepared using 61 mg of each labeled coat protein. 6.6 λ of an α-chymotrypsin solution (14.6 mg/ml in 0.1 M NH_4HCO_3, 8 mM deoxycholate (pH 9.0, 90% D_2O) was added to the 14-ml micelle sample and the digestion monitored at 276°K using ^{19}F NMR. After 20 h of digestion, the sample was loaded on a Sephacryl S200 column (350 ml) and run with 0.1 M NH_4HCO_3, 8 mM deoxycholate pH 9.0 buffer. Elution was followed by absorbance at 257 nm. Two peaks were resolved, one running at the void volume (the "micelles" peak) and one running at the internal volume (the "fragment" peak). The "micelles" peak was lyophilized to dryness and taken up in 6 ml of 0.1 M NH_4HCO_3(98% D_2O) pH 9.0. A 1.5-ml sample of this was sonicated for 1/2 h at 100 W power on a Braunosonic 1510 (Canadian Laboratory Supplies Limited, Edmonton, Alberta) with a titanium microprobe, cooled with ice water.

The "fragments" peak was partially lyophilized to a vol of 20 ml. This was added, with stirring, to 100 ml of 0.5 M formic acid and the sample was stirred for an additional 1/2 h. The precipitated deoxycholate was spun out at 9880 *g* in a Beckman JA14 rotor (Beckman Instruments, Fullerton, CA) for 20 min at 15°C. The supernatant was removed and respun for a further 10 min. The supernatant was then lyophilized to dryness, taken up in water and lyophilized again. The residue was dissolved in 0.8 ml of 10% pyridine, 0.3% acetic acid, pH 6.5 and subjected to paper electrophoresis. The sample was applied to Whatman 3 mm paper (Whatman Inc., Clifton, NJ) and electrophoresed at 3,000 volts for 45 min. After drying, strips were cut from each side and from the center of the paper. These were treated with a solution of 85% (1% ninhydrin in acetone)/15% (0.029 M cadmium acetate, 5.8 M acetic acid) and dried in an 80°C oven. Three major bands were obtained: one acidic and two basic. Each band was eluted with H_2O and dried. Samples for NMR were made by adding 1.4 ml of D_2O. 100 nmol quantities were removed for acid hyrolysis (6 N HCl, 0.1% phenol) and amino acid analysis.

NMR Methods

All ^{19}F NMR measurements were made at 254 MHz on a Bruker HXS-270 NMR spectrometer (Bruker Instruments Inc., Billerica, MA) operating in the Fourier transform mode. The specific acquisition parameters used for the spectra are given in the figure legends. Temperature variation was accomplished using the Bruker variable-temperature controller, calibrated against ice water. All chemical shifts are reported relative to 10 mM sodium trifluoroacetate in D_2O, pH 7. The linewidths reported are the observed linewidth minus the line broadening used by the computer as an exponential weighting function to increase the signal to noise ratio of the spectrum.

RESULTS

The ^{19}F NMR spectrum at 296°K of an equimolar mixture of the m-FPHE/m-FTYR-labeled proteins solubilized in DOC micelles is shown in Fig. 2. For comparison, the ^{19}F resonance for the m-FPHE amino acid by itself in solution is at −38.0 ppm, while that for m-FTYR by itself in solution is at −61.2 ppm. Two resonances near −61 ppm are observed for the m-FTYR-labeled protein, and we have previously assigned these resonances to the two TYR

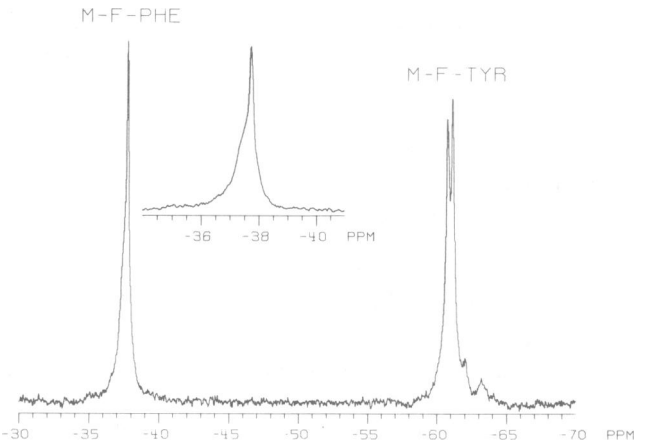

FIGURE 2 254-MHz ^{19}F NMR spectrum of deoxycholate micelles containing both m-FPHE- and m-FTYR-labeled M13 coat protein at 296°K. The micelles were prepared using 20 mg of each labeled phage. The spectrum was taken using a pulsewidth of 16 μs (66°), a sweepwidth of ±6,300 Hz, and 4K data points. 10,000 scans were collected with a delay between transients of 200 ms. The linebroadening used was 10 Hz. The inset is the m-FPHE peak expanded to illustrate its non-Lorentzian character.

positions in the sequence (positions 21 and 24). (The resonances for the labeled proteins in DOC micelles are too broad to permit resolution of the proton-fluorine nuclear spin-spin coupling, so that a single resonance is expected for each labeled position, even though these spectra were run in the absence of proton decoupling.) The small broad upfield shoulder of the main m-FMR resonances is due to different states of the protein being more stable at low temperatures (see below). Only one resonance is observed for the m-FPHE-labeled protein, even though there are three phenylalanines in the sequence (positions 11, 42 and 45). This opens the question of whether all three PHE positions have been equally substituted with m-FPHE, especially since the codons in the messenger RNA are different (see Fig. 1). The lineshape of the m-FPHE-labeled protein is distinctly non-Lorentzian (Fig. 2, inset) suggesting the existence of multiple overlapping peaks, and we shall see below that three resonances can be resolved.

To test the question of whether all three phenylalanines (11 and 42 coded for by UUU, and 45 coded for by UUC) were equally substituted by m-FPHE, the m-FTYR/m-FPHE micelles were subjected to α-chymotryptic digestion and the resulting fragments separated and analyzed. The ^{19}F NMR spectra showing the time course of the digestion are shown in Fig. 3. Chymotrypsin cleaves predominantly at phenylalanines and tyrosines. Because the tyrosines are partially protected by the bile salt molecules (4), the digestion should result in four fragments: acidic (1–11), micelle bound (12–42), most basic (43–45), and least basic (46–50).

The digestion time course of the m-FPHE spectra show two distinct kinetic phases (Fig. 4). Spectra *B* to *F* in Fig. 3 *a* show the decrease in intensity of what appears to be a narrower central component of the composite m-FPHE protein peak, and the concomitant appearance of a narrow peak upfield at −38.8 ppm which corresponds to free m-FPHE peptides. To determine what fraction of the total m-FPHE's are released in the initial burst phase, the area of the broad component of spectrum F at 76 min was compared to the total area of spectrum *A*. It was found that one-third of the m-FPHE's were initially cleaved. At longer times (spectrum *G* in Fig. 3 *a*), the broad protein resonance slowly disappears to a minimum. The area of the broad component of spectrum *G* was 22% of the total area of spectrum *A*. In contrast to the pronase digestion results reported previously (4), the tyrosine residues do not become exposed. There does not appear to be any release of m-FTYR from the micelles, indicated by the lack of

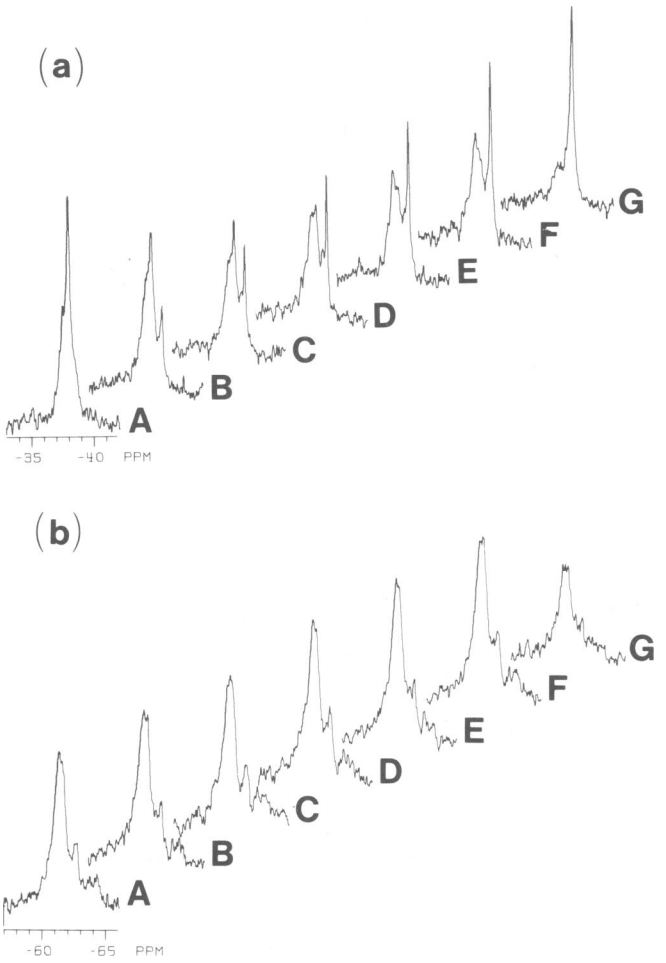

FIGURE 3 254-MHz ^{19}F NMR spectra of the chymotryptic digestion of deoxycholate micelles containing both m-FPHE and m-FTYR-labeled M13 coat protein at 276 K. The micelles were prepared using 26.5 mg of each labeled phage, following the procedure outlined in Methods for the chymotryptic digestion. Fig. 3 *a* shows the m-FPHE spectra while 3 *b* shows those of m-FTYR. Spectrum *A* was taken before the addition of chymotrypsin. Spectra *B–G* were taken at the following times after chymotrypsin addition: *B*, 8.5 min; *C*, 25.4 min; *D*, 42.4 min; *E*, 59.4 min; *F*, 76.3 min; *G* 1,269 min. The spectra were collected with a pulsewidth of 16 μs (66°), 4K data points, and a sweepwidth of ±5,000 Hz. 2,500 scans were collected for each spectrum with a delay time of 200 ms between each transient and a delay of four transients before data collection was started. The line broadening used was 20 Hz.

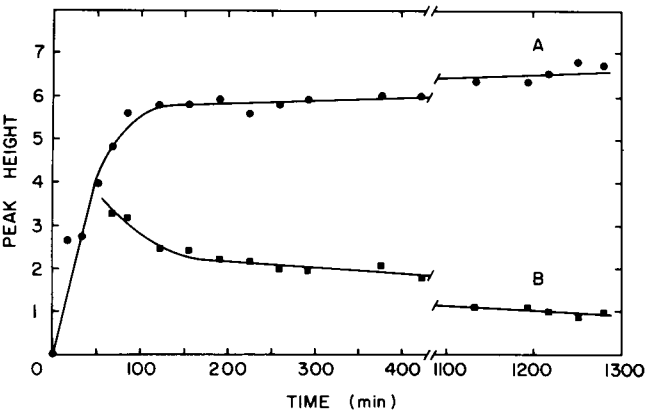

FIGURE 4 Peak heights of the 254 MHz ^{19}F NMR spectra of (*A*) m-FPHE released, and (*B*) m-FPHE in micelle bound protein, plotted as a function of time after chymotrypsin addition. These data were derived from the spectra shown in Fig. 3.

appearance of a narrow peak corresponding to free m-FTYR-containing peptides (see Fig. 3 *b*). The intensity of the bound m-FTYR peak does, however, decrease after a long time (spectrum *G*). This is due to linewidth and T_1 changes caused by aggregation.

The cleavage fragments were isolated as described in the methods section and their ^{19}F NMR spectra are shown in Fig. 5. The amino acid composition of each peptide fragment was determined and corresponded to the assignments given in Fig. 5.

A study of the ^{19}F NMR spectrum for the proteins solubilized in DOC micelles as a function of temperature confirmed the presence of m-FPHE at all three positions (see Fig. 6 *a*). At 327°K the m-FPHE resonance is resolved into three components: a broad resonance at −37.0 ppm and two partially resolved narrower peaks at −37.4 and −37.5 ppm. Computer simulation shows that they have approximately equal areas. The broad resonance has a linewidth of ~ 145 Hz while the narrow lines are each estimated to be 35 Hz wide. The effect of temperature on the m-FTYR resonances is shown in Fig. 6 *b*. The linewidths at 327°K are 68 Hz and 47 Hz for the downfield and upfield resonances, respectively. At 277°K there seems to be an alternative protein conformation present, indicated by the extra m-FTYR peaks that disappear at higher temperatures.

The ^{19}F NMR spectrum of equimolar quantities of the m-FPHE- and m-FTYR-labeled coat protein reconstituted in sonicated and sized phospholipid vesicles is shown in Fig. 7. The resonances from the m-FPHE's at −38.1 ppm and the m-FTYR's at −60.9 ppm are much broader in the vesicle preparation compared to the linewidths of

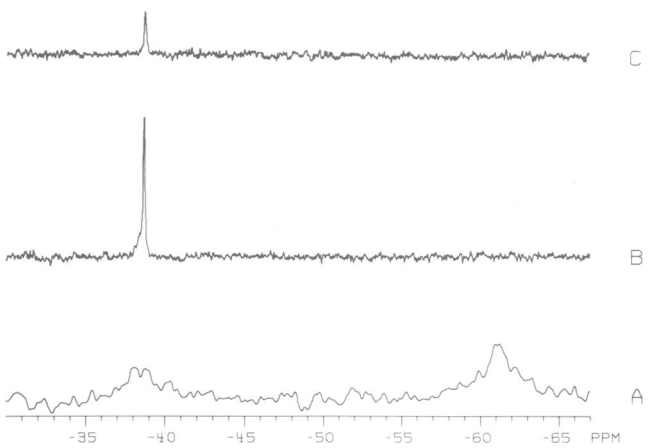

FIGURE 5 254 MHz ^{19}F NMR spectra of the fragments isolated after the digestion of deoxycholate micelle solubilized M13 coat protein with chymotrypsin. (*A*) micelle-bound fragment (0.3mM) (amino acids 12–42), 25,000 transients, 75-Hz line broadening. (*B*) the acidic fragment (3.7 mM) (amino acids 1–11), 2,500 transients, 10-Hz line broadening. (*C*) the most basic fragment (1.1mM) (amino acids 43–45), 2,500 transients, 10-Hz line broadening. All three spectra were collected with a pulsewidth of 16 μs (66°), a sweepwidth of ±5,000 Hz, and 4K data points. There was a delay of 200 ms between transients.

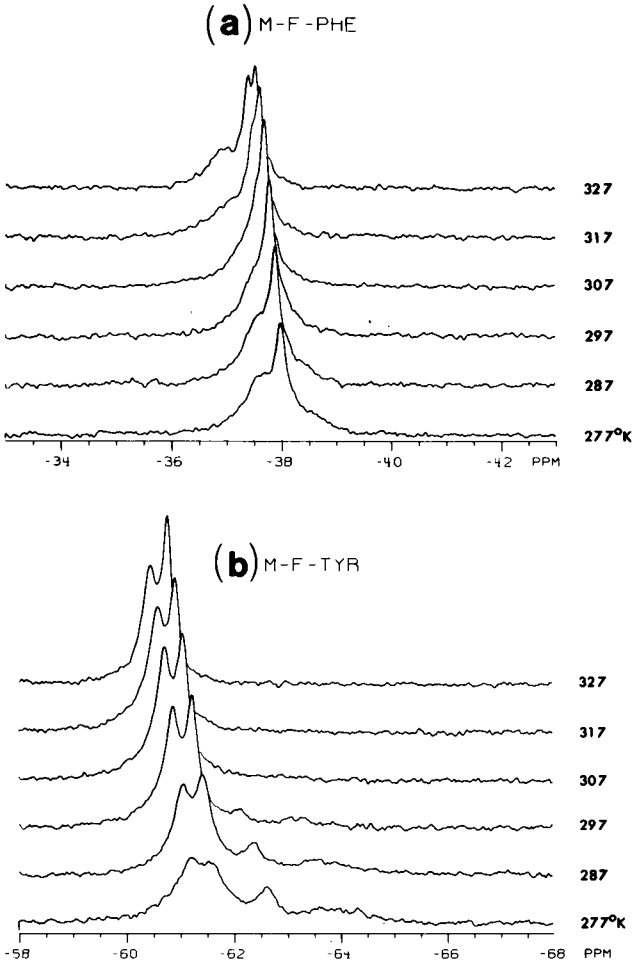

FIGURE 6 254 MHz ^{19}F NMR spectra of deoxycholate micelles containing both m-FPHE and m-FTYR labeled M13 coat protein as a function of temperature. The micelles were prepared using 20 mg of each labeled phage. *a*, m-FPHE spectra; *b*, m-FTYR spectra. Spectra were acquired using a pulsewidth of 16 μs (66°), a sweepwidth of ±6,300 Hz, and 4K data points. 5,000 scans with a delay of 200 ms between each transient were collected at each temperature. 10-Hz line broadening was used.

DOC micelle-bound protein peaks. Using the DISPA method of analysis (8), we find that the m-FTYR peak appears as a single Lorentzian resonance (with a linewidth of 263 Hz) within experimental error, i.e., the two m-FTYR are in indistinguishable environments. The m-FPHE resonance shows a tendency to deviate from a single Lorentzian in the DISPA plot, but is well approximated by a single line with a width of 228 Hz.

Vesicles prepared using both m-FPHE- and m-FTYR-labeled coat protein were subjected to digestion by pronase and α-chymotrypsin under the conditions described for Fig. 7. There was no detectable change in the ^{19}F NMR spectrum of either the m-FPHE or m-FTYR resonance after 65 h with pronase and 40 h with α-chymotrypsin, and no detectable presence of resonances corresponding to free m-FPHE or m-FTYR peptides (data not shown).

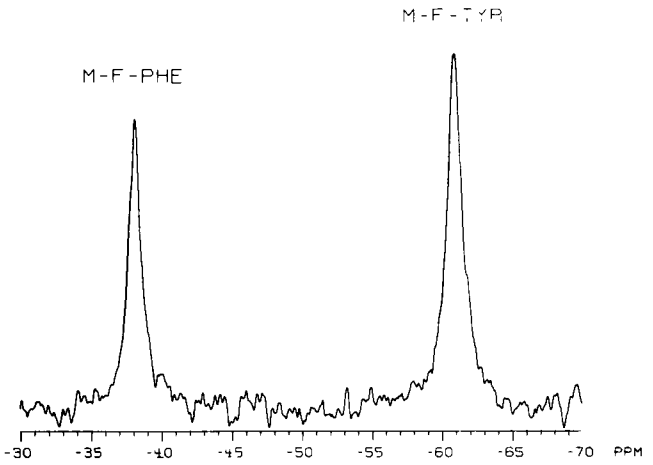

FIGURE 7 254 MHz ^{19}F NMR spectrum of synthetic vesicles containing both m-FPHE- and m-FTYR-labeled M13 coat protein. The spectrum was collected using a pulsewidth of 16 μs (66°), a sweepwidth of ±6,300 Hz, and 4K data points, with a delay of 250 μs between transients. It is the result of 340,000 scans using 50Hz linebroadening. For the pronase digestion, the solution was made 0.025 mg/ml pronase for the first 20 h at 297°K, and then made 0.15 mg/ml. For the α-chymotrypsin digestion, the solution was made 0.18 mg/ml chymotrypsin for the first 20 h at 297°K, and then the temperature was raised to 309°K.

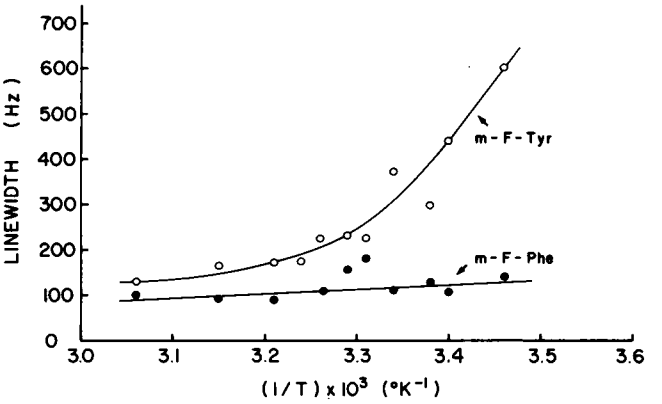

FIGURE 8 ^{19}F NMR linewidths for phospholipid vesicles containing both m-FPHE- and m-FTYR-labeled coat protein plotted as a function of reciprocal temperature. Acquisition parameters are as in Fig. 7. All spectra were 50,000 scans except 282°K, which was 100,000 scans.

The effect of temperature on the linewidths of the m-FPHE and m-FTYR resonances in reconstituted vesicles is shown in Fig. 8. The linewidth of the m-FTYR resonance broadens dramatically below the phase transition of the lipids (24°C), but the linewidth of the m-FPHE resonance broadens only linearly with 1/T.

The ^{19}F NMR spectrum of reconstituted vesicles containing 0.1% by weight 10-fluoropalmitic acid (with respect to total weight of lipids) is shown in Fig. 9. Three resonances are present, with the new resonance at −104 ppm corresponding to the 10-fluoropalmitic acid. This resonance provides a method for the simultaneous measurement of the fluidity of the vesicle lipids (2).[1] This resonance broadens dramatically at lower temperatures, and we will have to incorporate more labeled fatty acid to be able to follow the resonance accurately over the whole of the phase transition.

DISCUSSION

Here we have used ^{19}F NMR to probe the environment, exposure, and mobility of fluorine-labeled amino acid residues in the M13 coat protein. Our approach has been to compare the fluorophenylalanine residues, which are in the hydrophilic terminal regions of the sequence, with the fluorotyrosine residues, which are in the hydrophobic middle region of the protein, and to make this comparison for the protein either solubilized in DOC micelles or reconstituted in phospholipid vesicles.

The chymotryptic digestion of the micelle solubilized proteins shows an initial rapid appearance of a narrower resonance corresponding to m-FPHE-containing peptides. During this initial kinetic phase, the composite broad m-FPHE peak loses its narrower central component (see Fig. 6 *a*, 277°K for expanded spectrum), which corresponds very closely to one-third of the total area. (We know the micelle-bound resonance to have a much shorter T_1, 0.40 s, than free m-FPHE, and expect the T_1 to stay relatively constant and short during the initial cleavage. Therefore we believe these relative area measurements to be an accurate reflection of concentration changes.) The final area of the broad resonance decreases to ~ 22% of the initial total area. The dimer model for the micelle-bound protein (7) coupled with the sites of chymotryptic cleavage (Fig. 1) suggest that the first m-FPHE released is PHE 11 and that the last m-FPHE remaining is PHE 42. We feel that the reason the final area is < 33% is that aggregation of the micelles occurs at long times because of extensive removal of the charged termini of the protein (15). Evidence for aggregation was obtained during the separation of fragments on a S200 column. The micelles were eluted at the void volume, whereas before digestion they elute within the fractionation range of the gel.

Comparison of the micelle spectra as a function of temperature show that at low temperatures the narrow component of the m-FPHE resonance corresponds to one PHE, whereas at higher temperature the two narrow peaks correspond to a total of two PHE's. The digestion experiments suggest that the narrower resonance at low temperatures is PHE 11. We cannot say which additional resonance sharpens with temperature but the most likely is PHE 45 because it is nearer the end of the polypeptide.

In previous studies of the protein in vesicles (3–5), we drew two major conclusions relative to the fluorotyrosyl residues. First, while the fluorotyrosyl residues are only partially buried from solvent exposure and can eventually

[1]McDonough, B., McElhaney, R., and Sykes, B. D., personal communication.

be released with pronase in the DOC micelles, they are completely buried from solvent or lactoperoxidase exposure in the phospholipid vesicles and are not influenced by pronase digestion. Second, we rationalized the NMR linewidths, spin-lattice relaxation rates, and nuclear Overhauser enhancements in terms of a physically reasonable model and derived correlation times for the motion of the fluorotyrosyl sidechains which were similar to those for tyrosine residues in typical globular proteins. These motions were, however, very strongly influenced by the liquid crystal to gel phase transition of the lipids. This can be seen by the upper curve in Fig. 8 wherein the linewidth of the fluorotyrosyl residues rapidly increases below the phase transition. The linewidth is dominated by the chemical shift anisotropy contribution at these field strengths (3), which in turn are very strongly determined by the sidechain mobility of this residue; thus it can be concluded that the gel phase of the lipids greatly inhibits the sidechain mobility of the fluorotyrosyl residues. Another contribution to the line broadening which has not been separated out in the gel state but undoubtedly is also increased is the intermolecular dipolar interaction between the protons of the lipid sidechains and the buried fluorine nuclei.

Against this background one might have expected *a priori* that the hydrophilic terminal regions of the protein would be much more mobile and consequently that the fluorophenylalanine residues would have much narrower linewidths. One might also have expected these portions of the proteins to be much more susceptible to chymotryptic or pronase cleavage. Neither of these expectations is realized for the reconstituted vesicles. The linewidths of the fluorophenylalanyl residues are only slightly narrower than those of the fluorotyrosines, and there was no evidence of cleavage of the portions of the protein containing these fluorophenylalanines. The broad linewidths indicate that the terminal regions are not freely moving in solution but are either attached to the membrane surface or are significantly structured to the extent that their overall movement is constrained to the motion of the vesicle as a whole. These reasons may also provide an explanation for the resistance found to proteolytic digestion. The structure or lipid headgroup interaction may protect the cleavage sites or prevent dissociation of the cleaved peptides. Alternatively, it is possible that the protein is not present on the external surface of the vesicles. One model for this would be that the protein is in a "U-shaped" conformation (14, Fig. 5 *b*) with both ends on the inside of the vesicles. The other conformation that would prevent protein exposure on the surface of the vesicle would be if the protein molecules were lying perpendicular to the plane of the lipid bilayer, within the bilayer, possibly as dimers. This is ruled out, however, by the lower curve in Fig. 8 which shows that the linewidths of the fluorophenylalanine residues are not influenced by the transition to the gel state of the lipids but rather increases gradually with $1/T$. This is just what one would expect for a phenylalanine residue in a globular protein or in a structured terminal region of the coat protein which is attached or anchored in a phospholipid vesicle. Future experiments will be aimed toward following the motional behavior of the various regions of the protein while simultaneously monitoring the fluidity of the lipids with the fluorine-labeled fatty acid probe (see Fig. 9), and to comparing the reconstituted vesicles to biological membranes.

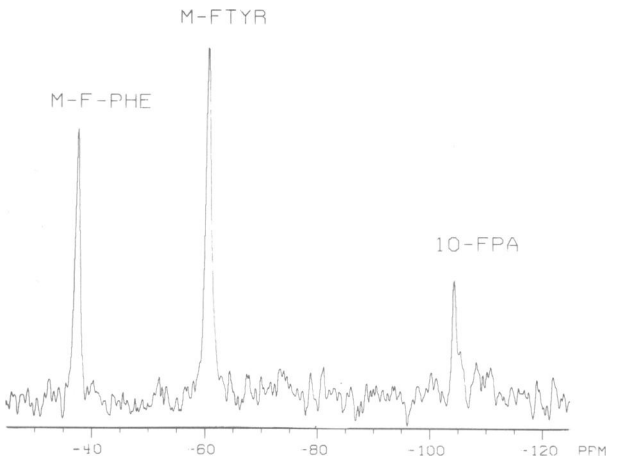

FIGURE 9 254 MHz ^{19}F NMR spectrum of synthetic vesicles containing m-FPHE and m-FTYR coat proteins as well as 0.1% 10-fluoropalmitic acid at 317°K. The spectrum was collected using a pulsewidth of 12 μs (49°), a sweepwidth of ±50,000 Hz, and 8K data points. It is the result of 50,000 scans, using a line broadening of 100 Hz.

NOTE ADDED IN PROOF

We have been concerned about the lack of susceptibility of vesicle-associated coat protein to chymotryptic digestion (Fig. 7). In recent experiments we prepared vesicles by a modified cholate dialysis procedure without urea. Vesicles prepared in this manner are indistinguishable from those we have previously studied. However, the protein in these vesicles is sensitive to chymotryptic cleavage. We isolated the peptide fragments released and found that about half of both the N-terminal peptide (1–11) and the two C-terminal peptides (43–45 and 46–50) were released, indicating that both termini are exposed on the outer surface. Our inability to observe chymotryptic digestion in Fig. 7 may be due to conformational changes or protein modification induced by urea.

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DISCUSSION

Session Chairman: Patricia C. Jost *Scribe*: Thomas Frielle

JOST: We'll begin with two questions from a referee, Robert Griffin: First, "has an independent experiment been performed to characterize the dynamic behavior of fluorophenylalanine and fluorotyrosine residues relative to that observed for the corresponding protonated residue?"

SYKES: We do not have an independent, biophysical measurement showing that the fluorotyrosine residues flip or rotate in the same way as normal tyrosine residues. There is, however, a large amount of information about the biological behavior of these residues. The fluorotyrosine and fluorophenylalanine phage molecules are infectious. The work of Schlesinger and co-workers has shown that the kinetics and mechanism of alkaline phosphatase are unchanged by substitution by these residues.

JOST: Robert Griffin's second question is: "Recent deuterium NMR results suggest that the phenylalanine and tyrosine residues in protein rings are executing twofold flips rather than continuous diffusion about the β–γ bond. Can the ^{19}F, T_1, and T_2 and Nuclear Overhauser Effect results be explained with this model for molecular motion? Is it possible with these data to differentiate clearly between these two modes of action?"

SYKES: With this question in mind, we have calculated the linewidth expected for these two models for the internal motion of these rings. The predicted linewidth for an immobilized ring in a vesicle of a size similar to ours is ~900 Hz, while we observe a linewidth of ~300 Hz. A linewidth in the 300 Hz region is predicted for free diffusional motion of the ring. It was on this basis that we published previously that the ring is rotating faster than $10^8 s^{-1}$. We then used the T_1 and the NOE measurements to fix the exact range of the rate of internal motion. A two-state jump model gives much less line narrowing. It therefore appears that the simplest motional models might exclude the twofold flips. However, one could imagine another motional model where the rings are restricted to twofold flips but the protein is spinning about its axis. That would decrease the linewidth. The answer really is that we cannot clearly differentiate between the two models but our data suggest that diffusional motion of the ring is more likely.

FEIGENSON: Your reconstitution of M13 coat protein into vesicles involves extensive dialysis. In addition, the lipids used are predominantly DMPC and DPPC. These factors have been reported to result in the polymeric β-form of the coat protein. If the reconstituted protein were strongly self-associated, this could increase the fluorophenylalanyl ^{19}F linewidth. Have you found, for example by CD, that the reconstituted protein is either in the α- or the β-form?

SYKES: We have not performed the CD measurements that would determine the conformational form of the reconstituted coat protein. However, a number of people, for example Keith Dunker, are observing what they think to be two states of the vesicle-bound coat protein. One is an aggregated state and one is nonaggregated, possibly a helical, state. We are approaching a consistency in the sense that previous preparations may well have been leading us to the "aggregated" state where we were not observing chymotryptic digestion. Now, in the absence of urea, we are observing a state in which the protein is digested in the expected manner. What must be understood is what role the urea was playing. Was it influencing the conformation of the protein during reconstitution, or was there carbamoylation of lysine side chains, which would actually change the state of the protein?

DUNKER: What is your lipid:protein ratio in these vesicles?

SYKES: It's of the order of 100 to 120 lipids/protein molecule.

DUNKER: In our experience, the protein would probably be in the α- rather than the β-form even when using DMPC and DPPC.

SYKES: This is a lipid ratio which is high compared to what we've seen this morning in the sense that M13 coat protein is a much smaller protein

and therefore the protein should be present in a sea of lipids. However, there exist the problems of lack of digestibility and possible aggregation in the initial preparations. The other possibility is the U-shaped conformation suggested by Wickner, in which both hydrophilic ends face the interior.

WOLBER: What kinds of reconstitution procedures have you tried? For instance, have you tried the detergent dilution method of Wickner that at the phase transition of DMPC results in a transmembrane orientation?

WEINER: We did originally try Wickner's cholate dilution procedure, but the resulting spectra were too broad to interpret, so we didn't pursue it any further. The cholate dialysis procedure with urea, however, was successful.

WOLBER: In the case of urea, does it appear that the orientation of the protein is transmembrane?

WEINER: In the case of urea, it's not clear. Without urea, it is apparent that we are digesting about half of the N-terminal and half of the C-terminal domains. You could say that there exist two types of U-structures or you could say that there exists a transmembranous arrangement. We can't differentiate between the two possibilities.

WOLBER: Considering the samples made according to Wickner's procedure, were the experiments performed above or below the phase-transition temperature?

WEINER: They were done at about room temperature, which is near the phase-transition temperature of DMPC.

WOLBER: I'd like to comment that care must be taken when following the Wickner procedure. Unless one is careful, one does not always get reproducible removal of the cholate during the subsequent washes after the detergent dilution. When we did the washes, there tended to be a ring of foam at the top of the tube. Unless we were very careful to aspirate that off, terrible samples resulted.

WEINER: Have you followed that with ^{14}C-labeled deoxycholate?

WOLBER: Once, when we were careful to aspirate off this ring. In that case, our lipid:protein ratio was 60:1 and our protein:deoxycholate ratio was 17:1. To our satisfaction, the deoxycholate was very low.

TRUDELL: You interpreted linewidth broadening only in terms of motion. Have you considered the possibility that ^{19}F spectra exhibit large chemical shifts depending on solvent? What if the fluorine were moving between two regions of slightly different dielectic constants?

SYKES: I'm not sure if I know what you mean. We are aware of the sensitivity of the fluorine chemical shift to solvent. We have in fact used this "solvent isotope shift" as a feature to distinguish whether our residues are buried in the lipid bilayer and not exposed to solvent or are on the surface and exposed to solvent. Are you suggesting that the greater linewidth might be due to some kind of two-site exchange between two different environments?

TRUDELL: If you make the sites different in dielectric constant, do you observe a difference in the width of the apparent center peak?

SYKES: Looking at the micelle peak, we were hoping to resolve three peaks from the three FPHE, but what we saw was an obviously heterogeneous single resonance. One component disappeared very quickly during digestion and this disappearance was matched by the appearance of peptide containing residues 1–11 in our separation of peptides released using electrophoresis. We used that result to assign the various components of this heterogeneous peak. If we increase the temperature, the peak does change into a more resolved set of three peaks. So there was no case where we felt the need to invoke chemical exchange between two forms to explain the linewidth. I think that if, as the temperature was increased, we saw a dramatic line narrowing, then we might have had to consider an exchange broadening.

TRUDELL: I performed the opposite experiment where I deliberately used two different environments and watched the exchange between them. In fact, I observed the exchange of the central line that one sees during rapid exchange. This exchange is influenced by the difference in the two dielectric constants.

SYKES: When you raised the temperature did you see the separate resonances from the two forms coalesced into one resonance?

TRUDELL: Yes.

SYKES: We didn't notice a temperature dependence which would be indicative of exchange.

MOORE: This is such a simple, short protein that you should be able to see quite a bit with proton NMR, particularly in micelles. Are there any data of that kind that would show us what part of the protein is actually in the lipid, particularly with reference to the interesting U-shaped structure about which you speculated?

SYKES: We have performed proton NMR spectroscopy on the micelles and on the vesicles. There are several alanine methyls which are in the exposed hydrophilic portions of the protein. They appear as narrow peaks in the micelle preparations even in the presence of the broad background from the deoxycholate residues. We take this result to be consistent with the model where the hydrophobic part is covered by micelle but the ends are rather freely moving in solution. The idea is that if you've got three or four links of internal rotation, you see very narrow lines. But, as you move to the vesicles, you can see no narrow proton NMR resonances rising above the background of the lipid resonances. This supports our feeling that even the exposed portion of the peptide is structured in some way.

WEINER: Those experiments were done with vesicles prepared in the presence of urea, and we may have had a different, possibly an aggregated, structure.

SYKES: Now that we can reconstitute properly digestible protein we should repeat these ^{1}H NMR experiments. That's a very good point.

L. BROWN: In principle, micelle systems are highly dependent upon pH, ionic strength, and temperature. You have interpreted multiple peaks in your spectra as being due to different residues. Do you have any idea how homogeneous your micelle systems are? Is it possible that the multiple peaks are due to polymers of your protein, different sized micelles, or a different organization of the micelles?

SYKES: In the micelle spectrum (see Fig. 6) we see two clear tyrosine resonances at higher temperatures. As we went towards lower temperatures we did get small resonances on the upfield side of the main pair of resonances. I think that we did indicate that those must be due to other states of the protein in the micelle. We use the micelle as a training ground as we head toward the vesicle, and there are differences. For example, the tyrosines are exposed in terms of the solvent isotope shift where they are not in the vesicles.

L. BROWN: It's a more fundamental question than that, because you have tried, particularly with the phenylalanine, to assign those different peaks to different residues to show that you do have labeling of all three. If they were to arise from a monomer or dimer of protein in your micelle, then your interpretation would not be correct.

SYKES: That's right, but the time-course of the release of peptides very nicely matches the time-course of the removal of fluorophenylalanine resonances, particularly the narrow component corresponding to FPHE 11. With the others it's not quite so clear because they are both broader. I think that if there were two states you might expect that one state might be digested completely, while the other is less rapidly digested. The sequential order of release of the peptides is the best information we have.

L. BROWN: You said that in the deoxycholate micelles you had sharp lines for alanine methyl groups, but you had quite broad lines for fluorotyrosines and fluorophenylalanines. Do you have some idea what would account for the large difference in linewidths?

SYKES: All of the ^{19}F NMR linewidths are broader than the corresponding proton linewidths because of chemical shift anisotropy, a relaxation mechanism not present to a great extent in protons. We do not have a direct comparison of the proton linewidths

TROY: If you reconstitute your ^{19}F-labeled protein into purified *E. coli* inner membrane, do you still observe chymotrypsin susceptibility, and if so, at both ends?

SYKES: We have not done that. We have thought about using mutants that prevent reassembly of the phage and accumulate the protein in the membrane, but we have not done those experiments.

TROY: Perhaps that might be fruitful because the conformation and orientation within the membrane both seem to be rather interesting. Those experiments might be a way of looking at it. Another question: Is it correct that when you infect, there is a 20% increase in *E. coli* cardiolipin synthesis?

WEINER: According to the work of Webster's group, there is an increase in cardiolipin biosynthesis when you infect.

TROY: If that's true then the question is twofold. Does that help the phage get into the membrane in order to get out of the cell? And what happens to the orientation of the protein within the membrane when you construct vesicles using varying concentrations of cardiolipin? Does that affect its chymotrypsin susceptibility?

WEINER: We haven't done those experiments beyond some very early work where we didn't have cardiolipin in our system, and we weren't getting much incorporation of the protein into the vesicles. One of the reasons we used cardiolipin was because of Webster's results which indicated that cardiolipin biosynthesis increased. So we put some cardiolipin in and found that it also kept the vesicles from aggregating because we were adding an acidic phospholipid. We never pursued it because other things became more important and the amount of time required to collect one piece of data was fairly substantial, but we are improving our sensitivity. We have not varied phospholipid content, but there are certainly many questions one could ask in terms of varying the phospholipid composition.

To get at your previous question, did you mean incorporate the protein into reconstituted vesicles with *E. coli* phospholipids, or did you mean insert it into natural membranes?

TROY: Well, you could try both. You might try to incorporate the protein into isolated membrane or you could take the lipids from the inner membrane and reconstitute it. I think that either procedure might be of some interest.

JOST: How would you perform the first experiment?

TROY: You might try to insert the protein directly into membranes, perhaps in the presence of a small amount of detergent. Alternatively, brief sonication may assist. The question is, how well will it intercalate into the membrane? I don't know this, but you might also try passing ^{19}F-labeled protein plus isolated membranes through the French-pressure cell. The other approach would be to isolate the inner membrane at a point in the 20-min life-cycle of the phage when there is an increased amount of the protein in the membrane.

WEINER: That experiment we could do. Wickner has done experiments like that to look at the orientation of the protein in the membrane and from these we know that the N-terminus faces out and is accessible to proteolytic digestion. That is how it was demonstrated originally.

TROY: Is there any reason to think that the cardiolipin might perhaps be a preferred boundary lipid for your protein?

WEINER: We have considered that idea and thought about doing some labeling experiments, but we haven't done them. It is certainly a possibility.

HUDSON: I believe that Overath's group (Pluschke et al. 1978. *J. Biol. Chem.* 253:5048) has found that a cardiolipin-deficient mutant infected by this phage grows perfectly well.

STAROS: This question concerns the rotational correlation calculations which you presented, distinguishing between the two-state flip and the diffusional mode for phenylalanine. In at least one case, in a deuterium experiment, I think that it has been observed that if the β-position of phenylalanine in a particular protein is labeled with deuterons, no α-β-rotation is observed, but the two-state flip around the β-α-bond is observed for the phenyl ring. If you had free rotation around the α-β bond, wouldn't your calculation approach the diffusional model whether or not the ring were subject to a two-state mode?

SYKES: We started originally by decomposing the relaxation times T_1 and T_2 (using the frequency dependence of T_2 and the Nuclear Overhauser Effect, respectively) into dipole-dipole and chemical shift anisotropy contributions. The chemical-shift anisotropy contribution was then calculable by a model assuming a diffusional motion of the ring and some wagging of the α-β-bond. We deduced the frequency of the α-β-bond wagging from the proton-fluorine dipole interaction which is not sensitive to the ring-flipping motion. That led us to the conclude that ring motional characteristics in membrane-bound proteins are about the same as in any globular protein. This motion is on the nanosecond time scale and the wagging may be on the 50-ns time scale. You would probably put in another motion, as you suggest, and calculate the results, but it doesn't seem reasonable that the α-β-bond would be spinning freely and the β-α restricted to a two-site flip. It is a physically less reasonable model, but I doubt that it could be mathematically excluded.

STAROS: The two -state flip and the diffusion models might correspond to different ways in which the hydrophobic protein interface interacts with its lipid environment. You might be observing a different tightness in the interaction of protein with lipid in those cases where you observe two-state as compared with those cases where you observe a diffusional mode.