NUCLEAR MAGNETIC RESONANCE OF THE FILAMENTOUS BACTERIOPHAGE fd

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ABSTRACT The filamentous bacteriophage fd and its major coat protein are being studied by nuclear magnetic resonance (NMR) spectroscopy. ^{31}P NMR shows that the chemical shielding tensor of the DNA phosphates of fd in solution is only slightly reduced in magnitude by motional averaging, indicating that DNA-protein interactions substantially immobilize the DNA packaged in the virus. There is no evidence of chemical interactions between the DNA backbone and the coat protein, since experiments on solid virus show the ^{31}P resonances to have the same principal elements of its chemical shielding tensor as DNA. ^{1}H and ^{13}C NMR spectra of fd virus in solution indicate that the coat proteins are held rigidly in the structure except for some aliphatic side chains that undergo relatively rapid rotations. The presence of limited mobility in the viral coat proteins is substantiated by finding large quadrupole splittings in ^{2}H NMR of deuterium labeled virions. The structure of the coat protein in a lipid environment differs significantly from that found for the assembled virus. Data from ^{1}H and ^{13}C NMR chemical shifts, amide proton exchange rates, and ^{13}C relaxation measurements show that the coat protein in sodium dodecyl sulfate micelles has a native folded structure that varies from that of a typical globular protein or the coat protein in the virus by having a partially flexible backbone and some rapidly rotating aromatic rings.

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy can contribute to the solution of important structural problems in molecular biology that are difficult to approach with diffraction techniques. Two such problems are part of the filamentous bacteriophage system. One of these is the packaging and folding of nucleic acids in nucleoprotein complexes such as chromosomes or viruses, where the difficulties arise not only from the large size and chemical complexity of the particles, but also from the disorder that may be present among the nucleotides even when the complexes are macroscopically ordered. Another is the structure of membrane bound proteins which are not stable outside of a lipid environment and cannot be crystallized.

Both of these areas are of considerable importance to the understanding of the filamentous viruses because the complete virion, which consists of only coat proteins and DNA, is assembled at the cell membrane utilizing coat protein molecules that reside within the membrane bilayer (1). In addition to the difficulty in studying these structures by other techniques, fd is an attractive subject for NMR studies because of its relatively small size, simple organization, small coat protein subunits, experimental tractability, and the extensive prior work including x-ray diffraction studies on fibers of fd and related viruses. Several reviews on filamentous bacteriophages summarize the available information (2–5).

fd is a long flexible filament with dimensions 9 × 900 nm in solution (6). It is constructed from 2,700 copies (6) of the 5,000 mol wt major coat protein (7) which surround the

single-stranded circular DNA of 6,400 nucleotides (8). Several copies of a minor coat protein are located at one end of the filament. The total particle weight of the virion is 16.4×10^6, of which 88% is the major coat protein. Fibers of fd give x-ray diffraction patterns that have been used for structural studies. The analysis through molecular model building has been carried out for fd and other filamentous viruses by Marvin and coworkers (9–11) and recently by Makowski and Caspar (12). The picture that emerges from the x-ray diffraction studies is that the DNA is extended lengthwise in a closed tube of coat proteins which are arranged in an overlapping helical array like scales on a fish (11).

Even though a reasonable model for the coat protein arrangement exists and can be further refined, there is little information available from any source on the structure of DNA in the virus or the nature of the DNA-protein interactions. The x-ray data show no resolved spots due to DNA diffraction, which could be due to the DNA being disordered or moving relative to the coat protein shell. An important constraint on any model of DNA-protein interactions in fd is the nonintegral (2.3) ratio of nucleotides to coat protein monomers (6). Photochemical cross-linking experiments indicate the presence of a specific DNA hairpin structure in fd at a fixed position of the DNA sequence in the virus (13).

During fd infection of *E. coli* the major coat protein is synthesized in the cytoplasm as a precursor procoat protein with an N-terminal 23 amino acid leader sequence; however, it is processed to the mature coat protein in the cell membrane where it is stored before being incorporated in the virus (14, 15). The major coat protein of fd has 50 amino acids in a single polypeptide chain; the sequence is characterized by a 19-amino-acid hydrophobic core with hydrophilic terminal regions (7). Because of the large number of hydrophobic side chains the protein is insoluble in water; it is soluble, however, in the presence of lipids or detergents (16, 17).

Assembly of the virus particle takes place as the DNA is extruded through the cell membrane, apparently with the coat protein being wrapped around the DNA as it passes through the membrane containing the stored coat protein (31). Over the last few years there has been some controversy over the extent of the conformational change that the coat protein undergoes during viral assembly (17, 18). Circular dichroism studies assign a conformation with ~50% α-helix to the coat protein in phospholipid vesicles or detergent micelles and a conformation with ~90% α-helix to the coat protein in the virus (17). Green and Flanagan (19) have applied the statistical method of Chou and Fasman (20) and the stereochemical method of Lim (21) to predict the secondary structure of fd coat protein from its primary sequence; while the first approach predicts a β-sheet for the hydrophobic midsection of the protein, the second predicts a highly α-helical protein with no β-sheet. These experimental and theoretical findings suggest the possibility of an enormous change in protein structure as a consequence of virus assembly; certainly a change in 19-amino-acids from β-sheet to α-helix would have to be considered a major conformational change in any protein, especially for one with only 50 amino acids. Large changes in protein structure have not been observed in the assembly of other viruses, so it is of some interest to compare the membrane-bound form of the coat protein to the coat protein of the virus (22).

We are using NMR spectroscopy to study the structure and dynamics of the coat protein and DNA of fd to describe these macromolecules and their interactions in the supramolecular structure of the virus. A wide range of NMR techniques are being employed because the materials of interest range from a low molecular weight protein or single-stranded DNA in solution with rapid motions, to highly ordered membrane or virus gels, to solid samples. By

observing ^{13}C, ^{1}H, ^{2}H, and ^{31}P resonances and employing various decoupling, spinning, and pulsing procedures, specific nuclear spin interactions from individual chemical sites can be studied. The interactions of interest include the chemical shift, dipole-dipole couplings, and nuclear quadrupole couplings.

MATERIALS AND METHODS

fd is grown on *E. coli* K3300 F^+ in a buffered super rich media of beef tryptone and yeast extract (Difco Laboratories, Detroit, Mich.). A colony of *E. coli* is grown for 14 h in 50 ml of rich media, transferred to the preculture which is 500 ml of rich media, grown for another 8 h, and then used to innoculate a 10 l growth. The cells are grown under oxygenation to an optical density of 2 to 4 at 550 nm before infection with fd at a multiplicity of 10. Antifoam B is used to control excessive foaming in the culture. After 4 more hours of growth the yield of virus plateaus at 1-2 $\times$ 10^{13} plaque forming units (PFU) per milliliter.

The cells are removed from the growth medium by centrifugation. The virus is precipitated from the supernatant with polyethylene glycol (Sigma-6000; Sigma Chemical Corp., St. Louis, Mo.), resuspended in distilled water, and banded on cesium chloride (EM Laboratories, Inc., Elmsford, N.Y.; reagent grade) block gradients. Typically, a yield of 2 g of purified virus from a 10-l growth is obtained as measured by PFU and optical density at 268 nm using an extinction coefficient of 3.84 $mg^{-1}cm^2$. The virus is stored at 4°C in distilled water after extensive dialysis.

Labeled amino acids were incorporated into fd by growing the infected *E. coli* on chemically defined medium. The M9 minimal salts were supplemented with 1 μg/ml thiamine-HCl, 1% glycerol, and the labeled amino acids (23). During growth, the pH was monitored and adjusted with KOH to be in the range 7.0–7.5. On defined medium, the final virus concentration is typically 5 $\times$ 10^{12} PFU/ml and gives 400 mg of purified virus.

To solubilize the coat protein from 1 g of fd, 2 g of sodium dodecyl sulfate (SDS) (Sigma Chemical Co.) recrystallized from ethanol are added to a 22 mg/ml virus solution. This solution is then made 40 mM in borate at pH 9.0. ~200 λ of chloroform is added and the milky solution is incubated at 40-50°C for 30 min to complete the solubilization of the coat protein which can be followed visually by the decrease in turbidity of the solution. The dissolution of the phage particle can be more accurately followed by a hyperchromic and hypsochromic shift in the λ_{max} of the UV spectrum of the sample. The coat protein and the viral DNA can be separated by column chromatography using Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, N.J.). On a column 150 $\times$ 2.5 cm equilibrated with 10 mM SDS, 40 mM borate, pH 9.0, and 8% glycerol, ~150 mg of material can be purified. Fractions from the Sephacryl column are pooled and concentrated on a stirred cell (Amicon Corp., Scientific Sys. Div., Lexington, Mass.; model 402) using a DM5 filter with a 5,000-mol wt cutoff. Concentrations of the coat protein were determined spectrophotometrically at a wavelength of 278 nm using an extinction coefficient of 1.65 $mg^{-1}cm^2$. After removal of glycerol by washing the concentrated solution in the stirred cell the coat protein in SDS is stored as a lyophilized powder. All pH values reported are meter readings. pH represents values of solutions in H_2O, and pH* represents values of solutions in D_2O.

The ^{13}C NMR spectra of fd and coat protein in SDS micelles were obtained on a Nicolet NT-150 spectrometer (Nicolet Instrument Corp., Madison, Wis.) at 37.7 MHz in 20 mm sample tubes using broadband proton decoupling. Because of dynamic range problems arising from observing 6 mM protein carbons in the presence of large amounts of detergent, 10^3 free induction decays were coadded, Fourier-transformed, and averaged in blocks. Samples were prepared by dissolving lyophilized coat protein SDS powder in 10 ml of $^{2}H_2O$ at pH 9.0. All ^{1}H NMR spectra were taken at 360 MHz on a Bruker WH-360 spectrometer (Bruker Instruments, Inc., Billerica, Mass.). Rapid scan correlation spectroscopy was used to avoid interference from the detergent resonances. The ^{31}P NMR spectra of fd in solution and as a solid were obtained on a homebuilt double resonance spectrometer with a magnetic field of 3.5 T at 61 MHz using proton decoupling fields of 1.2 mT. The ^{31}P NMR spectrum of the fd DNA in solution was obtained at 145 MHz on the Bruker WH-360 spectrometer in a 10 mm sample tube. The ^{2}H NMR spectra labeled fd was taken on a homebuilt spectrometer with a magnetic field of 5.8 T at 38 MHz.

RESULTS

^{31}P NMR of fd

The only phosphorus atoms present in fd are located in the phosphodiester linkages of its DNA. The ^{31}P NMR data presented here are interpreted in terms of the chemical shift properties of the DNA. Fig. 1 contains proton decoupled ^{31}P NMR spectra of fd DNA; these spectra are "high resolution" by the usual criterion of containing only chemical shift information that is not obscured by dipolar or other broadening mechanisms. Figs. 1 *A* and *B* are data on the DNA in the virion, while Fig. 1 *C* contains the ^{31}P NMR spectrum of single-stranded fd DNA in solution.

The ^{31}P NMR spectrum from solid lyophilized fd is shown in Fig. 1 *A*. This chemical shift powder pattern is typical of that found for phosphodiesters and is essentially identical to that observed for solid samples of double stranded calf thymus DNA, single-stranded fd DNA, and nucleotides 24, 25). This pattern is that of a nonaxially symmetric shielding tensor, the principal values of which can be measured directly from the spectral discontinuities as σ_{11} = 85, σ_{22} = 22, and σ_{33} = −109 ppm from 85% phosphoric acid; these values are within experimental error of those of DNA in the absence of proteins. We have also obtained the proton decoupled ^{31}P NMR spectrum of this solid fd sample spinning rapidly at the magic angle (θ = 55°) with respect to the magnetic field. This procedure removes the chemical shift

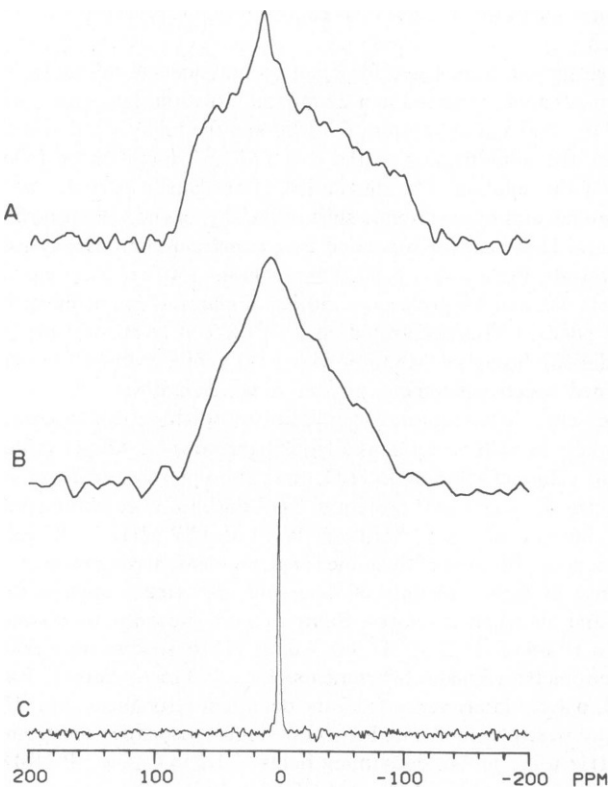

Figure 1 ^{31}P NMR spectra of fd virus at 30°C. (*A*) Lyophilized fd powder with 1.2 mT proton decoupling (60 MHz). (*B*) 50 mg/ml virus solution, pH 7, with 1.2 mT proton decoupling (60 MHz). (*C*) Single-stranded fd DNA in solution free of coat protein and without proton decoupling (145 MHz).

anisotropy, giving an isotropic chemical shift spectrum which in this case is a single line at −0.9 ppm.

Figs. 1 *B* and *C* contain spectra of fd virus and fd DNA in solution; they illustrate the influence of hydration and the resulting motion on the ^{31}P chemical shielding tensor of Fig. 1 *A*. The narrow resonance of Fig. 1 *C* is indicative of complete motional averaging for single stranded DNA in solution. There is only limited reduction of the tensor for the DNA in the virus as seen in Fig. 1 *B*. The breadth of this resonance is due to incompletely averaged inhomogeneous chemical shift anisotropy shown by pulsed, spinning, and field dependent experiments.

The static chemical shift properties of the DNA in fd and double-stranded calf thymus DNA are the same within experimental error. However, their solution properties as determined by ^{31}P NMR are quite different. The viral phosphate resonance shows only a small influence of molecular reorientation. Double-stranded DNA has a ^{31}P linewidth due to chemical shift anisotropy relaxation, since the linewidth increases linearly with the square of the magnetic field strength. By identifying that part of the total linewidth due to chemical shift anisotropy relaxation with decoupling experiments, an effective isotropic reorientation time of $\sim 2 \times 10^{-6}$ s at 30°C can be calculated for DNA phosphate backbone motion. This is substantially more rapid motion than the data of Fig. 1 *C* indicate can be present in the DNA of fd.

^{1}H NMR of fd

^{1}H, ^{2}H, and ^{13}C NMR have been used to study the major coat protein of fd in the assembled nucleoprotein complex and in the protein-detergent complex. Since the virus is 88% by weight coat protein, natural abundance ^{13}C and ^{1}H NMR spectra are dominated by protein resonances. The ^{2}H NMR studies rely on isotopically labeled coat protein.

The ^{1}H NMR spectrum of fd in $^{2}H_2O$ solution is given in Fig. 2 *A*. Only a small percentage of the protons in the virus give rise to signals in this conventional Fourier transform spectrum at 360 MHz, especially in the aromatic region 6–8 ppm. The upfield resonances (1–4 ppm) that correspond to aliphatic protons do show some resolvable features. Exhaustive purification, control experiments, and the observation of broad ^{31}P, ^{13}C, and ^{1}H signals for the same samples indicate that these upfield resonances are from amino acid side chains of intact virions rather than impurities or broken up particles.

The ^{1}H NMR spectrum of fd coat protein in SDS micelles (Fig. 2 *B*) can be accounted for with contributions from all of the amino acids with many resolved resonances. The differences in linewidths, particularly in the aromatic region, between Figs. 2 *A* and *B* dramatically demonstrate the restrictions on motional averaging in the coat protein that accompany virus formation.

^{2}H NMR of fd

We have prepared fd samples with deuterons substituted for hydrogens at specific locations by synthesizing ^{2}H labeled amino acids and then growing fd infected *E. coli* on defined media containing one or more labeled amino acids. ^{2}H NMR often gives a readily interpretable view of molecular dynamics because it has spin 1 and its behavior is dominated by its quadrupole moment.

^{2}H NMR spectra of fd labeled at various positions have partially averaged quadrupole powder patterns indicative of restricted rotation of side chains.

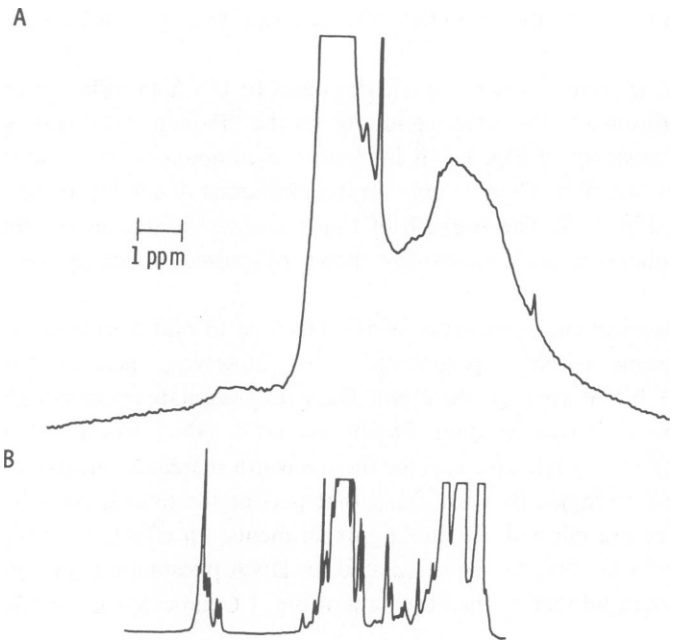

Figure 2 ^{1}H NMR spectra of fd virus and its coat protein at 360 MHz. (*A*) 3 h of signal averaging a 50 mg/ml virus solution in D_2O, pH* 7.0, at 30°C; the two off-scale peaks are residual HOD and a small amount of polyethylene glycol. (*B*) 2 mM coat protein in SDS micelles and D_2O, pH* 9.0, 45°C from 256 scans with a 1-s recycle delay; the off scale peaks are SDS and HOD.

Natural Abundance ^{13}C NMR of fd

Fig. 3 contains ^{13}C NMR spectra of fd in solution and of the coat protein in SDS micelles. This spectral comparison is similar to the one in Fig. 2. In spite of long signal averaging on a very concentrated and large sample of fd in solution very little resonance intensity is observable in Fig. 3 *A*. High power proton decoupling and magic angle sample spinning experiments show that the severe broadening is due to ^{1}H–^{13}C dipolar couplings and ^{13}C chemical shift anisotropy. These static linebroadening mechanisms reflect the lack of motional averaging in such a large particle as a filamentous virus. The lack of resolved aromatic (110–160 ppm) and carbonyl (170–180 ppm) carbon resonance intensity is particularly obvious when the protein spectrum of Fig. 3 *B* is compared to the virus spectrum of Fig. 3 *A*. As in the ^{1}H NMR case, some aliphatic carbon intensity is observed and partially resolved in the upfield part (10–40 ppm) of the ^{13}C spectrum of the virus.

NMR of Exchangeable ^{1}H Resonances of fd Coat Protein in SDS Micelles

The low field ^{1}H NMR spectrum of the coat protein solubilized in SDS is shown in Fig. 4. ~54 exchangeable protons are observed in H_2O at pH 8.4 and 30°C as shown in Fig. 4. Within 5 min of the addition of ^2H_2O at pH*7.4 and 30°C to a sample lyophilized from ^1H_2O, the number of exchangeable protons in this region is reduced to 22. When the pH is adjusted to 10.0 and the sample is heated to 45°C for 30 min, all of the nitrogen- and oxygen-bonded protons are exchanged for deuterons. The results are shown in Fig. 4 *B*.

The intensity of the exchangeable protons at pH*7.4 and 30°C has been followed as a function of time. From Fig. 5 it can be seen that there are large differences in exchange rates among the 22 more slowly exchanging protons. About half of these protons exchange with a

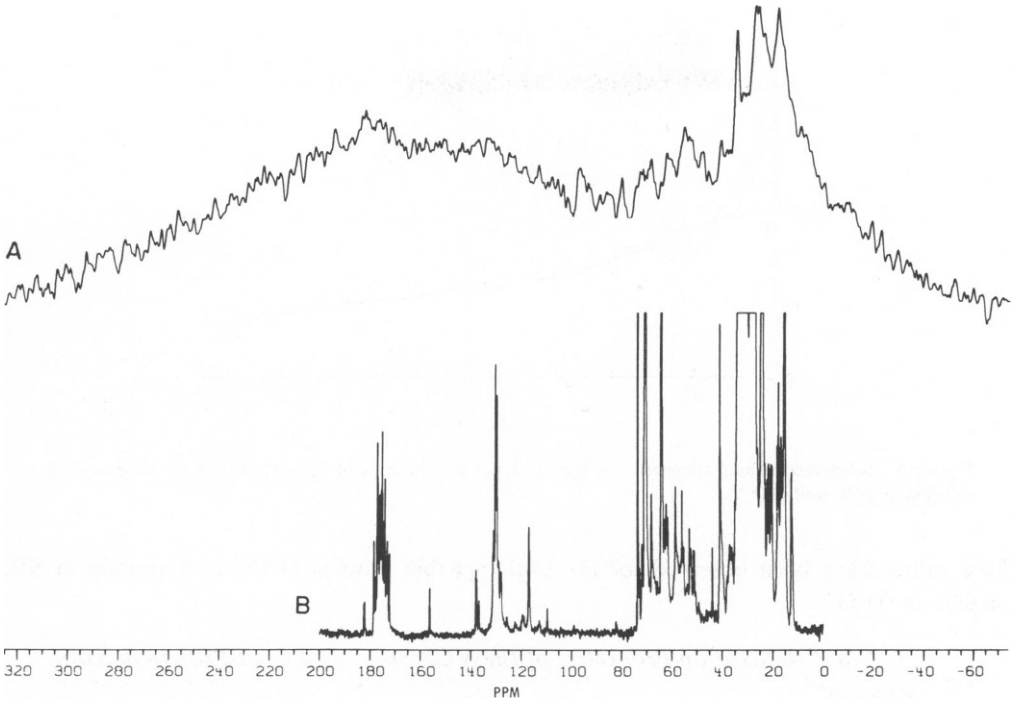

Figure 3 ^{13}C NMR spectra of fd virus and its coat protein at 38 MHz. (*A*) 50 mg/ml virus solution in D_2O, pH* 7.0, 30°C after 36 h of signal averaging. (*B*) 7 mM coat protein in SDS micelles and D_2O at 50°C, pH* 9.0, after adding 70 blocks of 1,024 scans; the off-scale peaks are detergent and glycerol.

half life of 10 min, a few near 40 min, and the remaining eight with a half life longer than the 7-h experiment. This range of exchange rates is typical of a globular protein (26). Also typical of a globular protein is the change in chemical shift of protons as a function of temperature. For hydrogen bonded protons a chemical shift change of 0.002 ppm/°C is observed, while in the absence of hydrogen bonding a rate of about 0.007 ppm/°C is measured (26). Rates near

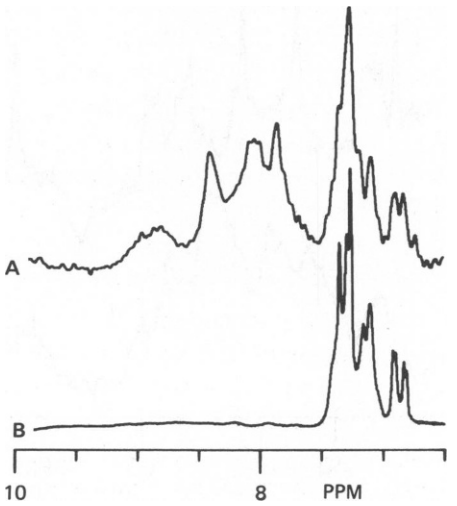

Figure 4 ^1H NMR correlation spectra of the aromatic region of fd coat protein at 360 MHz. (*A*) In H_2O at 30°C and pH 8.4. (*B*) In D_2O, pH* 7.4, at 30°C after the sample was incubated at a pH* of 10 at 45°C for 30 min to exchange protons.

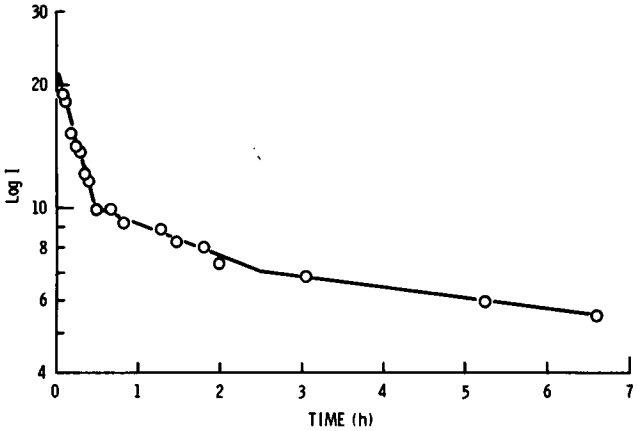

Figure 5 Integrated proton intensities of the exchangeable protons in fd coat protein vs. time in D_2O solution at 30°C and pH* 7.4.

these values have been observed for the exchangeable protons of the coat protein in SDS micelles in ^1H_2O.

^{13}C NMR Relaxation Properties of the α Carbons of fd Coat Protein in SDS Micelles

The α-carbon region of the natural abundance ^{13}C NMR spectrum of the coat protein in SDS is compared with that of a typical globular protein, lysozyme, in solution in Fig. 6. For the coat protein, there are at least two classes of carbon resonances in this region; the majority of lines are broad and overlapping just as they are in the spectrum of lysozyme. However, unlike lysozyme there are about 8 α-carbons that display sharp lines. These few sharp resonances in the coat protein spectrum can also be distinguished on the basis of T_1 and nuclear Overhauser

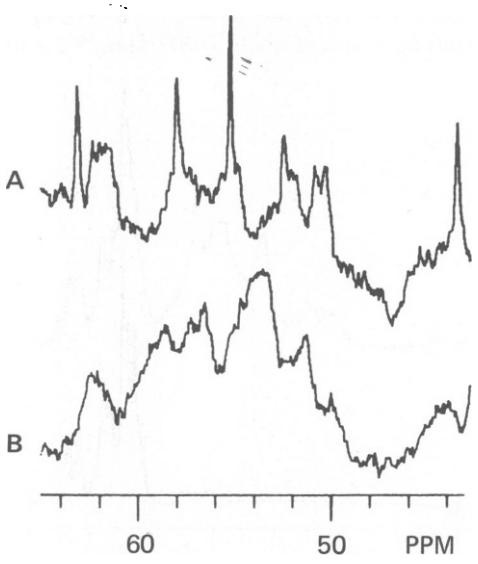

Figure 6 The α-carbon region of the ^{13}C NMR spectra at 38 MHz. (*A*) 7 mM fd coat protein in SDS and D_2O at 50°C and pH* 9.0. 81 blocks of 1,024 scans were obtained with a 2-s recycle delay. (*B*) 14 mM lysozyme in D_2O at a pH* of 4.5 and a temperature of 35°C. 3,600 scans were collected with a 2-s recycle delay.

TABLE I
T_1 AND NOE VALUES FOR SELECTED CARBONS FROM THE ^{13}C NMR SPECTRA OF FD COAT PROTEIN AT pH 9.0 AND 35°C.

^{13}C Resonance	T_1	NOE
	(*ms*)	
Broad αC	110	1.1
Sharp αC	~250	2.3
Phe C^{ϵ}, C^{δ}	140	2.0
Tyr C^{ϵ}	150	1.5

enchancement (NOE), as shown in Table I. The narrow carbon resonances of the coat protein have long T_1*s* and more than the minimal NOE while the broad resonances have short T_1*s* and minimal NOE (27).

^{13}C NMR of the Aromatic Residues of fd Coat Protein in SDS Micelles

The aromatic region of the natural abundance ^{13}C NMR spectrum of the coat protein in Fig. 7 has resonances from the two tyrosines, three phenylalanines, and one tryptophan of the protein. Chemical shift dispersion is apparent for the tyrosines and phenylalanines. The C_ζ resonances of the tyrosines are split into a doublet near 156 ppm and the C_γ lines of the three phenylalanines are resolved near 138 ppm. The protonated phenylalanine carbons dominate the spectrum at 130 ppm as very sharp lines, especially compared with phenylalanines of a globular protein and with those of the protonated tryptophan carbons of the same spectrum at

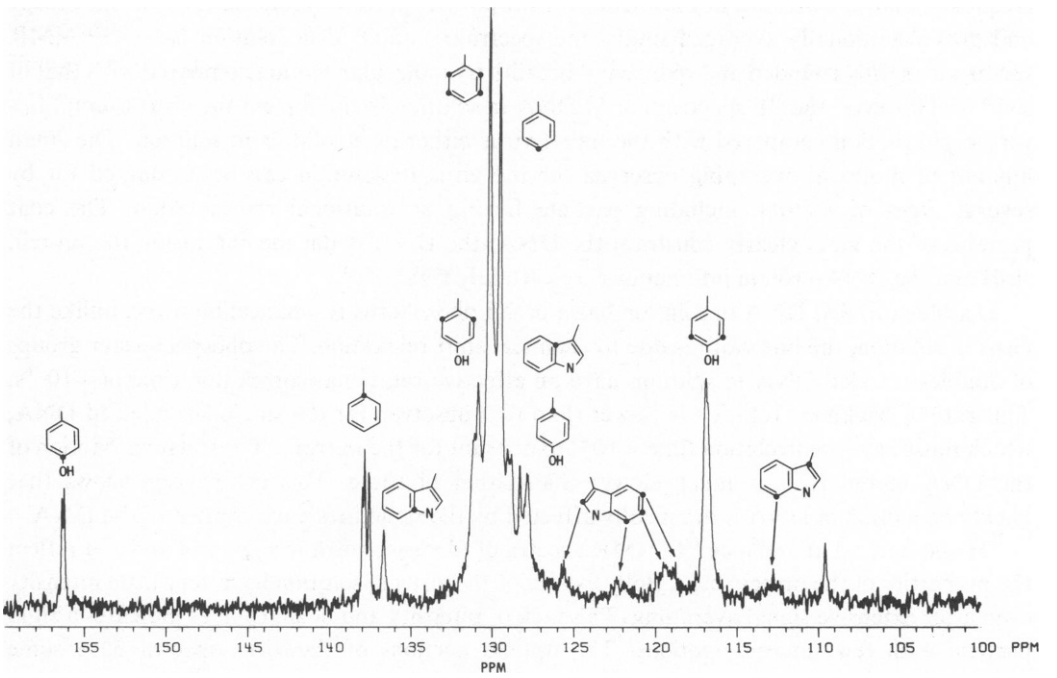

Figure 7 Aromatic region of the ^{13}C NMR spectrum at 38 MHz of fd coat protein in SDS and D_2O at 50°C and pH* of 9.0. 81 blocks of 1,024 scans at a 2-s recycle delay were summed. Resonances from three phenylalanines, two tyrosines, and one tryphophan are identified by the boldface dot.

113, 119, 122, and 126 ppm which are broad. The linewidth of the tyrosine C_ϵ at 117 ppm is intermediate between the sharp phenylalanine resonances and the broad tryptophan resonances. The relaxation parameters for the aromatic carbons are listed in Table I.

DISCUSSION

These NMR experiments provide information on the properties of fd, its major coat protein, and its DNA. The purpose of the study is to compare the assembled and dissociated subunits to understand the structural changes that accompany virus assembly. The data presented in Figs. 1–3 lead to a model of fd based primarily on qualitative descriptions of the molecular dynamics of identifiable sites. At the present early stage of this research, the model is necessarily of low resolution; however, extensions of the work using isotopic labels and oriented fibers of the virus can provide the means for a detailed structure determination. Figs. 1 *C*, 2 *B*, 3 *B*, 4, 6, and 7 can be interpreted to give a view of the properties of the viral components before viral assembly.

^{31}P NMR of the virus is selective for the phosphodiester backbone of the single-stranded DNA in the interior of the virus. The ^{31}P chemical shielding properties give structural and dynamical information on the DNA. The principal values and angles of a chemical shielding tensor and, less directly, the isotropic chemical shift reflect the electronic and steric properties of an atomic site. Since the ^{31}P chemical shift powder pattern of solid fd is nearly identical to that observed for DNA or nucleotides in the absence of proteins, there is no evidence that the packaging of the DNA in the virus coat alters the chemistry or geometry of the phosphodiester backbone. The magnitude of the anisotropy and the apparent symmetry of the chemical shielding tensor are affected by molecular and macroscopic motion. Motions more rapid in frequency than the breadth of the chemical shift powder pattern would remove the anisotropy and give a motionally averaged single line spectrum. Intact fd in solution has a ^{31}P NMR spectrum slightly rounded and reduced in breadth by molecular motion compared with that of solid fd. However, the ^{31}P spectrum of fd DNA in solution isolated from the virus exemplifies very rapid motion compared with the intact virus either as a solid or in solution. The small amount of motional averaging observed for the virus in solution can be accounted for by several types of motion, including particle flexing or rotational reorientation. The coat proteins of the virus clearly constrain the DNA; the DNA is not moving inside the protein shell and the DNA-protein interactions are certainly rigid.

Double-stranded DNA in solution has a broad phosphorus resonance; however, unlike the virus in solution, the linewidth is due to chemical shift relaxation. The phosphodiester groups of double-stranded DNA in solution have an effective rotational correlation time of $\sim 10^{-6}$s. This rate of backbone rotation is slower than that observed for the single-stranded fd DNA, which must have a correlation time $<10^{-9}$ to account for the narrow ^{31}P resonance. Motion of the DNA in the virus is much slower than either of these. This comparison shows that backbone motion in DNA is drastically affected by the structural environment of the DNA.

^{1}H and natural abundance ^{13}C NMR spectra of fd virus shown in Figs. 2 *A* and 3 *A* reflect the properties of the protein coat. Both spectra of the virus in solution have very little intensity even after extensive signal averaging. This lack of intensity and of sharp lines indicates a rigid particle with few internal motions. The upfield portions of the virus spectra have some partially resolved resonances that must arise from relatively mobile aliphatic side chains; similar findings have been reported for the ^{1}H NMR spectra of tobacco mosaic virus coat protein oligomers (28).

The NMR results for fd in solution give a dynamical view of the assembled particle. The phosphorus results indicate that the interior of the virion is immobile and that the filament itself is rigid with a small amount of flexing or rotation. The ^{1}H, ^{2}H, and ^{13}C NMR data are all consistent with this view of the virus particle. The conclusion that fd has immobilized coat protein subunits with some flexible aliphatic side chains is reinforced by finding three nuclei which are affected by different spin interactions that yield consistent results. These results provide a qualitative view of the virus as a tightly knit aggregate of proteins constraining the DNA in the interior and forming a rigid filamentous virus particle.

The ^{1}H NMR spectrum of the coat protein in SDS micelles shown in Fig. 2 indicates that there is far more motion present in the coat protein in this environment than in the assembled viral coat protein. None of these aromatic resonances were observed in the intact virus. The ϵ-protons of the two tyrosines are resolved in the region around 6.8 ppm despite the fact that they are separated by only two amino acids in the polypeptide chain. The separation of these resonances means that the electronic environments of the rings are different. The p*Ka*'s of the tyrosines have been measured by recording ^{1}H NMR chemical shifts vs. pH, and both have been found to be ~12.5, indicative of similar chemical environments that are extremely hydrophobic.

The exchangeable proton and aromatic regions of the ^{1}H NMR spectrum of the SDS solubilized protein in H_2O at pH 8.4 and at a temperature of 30°C are shown in Fig. 4 *A*. ~54 exchangeable protons are observed in this region between 6.5 and 10 ppm from DSS. Several groups of exchangeable protons will most likely not be observed under these experimental conditions, such as the hydroxyl protons of serine and threonine, which normally exchange very rapidly and are rarely, if ever, observed in spectra of native globular proteins. The ϵ-amino protons of lysine and the amino terminus of the polypeptide chain will not be observed because their normal p*Ka*'s are so close to that of the samples' pH. The protons that account for the exchangeable proton intensity are most likely the 48 backbone amide protons, the two sidechain amide protons of the glutamine residue, the indole N–H of the tryptophan, and the two phenolic OH's from the tyrosine side chains buried in the hydrophobic environment. This sums to 53 exchangeable protons and is in excellent agreement with the observed integration value.

Backbone amide proton exchange rates for a random coil in an aqueous medium at pH 8.4 and 30°C based on equations of Englander et al. (29) are slow enough so that they will be observed. However, at 60°C the calculations indicate that these random coil amide protons should no longer be present. At 60°C only 46 of the 54 exchangeable protons observed at 30°C are present. The two sidechain amide protons of glutamine are no longer observed at their high field position. However, the other sidechain exchangeable protons should still be observed resulting in the observation of 43 of the 48 backbone amide protons. Proteolytic digestion studies by Woolford and Webster (30) on fl coat protein, which has the same amino acid sequence as fd, in deoxycholate indicate that approximately eight amino acids from each terminus of the polypeptide are susceptible to cleavage and hence are freely exposed to the aqueous media. All except for about five of these amino acids must be involved in some secondary structure that reduces the rate of exchange of the backbone amide protons to explain our results. The predictions of Green and Flanagan (19) based on the rules of Lim (21) indicate that all but seven amino acids in these terminal regions will be α-helical, and of these, one is the amino terminus and another is a proline residue which does not have a peptide N-H. Thus, these predictions indicate secondary structure in part of the terminal regions in full agreement with our results.

Further evidence for hydrogen bonding has been observed by the exchangeable proton chemical shift dependence on temperature. Two multiproton resonances shift at a rate of 0.002 ppm/°C over the temperature range of 25–90°C; this is indicative of hydrogen bonding. However, three multiproton resonances shift at a rate of ~0.006 ppm/°C over the same range of temperature, indicative of no hydrogen bonding. Fig. 4 *B* is the ^{1}H NMR spectrum of the SDS solubilized coat protein in D_2O, pH* 7.4, and at a temperature of 30°C after the sample had been at a pH* of 10.0 and incubated at 45°C for 30 min to exchange all exchangeable protons for deuterium. However, 22 exchangeable protons are observed at a pH* of 7.4 and a temperature of 30°C when D_2O is added to a lyophilized sample of coat protein and SDS that has not been exposed to D_2O before. As a result, more than half of the exchangeable protons and hence many of the peptide amide protons have an exchange rate half-life of <10 min. Among those exchangeable protons observed in D_2O there is a wide range of rates from a half-life of 10 min to something greater than the length of the 7 hour exchange experiment. Similar rates have been observed in native globular proteins and for mellitin in micelles (31). This range of rates cannot be explained only by the presence of detergent molecules forming a micelle. Present knowledge of micelles indicates that the outer aqueous region or Stern region of the micelle is ~80% of the micelle volume and in this region exchange rates might be slowed by as much as a factor of 10, while the core of the micelle is very hydrophobic where rates could be slowed by a large factor. This two-state model for micelles cannot explain the range of rates with half-lives from 1×10^{-5} to 10 min to >420 min. The protein must have highly structured regions to account for these observed rates of hydrogen exchange.

The results of the ^{1}H NMR experiments on the coat protein in a model membrane environment indicate that portions of the polypeptide chain are structured. At least some hydrogen bonding is implied by the temperature dependence of the chemical shifts of the exchangeable protons. Accounting for the number of exchangeable protons in H_2O solutions leads one to the conclusion that part of the terminal sequences possessed structure, as there appears to be a small mobile tail sequence.

The natural abundance ^{13}C NMR spectrum of the coat protein in SDS at pH* 9.0 and 50°C is shown in Fig. 3 *B*. As in the ^{1}H NMR spectra comparison, there is a dramatic decrease in linewidth of all parts of the carbon spectrum upon solubilizing the coat protein in micelles. The methyl and methylene region of the coat protein spectrum is dominated by detergent resonances as well as a large peak at 70 ppm. However, the low field regions of the spectrum and most of the α-carbon region are free from interfering lines.

The α-carbons in the region from 40 to 65 ppm exhibit at least two classes of motion based on three relaxation parameters. The broad resonances with short T_1s and minimal NOE values are characteristic of a rigid α-carbon backbone like that found for native globular proteins. From T_1 and NOE data of these broad resonances which form the bulk of the intensity in the α-carbon region a rotational correlation time of ~15 ns for the micellar complex was obtained. Also present in the α-carbon region and superimposed on the broad resonances are a number of sharp resonances with long T_1s and significantly large NOE values. These characteristics are indicative of rapid motion of some α-carbons; since the T_1 and NOE data do not fit calculations for isotropic reorientation this motion is anisotropic. There are approximately eight α-carbons with a large degree of motion that would probably not be involved in a structured region of the polypeptide. This number is in good agreement with the six terminal amino acids that do not possess secondary structure based on the rules of Lim (21).

The aromatic resonances are assigned in Fig. 7. The phenylalanine and tyrosine

protonated carbons have resonances that are sharper than that typically observed in globular proteins and these have relatively long T_1s and nonminimal NOE values, as seen in Table I. These rings therefore have significant amounts of internal mobility and, as for the sharp α-carbons, the motion is anisotropic. The protonated carbons of the tryptophan, however, are broad and indicative of a correlation time of ~ 20 ns, in close agreement with the bulk of the α-carbons. So while the phenylalanine and tyrosine rings have a great deal of motion, the tryptophan ring which is only one amino acid removed from one of the tyrosines is immobile. As in the ^{1}H NMR spectra there is chemical shift inequivalence of specific atomic sites, the tyrosine ζ-carbons form a doublet, and the three phenylalanine γ-carbon resonances are resolved. Again this is indicative of different environments for the rings which can be explained by a structured polypeptide.

The coat protein in SDS micelles appears to be a good model for the study of membrane proteins. Circular dichroism studies (17) show that large amounts of α-helix are present when the protein is solubilized in detergents or lipid vesicles, and this agrees with the predictions of secondary structure from the polypeptide sequence. Our NMR results indicate that the polypeptide backbone is rigid, as it would be in a structured protein, except for a few α-carbons, which most likely exist as a structureless N-terminus. The chemical shift dispersion of specific atomic sites is also a strong argument for a structured polypeptide, since random coil sequences never show this inequivalence. Linewidth differences such as those observed in the aromatic region are also indicative of different environments, possibly generated by a structured polypeptide. Unlike globular proteins, but possibly typical of membrane proteins, part of the backbone is mobile and unstructured. Also, some of the aromatic resonances indicate more mobility than is normal for globular proteins. While many of the overall spectral characteristics such as broad α-carbons, chemical shift inequivalency, and variation in aromatic resonance linewidths are reminiscent of globular proteins, this proposed membrane structure has some added features that can be distinguished and characterized by NMR.

The structure of the virus particle is that of a single-stranded circle of DNA restrained and encapsulated by the coat protein to form a stiff filament. Although there may be some flexibility to the filament it is considerably stiffer than double-stranded DNA. The coat protein peptide backbone is also immobile and the only detectible motion is in a few aliphatic side chains. The few rapidly moving α-carbons in SDS solubilized protein have had their motion drastically slowed down, as have tyrosine and phenylalanine rings in the assembled virus. Although our studies do not show if there is a β-sheet to α-helix transition, they do indicate dramatic dynamical changes in sidechain mobility and the formation of a structured terminal segment upon virus assembly.

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DISCUSSION

Session Chairman: Frederic Richards *Scribe*: Thomas A. Gerken

JELINSKI: In Fig. 7 you showed a spectrum of d6-phenylalanine. How do you explain the build up of intensity in the center of the phenylalanine powder spectrum? If you calculate the spectrum you expect for a C-D bond, you would expect a quadrupolar splitting of ~120 kHz or more. To me the bonded spectrum indicates motion. To what do you attribute that center portion of the spectrum of your labeled virus? Is that water in natural abundance or is it an $\eta = 1$ deuterium pattern?

OPELLA: There are complications in the interpretation of ^{2}H NMR spectra of d_5Phe and fd virus labelled with d_5Phe. Obviously, the polycrystalline sample of d_5Phe does not give a spectrum corresponding to immobilized C-D bonds. We know from ^{13}C NMR experiments that Phe in the solid state has substantial mobility of the ring, since ^{13}C-^{1}H dipolar couplings are reduced by motion.

The ^{2}H NMR spectrum of d_5Phe has splittings of 125 kHz as well as substantial intensity with much smaller splittings. Therefore, there is evidence for static and motionally averaged quadrupolar interactions. C-D bonds that have an angle of 180° with respect to the axis of rotation will not have their quadrupole couplings reduced by the motion. If the phenyl rings are making 180° flips about the β–γ bond, then the C_ζ–D interaction will not be affected while the C_δ–D and C_ϵ–D interactions will be partially averaged. Any motions other than 180° ring rotations present in the Phe residues of the virus in solution will reduce the largest ^{2}H quadrupole splittings.

The ^{2}H NMR spectrum of d_5Phe labeled virus in solution shows no reduction of the 125 kHz quadrupole splittings. Therefore, one or more of the three Phe rings per coat protein are immobilized in the virus. However, since the central part of the virus spectrum is not well resolved and therefore difficult to analyze, we cannot make statements with regard to ring flips from the present data. Future work with more careful exchange of residual HO^{2}H will enable us to describe the ring dynamics of the virus.

JELINSKI: In your paper you also mentioned that the ^{31}P linewidth of fd in solution was different from the ^{31}P linewidth for double stranded DNA in solution. Would you comment on that?

OPELLA: We have been studying DNA in a variety of situations. The ^{31}P resonance from high molecular weight DNA or DNA-protein complexes is typically very broad and needs to be analyzed for linebroadening mechanisms in order to describe the phosphates of the DNA samples. Double-stranded DNA in solution has its linewidth due mostly to chemical shift anisotropy relaxation induced by bending motion of the polymer. In contrast, the broad ^{31}P linewidth for fd in solution is due to unaveraged static chemical shift anisotropy.

DOBSON: I want to follow that point up. You are saying that the difference between solution and the solid is due to internal motion in the DNA. We've looked at the ^{31}P NMR of RNA in TBSV. We find that for this spherical virus the differences between the spectra in solution and the solid state are caused only by the overall molecular reorientation and not by any difference in the internal motion of the RNA.

OPELLA: Our ^{31}P NMR results for double-stranded DNA show evidence of bending modes of the DNA but not of rapid internal motions.

DOBSON: This is something associated with the particle as a whole then.

OPELLA: Since the ^{31}P chemical shielding tensor is only slightly rounded or averaged by motion, we see no evidence of rapid internal motions of the DNA packaged in the virus.

DOBSON: I'm asking about the slight rounding, actually. Is that due to molecular motion?

OPELLA: The rounding of the ^{31}P powder pattern of fd in solution is so slight and nonspecific that it is difficult to analyze. The field strength dependence and magic angle sample spinning on this resonance demonstrate that the linewidth is from unaveraged static chemical shift anisotropy. It looks like overall rotational diffusion of the virus particle can account for the spectrum, and there is no need to have additional modes of motion present.

MAKOWSKI: You showed a broad band of mobilities in the aromatic rings (Fig. 7). I don't have a good feeling for how this compares to what one would expect for a globular protein.

OPELLA: The mobilities of the aromatic rings of fd coat protein in SDS micelles differ substantially from those of globular proteins in that there is a relatively large range of rates of internal motions since some of the phenylalanine rings appear to be moving quite rapidly. This is based on the relaxation properties of the ring resonances of the ^{13}C NMR spectrum of the coat protein, where the Phe C_ϵ and C_δ resonances are extremely sharp with long T_1 and large NOE. In contrast, the protonated Trp carbons are very broad with short T_1 and small NOE, indicative of an immobilized residue.

MAKOWSKI: You are saying that there are some groups with much more mobility than you would expect for a globular protein, and that there are others that are very stationary?

OPELLA: In a active globular protein there are typically a few immobilized rings undergoing 180° flips or rotations at moderate rates.

MAKOWSKI: These virus particles tend to align parallel to the magnetic field lines. Is there any way this could have some effect on the interpretation of the spectra?

OPELLA: Yes. Magnetic field alignment of filamentous viruses could have a large effect on the NMR spectra. However, even though our samples exhibit orientation in a polarizing microscope, we have as yet seen no evidence for orientation in the NMR spectra.

TORCHIA: I'd like to return to Fig. 1 *b*. You seem to have good evidence that that's an inhomogeneously broad line. Your model is that you are rotating around the long axis of the virus to get that line shape. I don't understand why it's not actually symmetric.

OPELLA: Because the rotation is not fast compared to the 10 kHz chemical shift anisotropy.

TORCHIA: Well, if it's not fast then you're not going to get an inhomogeneously broad line because you're going to be having motions on the time scale of your interaction.

OPELLA: The rotation of the particle about its long axis is $10^4 s^{-1}$ or slower. This is not rapid enough to induce efficient relaxation.

WÜTHRICH: Did I understand correctly that you conclude that you see sizeable populations of spatial arrangements for the aromatic rings of pheylalanine and tyrosine which deviate from the "average" position in space and that there is no evidence for 180° flips about the C^β-$^\gamma$ bond?

OPELLA: In solution, the Phe rings of the fd coat protein undergo reorientation independent of the protein backbone. There is a range of motion of aromatic rings since the Trp looks to be immobilized and the Tyrs have properties intermediate between the Trp and Phes.

WÜTHRICH: What kind of motions of the aromatic rings do you think you are observing in your spectral data?

OPELLA: We know that parts of the polypeptide backbone are flexible, unlike in a globular protein. Most of the α-carbons have relaxation properties as expected for the overall tumbling time of the protein. A few of the α-carbons give sharp lines, have long T_1s, and have large NOEs, and therefore, give evidence for flexibility. The Phes may not be only reflecting phenyl group rotation, but also backbone segmental motion.

WÜTHRICH: Do you think you see rotations about the C^β-C^γ bond or do you feel rather that there are motions involving large time variations of the dihedral angle about the C^α-C^β bond?

OPELLA: The Phe rings may be undergoing several kinds of motion in order to have the narrow resonances and other relaxation properties observed in the ^{13}C NMR spectra of the coat protein in micelles.

NALL: I'd like to talk about Fig. 1 *b* again. Perhaps the problem Dr. Torchia had in mind was an explanation for the broadened powder pattern observed in the solution spectrum.

OPELLA: I think he is more worried that the lineshape is due to relaxation.

NALL: I would argue that this spectrum is not fully proton-decoupled due to the fact that the motions are so slow that complete decoupling of P^{31} from the protons is not possible.

OPELLA: The magic angle sample spinning spectrum gives narrow ^{31}P spinning sidebands. If the proton decoupling was inadequate, then these would be broad.

WILLIAMS: As I understand, you see a phenylalanine that is motionless in the intact phage and when you strip the coat protein from the phage it becomes very mobile. Is that correct?

OPELLA: Because there are three Phes in the coat protein, we have to soften that specific statement a bit. It is clear that one or more of the Phes is not moving in the assembled virus.

WILLIAMS: All right. In our Raman studies of fd phage we observed Raman hypochromism for the phenylalanine bands when we strip the coat protein from the phage. You could interpret that to mean that the phenylalanines are intercalating with the DNA bases or they are interacting with some other rings in the coat proteins and that when you denature the phage they are moving out free into solution. Do you have any feeling for what the environment of the phenylalanines are in the intact virus and in isolated coat protein?

OPELLA: The experiments are not yet giving structural information. The fd coat protein in SDS micelles is a native protein; it is not denatured.

TAINER: Is your coat protein in solution a monomer or a dimer?

OPELLA: The protein exists as a dimer in SDS micelles.

TAINER: So you envision two short helical segments stuck together somehow with most of the side chains exposed to solvent?

OPELLA: These NMR data do not show if the structure is helical.

TAINER: But the CD of coat protein in SDS solution suggests that it's α-helical.

OPELLA: CD suggests there is a lot of α-helix in the coat protein in its membrane bound form and in the intact virus. It further suggests there are substantial changes in helix content between these two states. Our NMR results have not yet given much information on this point. I think the protein is mostly α-helical with some regions —probably at the ends— that are very mobile.

TAINER: Have you tried building CPK models? You'll find that if you do that, there is asymetry such that one side of the α-helix of a monomer is very flat and the other side has most of the bulky hydrophobic side chains. I wonder if this sort of modeling might help you in looking at some of your data?

OPELLA: I think molecular model building will help in interpreting the NMR data. Other types of NMR experiments can give explicit structural information, especially where oriented fibers are employed.

NALL: When you took the spectrum of phage in solution using magic angle spinning, how did you prevent the phage from pelleting against the side of the spinner?

OPELLA: It does pellet.

NALL: So you are looking at a solid, where proton decoupling is expected to be efficient, and not at a solution, where it might be inefficient due to slow motions?

OPELLA: The sample is very hydrated and no spectral changes are observed after the virus goes into solution.

NALL: I'm suggesting that the solution spectrum in Fig. 1*b* could still be partially dipolar broadened since it is a spectrum of phage in solution and not of a solid pellet.

OPELLA: The field strength dependence of the linewidth suggests that an interpretation in terms of static CSA is correct.

NALL: The sharp features of a powder pattern can be smoothed due to dipolar interactions without large changes in the field strength dependence of the linewidth.

OPELLA: That is right. A refocussing experiment could differentiate these mechanisms of linebroadening.

LLINÁS: Have you looked at the rates of amide exchange when the protein is on the virus and when it is free in solution? That might tell you about a change in flexibility or overall conformational stability.

OPELLA: The rates of amide N-H exchange vary widely for the coat protein in micelles, including some protons which take longer than 10 h to exchange at 30°C. We cannot resolve any amide hydrogens for the intact virus in solution.