NMR Studies of the Structure and Dynamics of Membrane-Bound Bacteriophage Pf1 Coat Protein

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Filamentous bacteriophage coat protein undergoes a remarkable structural transition during the viral assembly process as it is transferred from the membrane environment of the cell, where it spans the phospholipid bilayer, to the newly extruded virus particles. Nuclear magnetic resonance (NMR) studies show the membrane-bound form of the 46-residue Pf1 coat protein to be surprisingly complex with five distinct regions. The secondary structure consists of a long hydrophobic helix (residues 19 to 42) that spans the bilayer and a short amphipathic helix (residues 6 to 13) parallel to the plane of the bilayer. The NH_2-terminus (residues 1 to 5), the COOH-terminus (residues 43 to 46), and residues 14 to 18 connecting the two helices are mobile. By comparing the structure and dynamics of the membrane-bound coat protein with that of the viral form as determined by NMR and neutron diffraction, essential features of assembly process can be identified.

THE TECHNICAL DIFFICULTIES ASSOCIATED with NMR studies of proteins in membrane environments can be overcome with a concerted approach that uses the methods of solution NMR spectroscopy on proteins in micelles and those of solid-state NMR spectroscopy on proteins in bilayers. Both methods make extensive use of isotopically labeled protein samples. In this approach the secondary structure of the protein is determined on the basis of distance measurements in micelles, while the arrangement of the major elements of secondary structure is derived from measurements of angular parameters in oriented bilayers. The dynamics of the protein are described by motional averaging of line shapes observed in solid-state NMR experiments, which occurs when large-amplitude motions occur more frequently than 10^4 Hz, and nuclear spin relaxation, which reflects motions that occur with frequencies near 10^9 Hz (*1*). We report the NMR structure of membrane-bound Pf1 coat protein, which has been compared to the structure of the coat protein in virus particles as determined by NMR and neutron diffraction (*2*).

Distance information on proteins in solution can be obtained from ^{1}H-^{1}H homonuclear nuclear Overhauser effect (NOE) measurements and can be used to determine secondary and tertiary structure (*3*). Since amide ^{1}Hs on adjacent residues in an α helix are proximate (2.8 Å) and give strong cross-peaks in two-dimensional (2-D) NOE experiments, they are particularly useful for determining the secondary structures of membrane proteins, which typically have large fractions of α helix. Previously we have shown that residues 30 to 40 of Pf1 coat protein in micelles are helical (*4*). In order to characterize the entire protein structure it was necessary to reduce the problems resulting from the broad lines and efficient spin-diffusion that accompany the slow reorientation of proteins in micelles, as well as the extensive spectral overlap that is a consequence of many residues having the same helical secondary structure. This was accomplished by labeling the protein uniformly with ^{2}H (80%) on all carbon sites, with ^{15}N (98%) on all nitrogen sites, and with ^{2}H (50%) on all exchangeable sites and by combining ^{1}H-^{15}N heteronuclear correlation and ^{1}H-^{1}H NOE spectroscopy (*5*). The 2-D ^{1}H-^{15}N correlation spectrum of uniformly ^{15}N-labeled Pf1 coat protein resolves the resonances from all amide sites and has been completely assigned (*4–7*). The combined 2-D ^{1}H-^{15}N correlation and ^{1}H-^{1}H NOE spectrum in Fig. 1 has NOE cross-peaks between all amide correlation peaks from adjacent residues within α helices. The tracing of two continuous pathways of NOEs among the various amide ^{1}H atoms directly maps out the two stretches of α helix in the protein (*3, 8*). The existence of these two α helices is strongly supported by the observation of many of the appropriate cross-peaks between amide ^{1}H and Cα ^{1}H resonances in both 2-D and 3-D spectra (*7*); for example, the Cα ^{1}H resonance from residue 36 has NOE cross-peaks to the amide ^{1}H resonances on residues 37, 39, and 40. The correlation peaks assigned to the amide sites in the NH_2- and COOH-terminal regions and the region between the two helices do not have ^{1}H-^{1}H NOE cross-peaks to other amide sites.

Solid-state NMR spectroscopy can determine the complete structures of immobile proteins, such as those embedded in lipid bilayers, when several spectral parameters are measured in order to characterize the angles between each of the peptide planes and the direction of sample orientation (*9*). Since the secondary structure of the membrane-bound form of Pf1 coat protein is established by the homonuclear ^{1}H-^{1}H

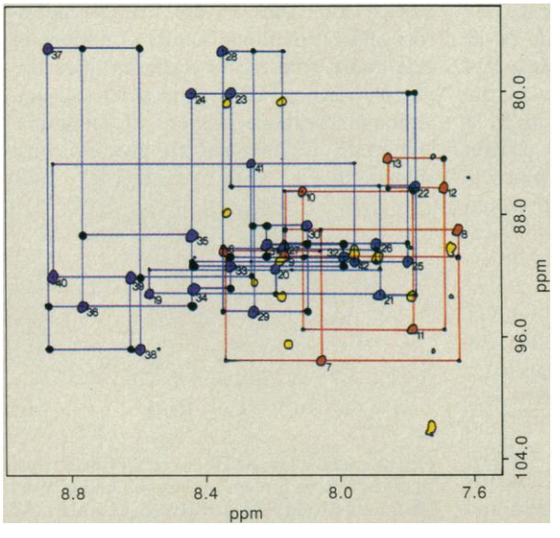

Fig. 1. Combined 2-D ^{1}H-^{15}N correlation and ^{1}H-^{1}H NOE spectrum of uniformly ^{2}H-, and ^{15}N-labeled coat protein in dodecylphosphocholin micelles in 50% H_2O–50% D_2O solution at 50°C and pH 5.0. Each amide site gives rise to a single correlation peak in the contour plot. The lines connect the cross-peaks between correlation peaks from adjacent residues. The blue lines and correlation peaks are associated with amide sites in the hydrophobic helix, the red lines and correlation peaks are associated with the amide sites in the amphipathic helix, and the yellow correlation peaks are from mobile amide sites. The assignments of the resonances from the residues in the helices are indicated with the numbers near the corresponding correlation peaks (*4–7*). The protein sample was obtained from a viral infection of the host bacteria *Pseudomonas aeruginosa* in a growth medium containing glycerol-d_5 (Merck Isotopes) as the carbon source and ^{15}N ammonium sulfate (EG&G Mound Applied Technologies) as the nitrogen source in 90% D_2O–10% H_2O (v/v). Purified virus particles (8.0 mg) were solubilized in 80 mg of dodecylphosphocholine-d_{38} (Merck Isotopes). The solution was maintained at 40°C, pH 8.5, in 50% H_2O–50% D_2O for 24 hours to establish the appropriate isotopic distribution on all amide sites. The pH was reduced to 5.0 prior to lyophilization and redissolving of the sample in 0.45 m of 2 mM citrate buffer, 50% H_2O–50% D_2O to give a 3 mM protein concentration. This spectrum was obtained on a Bruker AM500 NMR spectrometer with a 5-mm "reverse" probe. A total of 4096 data points were digitized during data acquisition interval t_2 for a spectral width of 5 kHz. The t_1 interval of 0.1389 ms was incremented 256 times. The mixing interval was 325 ms.

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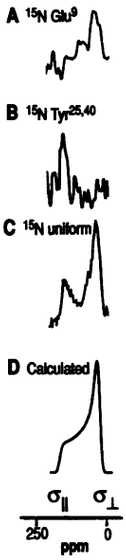

Fig. 2. Solid-state NMR spectra of ^{15}N-labeled Pfl coat protein in oriented phospholipid bilayers. The experimental spectra are compared to a calculated ^{15}N amide chemical-shift powder pattern with the positions of the principal components marked. The samples were obtained from viral infection of the bacteria on a defined medium. The spectra were obtained on a home-built 8.5-T spectrometer.

NOE cross-peaks in Fig. 1, a single spectral parameter, in this case the ^{15}N chemical shift, is sufficient to establish the orientations of the α helices relative to the plane of the bilayer. Solid-state ^{15}N NMR spectra of labeled Pfl coat-protein samples oriented in phospholipid bilayers are shown in Fig. 2. These spectra can be simply interpreted by using the nearly axially symmetric ^{15}N amide chemical shift tensor as a qualitative guide to the orientation of the peptide groups in the helices, since an amide N–H bond approximately parallel to the direction of the applied magnetic field has a ^{15}N resonance frequency close to that of the principal element $\sigma_{\parallel}$ and an amide N–H bond perpendicular to the field has a ^{15}N resonance frequency close to that of the principal element $\sigma_{\perp}$ (*10*). The spectrum in Fig. 2C of uniformly ^{15}N-labeled protein has two bands of signal intensity near the resonance frequencies associated with $\sigma_{\parallel}$ and $\sigma_{\perp}$ of the amide chemical shift tensor. Spectral resolution was greatly improved by using selectively ^{15}N-labeled protein samples. There is only one Glu residue (Glu^{9}) in Pfl coat protein (*11*). The spectrum in Fig. 2A of ^{15}N-Glu^{9}–labeled Pfl coat protein has a single line with a resonance frequency near $\sigma_{\perp}$, therefore the N–H bond of Glu^{9} and hence the amphipathic helix that includes this residue is approximately perpendicular to the direction of the magnetic field and parallel to the plane of the bilayer. The two Tyr residues in Pfl coat protein are in the hydrophobic helix, therefore the spectrum in Fig. 2B of ^{15}N-Tyr–labeled Pfl coat protein has overlapping resonance intensity near $\sigma_{\parallel}$. This result demonstrates that the N–H bonds of Tyr^{25} and Tyr^{40} (and the hydrophobic helix) are approximately parallel to the direction of the magentic field and perpendicular to the plane of the bilayer.

Solid-state NMR spectroscopy is particularly well suited for describing the dynamics of immobile proteins in unoriented samples (*12*), because powder pattern line shapes are strongly influenced by motional averaging. Effectively isotropic, large-amplitude motions that occur more frequently than the breadth of the relevant powder pattern are particularly easy to recognize because they result in narrow resonance intensity at an average position. Selectively ^{15}N-Gly–labeled Pfl coat protein has six amide ^{15}N resonances that contribute resonance intensity between 30 and 200 ppm in the spectra of Fig. 3, A and B, both of which have a narrow isotropic spectral component superimposed on the powder pattern. These sprectra demonstrate that the protein has at least one mobile Gly residue in both its membrane-bound form and its structural form in virus particles, although most of the glycines are rigidly held by the protein structure. Amide resonances from Gly^{15} and Gly^{17}, but not those from any other glycines, have negative ^{1}H-^{15}N heteronuclear NOEs in micelles (7), indicating that Gly^{15} and Gly^{17} account for the narrow resonance intensity in the spectrum in Fig. 3A and that all of the other glycines in the protein contribute to the powder pattern and are immobile, with the exception of Gly^{1} at the NH_2-terminus. The same analysis holds for the coat protein in the virus particles where Gly^{15} or Gly^{17} or both are mobile.

Solid-state ^{2}H NMR spectra of CD_3-labeled side chains are also sensitive indicators of local motions. The hop motions of side chains with rigid backbones characteristically alter powder pattern line shapes, although backbone motions of substantial amplitude are required to give isotropic signals. In Fig. 3, C to F, the ^{2}H NMR

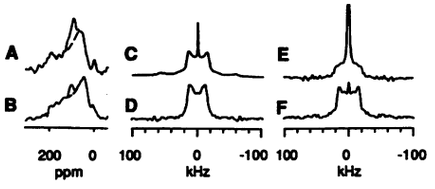

Fig. 3. Solid-state NMR spectra of selectively labeled Pfl coat protein in phospholipid bilayers (**A, C,** and **E**) and virus particles (**B, D,** and **F**). All of the samples are fully hydrated, stationary, and unoriented: (A and B) ^{15}N-Gly–labeled; (C and D) ^{2}H-Thr–labeled; and (E and F) ^{2}H-Leu–labeled. The samples were obtained from viral infection of bacteria on a defined medium. The ^{15}N NMR spectra were obtained on a home-built 8.5-T spectrometer and the ^{2}H NMR spectra were obtained on a home-built 5.9-T spectrometer. The dashed lines in the ^{15}N NMR spectra demonstrate the approximate line shape of the underlying powder pattern.

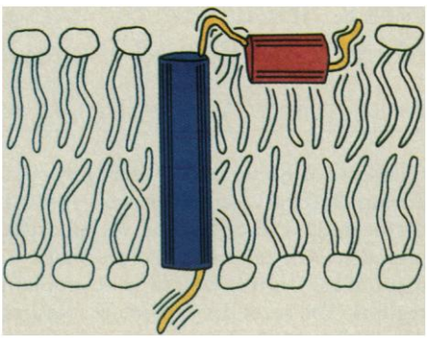

Fig. 4. Model of Pfl coat protein in lipid bilayers. The amphipathic helix parallel to the plane of the bilayer is red, the hydrophobic membrane spanning helix is blue, and the mobil terminal and loop regions are yellow.

spectra for two different, selectively deuterium-labeled proteins in phospholipid bilayers and in virus particles are compared. In both cases, narrow isotropic resonance intensity was observed in the membrane-bound form, but not in the viral form. A qualitative analysis of these spectra, where the presence of substantial isotropic resonance intensity indicates that the protein has one or more mobile residues of a particular type, strongly suggests that Thr^{5} near the NH_2-terminus and Leu^{43} near the COOH-terminus are mobile in the membrane-bound form, but not in the viral form of Pfl coat protein. Similar data indicate that Val^{2} and Ala^{46} are also mobile in the membrane-bound form of the protein, but not in the virus particles.

The results of the NMR experiments are summarized with the model of the protein in Fig. 4. There are five distinct regions in this small 46-residue membrane-bound protein. The protein has two α helices, as defined by the observation of the appropriate homonuclear ^{1}H-^{1}H NOEs in micelles in Fig. 1. The data in Fig. 2 demonstrate that the amphipathic helix is parallel to the plane of the bilayer and the hydrophobic helix is perpendicular to the plane of the bilayer. The narrow isotropic resonance intensity in the spectra in Fig. 3 indicates that the two rigid helices are connected by a mobile loop and that there are mobile residues at both the NH_2- and COOH-termini of the membrane-bound form of the protein. In contrast, the coat protein in the virus has structured NH_2- and COOH-terminal residues, but does retain the mobile internal loop connecting the two helices.

Pfl coat protein has both a long hydrophobic helix that spans the membrane bilayer as well as a helix that is relatively short, distinctly amphipathic, and parallel to the plane of the bilayer, as expected for a helix with its hydrophobic side in contact with

the lipid chains and with the hydrophilic side exposed to the lipid headgroups and solvent. The two helices are connected by a mobile loop and residues at both the NH_2- and COOH-termini of the protein are mobile. The structures of both photosynthetic reaction centers (*13*) and bacteriorhodopsin (*14*) are dominated by long hydrophobic membrane-spanning helices connected by loops, some of which contain relatively short amphipathic bridging helices. Bacteriorhodopsin has been shown to have mobile NH_2- and COOH-terminal regions as well as mobile internal loops (*15*). The dynamics of the coat protein from a different filamentous bacteriophage (fd and M13) has also been characterized in some detail, showing that it also has mobile NH_2- and COOH-terminal regions in its membrane-bound form (*16–18*). The mobile NH_2- and COOH-terminal regions as well as the mobile internal connecting loops are characteristic of membrane-bound proteins, as are the rigid hydrophobic bilayer spanning helices and amphiphatic bridging helices. Pf1 coat protein is a typical membrane protein.

REFERENCES AND NOTES

1. M. J. Bogusky, R. A. Schiksnis, G. C. Leo, S. J. Opella, *J. Magn. Reson.* **72**, 186 (1987).
2. R. Nambudripad, W. Stark, S. J. Opella, L. Makowski, *Science* **252**, 1305 (1991).
3. K. Wüthrich, *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
4. R. A. Schiksnis, M. J. Bogusky, P. Tsang, S. J. Opella, *Biochemistry* **26**, 1373 (1987).
5. K. Shon and S. J. Opella, *J. Magn. Reson.* **82**, 193 (1989). The essentially complete replacement of the ^{1}Hs bonded to carbons with ^{2}Hs and the dilution of the exchangeable amide ^{1}Hs by one half with ^{2}Hs result in sufficient narrowing of amide ^{1}H resonance linewidths and suppression of spin-diffusion so that the short internuclear distances between amide ^{1}Hs in an α helix result in observable NOE cross-peaks.
6. M. J. Bogusky, P. Tsang, S. J. Opella, *Biochem. Biophys. Res. Commun.* **127**, 540 (1985).
7. K. Shon and S. J. Opella, in preparation.
8. The interproton distances characteristic of the α-helix secondary structure that are observed are d_{NN}, $d_{\alpha N}$, $d_{\alpha N}$ (i, i+3), and $d_{\alpha N}$ (i, i+4).
9. S. J. Opella, P. L. Stewart, K. G. Valentine, *Q. Rev. Biophys.* **19**, 7 (1987); S. J. Opella and P. L. Stewart, *Methods Enzymol.* **176**, 242 (1989); L. E. Chirlian and S. J. Opella, *Adv. Magn. Reson.* **14**, 183 (1990).
10. G. S. Harbison *et al.*, *J. Magn. Reson.* **60**, 79 (1984); C. J. Hartzell, M. Whitfield, T. G. Oas, G. P. Drobny, *J. Am. Chem. Soc.* **109**, 5967 (1987); T. G. Oas, C. J. Hartzell, G. W. Dahlquist, G. P. Drobny, *ibid.*, p. 5962; Q. Teng and T. A. Cross, *J. Magn. Reson.* **85**, 439 (1989).
11. D. H. Rowitch and R. N. Perham, *J. Mol. Biol.* **195**, 873 (1987).
12. S. J. Opella, *Methods Enzymol.* **131**, 327 (1986).
13. J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, *Nature* **318**, 618 (1985); D. C. Rees, H. Komiya, T. O. Yeates, J. P. Allen, G. Feher, *Annu. Rev. Biochem.* **58**, 607 (1989).
14. R. Henderson *et al.*, *J. Mol. Biol.* **213**, 899 (1990).
15. M. A. Keniry, H. S. Gutowsky, E. Oldfield, *Nature* **307**, 383 (1984); J. L. Bowers and E. Oldfield, *Biochemistry* **27**, 5156 (1988).
16. T. A. Cross and S. J. Opella, *Biochem. Biophys. Res. Commun.* **92**, 478 (1980).
17. G. D. Henry and B. D. Sykes, *Biochemistry* **29**, 6303 (1990).
18. L. A. Colnago, G. C. Leo, K. G. Valentine, S. J. Opella, in *Biomolecular Stereodynamics III*, R. H. Sarma and M. H. Saarma, Eds. (Adenine, Guilderland, NY, 1986), pp. 147–158; G. C. Leo, L. A. Colnago, K. G. Valentine, S. J. Opella, *Biochemistry* **26**, 854 (1987).

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Membrane-Mediated Assembly of Filamentous Bacteriophage Pf1 Coat Protein

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Filamentous bacteriophage Pf1 assembles by a membrane-mediated process during which the viral DNA is secreted through the membrane while being encapsulated by the major coat protein. Neutron diffraction studies showed that in the virus most of the coat protein consists of two α-helical segments arranged end-to-end with an intervening mobile surface loop. Nuclear magnetic resonance studies of the coat protein in the membrane-bound form have shown that the secondary structure is essentially identical to that in the intact virus. A comparison indicates that during membrane-mediated viral assembly, while the secondary structure of the coat protein is largely conserved, its tertiary structure changes substantially.

THE COAT PROTEINS OF FILAMENTOUS bacteriophage are among the simplest examples of transmembrane proteins. Prior to assembly they are inserted into the inner host cell membrane with their NH_2-termini on the outside of the membrane and their COOH-termini on the inside (*1, 2*). During assembly, the basic amino acids near the COOH-terminus of the coat protein form electrostatic interactions with the DNA (*3*) and the proteins interact with one another to form the long (1 to 2 μm), flexible, 65 Å diameter virions with no incorporation of lipid. Characterization of the process by which the protein coat assembles around the viral DNA as it is secreted through the host cell membrane may aid in our understanding of a number of membrane-mediated processes, including assembly of membrane components and protein secretion. In this report we compare the structure of the coat protein of filamentous bacteriophage Pf1 in the intact virus particle [as determined by neutron diffraction (*4, 5*)] with its membrane-bound conformation [as determined by nuclear magnetic resonance (NMR) (*6*)]. This comparison provides information about the structural transformations that take place during membrane-mediated translocation processes.

Neutron diffraction from magnetically oriented gels of specifically deuterated virus particles has been used to determine the positions of 15 of the 46 amino acid residues in the coat protein of filamentous bacteriophage Pf1 (*4, 5*). A typical neutron fiber diffraction pattern from a native (unlabeled) specimen is shown in Fig. 1. Difference

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Table 1. Secondary structure of the Pf1 coat protein. The intact virion results are from neutron diffraction. Results on the coat protein in membranes are from NMR spectroscopy. Solid-state NMR spectroscopy shows that Gly^{15} or Gly^{17} or both are mobile in the surface loop of the virus.

Intact virion		Membrane	
Residues	Structure	Residues	Structure
		1–5	Mobile
2–12	α Helix	6–13	α Helix
13–19	Surface loop	14–18	Mobile
20–42	α Helix	19–42	α Helix
43–46	No information	43–46	Mobile