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Solid-State NMR Structural Measurements on the Membrane-Associated Influenza Fusion Protein Ectodomain

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Enveloped viruses such as HIV and influenza virus (IFV) are enclosed by a membrane which is obtained from an infected host cell. Infection of a new cell begins with joining or "fusion" of the viral and host cell membranes with an end result of a single membrane and the viral nucleocapsid in the host cell cytoplasm. Although membrane fusion is thermodynamically allowed, the rates of uncatalyzed membrane fusion are typically small. For this reason, enveloped viruses have fusion proteins in their membranes that bind to the host cell membranes and catalyze fusion.[1] This paper describes studies of a large and functional domain of the IFV hemagglutinin (HA) fusion protein and includes bacterial expression and isotopic labeling of the protein, characterization of its folding and fusion activity, and membrane incorporation and solid-state nuclear magnetic resonance (SSNMR) structural measurements.

The HA protein is composed of HA1 and HA2 subunits. HA1 lies completely outside the virus, while HA2 has a ~185 residue N-terminal ectodomain that lies outside the virus, a ~25 residue transmembrane domain, and a ~10 residue C-terminal endodomain that is inside the virus.[2] The IFV is taken into the host respiratory epithelial cell by receptor-mediated endocytosis, and the cell physiological processes lower the pH of the endosome to ~5. The HA1 and HA2 subunits dissociate, and a large HA2 structural change results in exposure of the ~20 residue N-terminal "fusion peptide" (IFP) region. The IFP binds to endosomal membranes, and membrane fusion occurs. There has been a pH 7.5 structure of the HA1/HA2 ectodomain complex crystallized from aqueous solution and a pH 4.4 structure of residues 34−178 of HA2 that forms the "soluble ectodomain" (SHA2) and which was also crystallized from aqueous solution.[2,3] In addition, there have been liquid-state NMR structures of IFP in detergent micelles as well as electron spin resonance measurements of motion and membrane insertion of specific residues of IFP and of a HA2 construct composed of residues 1−127.[4,5] The present work is on a "FHA2" full ectodomain construct composed of residues 1−185 of HA2 and an eight residue C-terminal tag (Figure 1A). SSNMR has the potential for providing high-resolution structural information for FHA2 in the physiologically relevant membrane-bound state and for addressing structural effects of factors that reduce fusion activity including neutral pH and mutations.

There have been some previous applications of SSNMR to other large bacterial and human membrane proteins as well as membrane-associated IFP, and our study builds on this work.[6−10] SSNMR requires efficient production of >10 mg quantities of isotopically labeled protein, and this was accomplished by FHA2 expression in *Escherichia coli* cells. Significant isotopic labeling requires expression in minimal media which lacks amino acids, but it was found that the purified FHA2 yield was ~0.1 mg/L fermentation culture for *E. coli* grown only in minimal media. The successful approach was growth to OD 7 in a rich LB medium followed by a switch to minimal medium composed of glucose, salts, and the labeled amino acids.[11] Although there has been progress in SSNMR assignment and structure determination of uniformly ^{13}C,^{15}N-labeled membrane proteins, it was decided to begin with amino acid type labeling so that assignment would be more straightforward.[12−14] FHA2 purification was done using 0.5% *N*-laurylsarcosine detergent, and FHA2 with >95% purity was obtained using a cobalt resin which bound the FHA2 histidine tag (Figure 1B). Yields of ~8 g cell mass and ~3 mg purified FHA2 per liter fermentation culture were obtained with this approach.

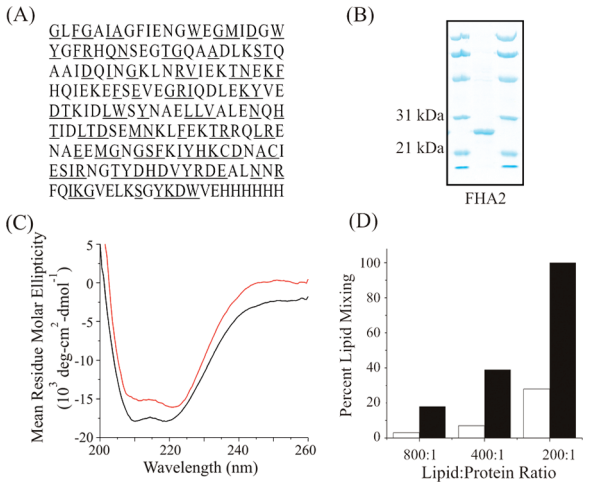

Figure 1. (A) FHA2 amino acid sequence from the influenza X31 strain. Each of the underlined residues is a first residue in a unique sequential pair. (B) SDS-PAGE gel of purified FHA2, MW = 22.5 kD. (C) Circular dichroism spectra at 4 °C of FHA2 in 0.5% BOG detergent at pH 5.0 (red line) and pH 7.4 (black line). (D) Final extent of lipid mixing in vesicles of LM3[7] induced by FHA2 at pH 5.0 (filled bars) and pH 7.4 (open bars).

The FHA2 was exchanged into a solution of 0.5% β-octylglucoside detergent (BOG) in 5 mM HEPES/10 mM MES ("HM buffer") at pH 7.4. The overall secondary structure as a function of pH was probed with circular dichroism (CD) spectroscopy (Figure 1C). Observation of CD minima at 208 and 222 nm at both pH 5.0 and pH 7.4 was consistent with a significant fraction of helical conformation. The θ_{222nm} value of −16 000 deg·cm^2/dmol at pH 5.0 correlated with ~50% of the residues in helical conformation and can be compared to the ~60% of the residues in helical conformation expected if the SHA2 and IFP regions of detergent-associated FHA2 have the same conformations observed in their respective structures. A common assay to probe fusion peptide-induced membrane perturbation is peptide-induced lipid mixing (LM) between different unilamellar vesicles. By this assay, FHA2 was a potent fusogen and worked at ~10-fold lower concentrations than has been observed for IFP (Figure 1D).[15,16] At FHA2/lipid ~ 0.001, the LM rate was >0.5 s^{-1} and is >10-fold larger than LM rates observed for fusion peptides at higher ratios.[15] There was also a striking pH dependence of FHA2-induced lipid

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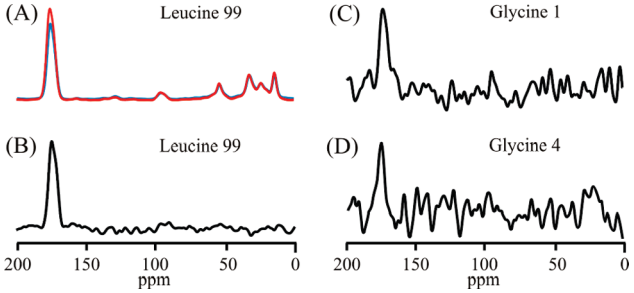

Figure 2. ^{13}C SSNMR spectra of membrane-associated FHA2. (A) REDOR S_0 (red) and S_1 (blue) spectra for a Leu-^{13}CO, Val-^{15}N sample and (B−D) S_0-S_1 spectra, respectively, representing ^{13}CO signals from the L99, G1, and G4 ^{13}COs and having peak chemical shifts of 178.0, 174.7, and 177.8 ppm, respectively. Signal averaging times were 1−3 days, and each spectrum was processed with 3 ppm line broadening and baseline correction. Variation in signal-to-noise in B−D was due to differences in amounts of material, FHA2/lipid, and signal averaging times.

mixing which correlated with previous IFP studies and with the pH of IFV fusion. Enhanced pH-dependent fusion has also been previously shown for other HA2 constructs.[15,17] The CD and lipid mixing results are consistent with folded and active FHA2.

Reconstitution of FHA2 into membranes for SSNMR began with formation of a thin film of di-*O*-tetradecylphosphatidylcholine (DTPC, 32 mg), di-*O*-tetradecylphosphatidylglycerol (DTPG, 8 mg), and BOG (160 mg). The film was dissolved in 5 mL of pH 5.0 HM buffer, and this solution was mixed with a 2 mL solution containing FHA2 (5 mg) and pH 7.4 HM buffer with 0.5% BOG. BOG removal was achieved by dialysis at 4 °C into pH 5.0 HM buffer with a membrane having 3 kD cutoff and with one buffer change over 3 days. A hydrated membrane pellet was obtained by centrifugation by the slurry at 50 000*g* for 4 h.[18] In addition, ether- rather than ester-linked lipids were used for these initial SSNMR studies to reduce the natural abundance carbonyl signal and simplify spectral interpretation.

In order to obtain residue-specific SSNMR spectra, ^{13}CO and ^{15}N labelings were, respectively, chosen for the first and second residues of a unique sequential pair in FHA2. As displayed in Figure 2A, an unfiltered ^{13}C "S_0" spectrum was obtained with the rotational-echo double-resonance (REDOR) sequence while signals from ^{13}COs directly bonded to ^{15}Ns were attenuated in the REDOR S_1 spectrum.[19] Figure 2B−D displays $S_0 - S_1$ difference spectra which were filtered ^{13}CO signals from different unique sequential pairs. ^{13}CO labeling of >70% was suggested by comparison of ^{13}CO/natural abundance ^{13}Cα intensity ratios in S_0 spectra, and >70% ^{15}N labeling was suggested by comparison of S_0 and S_1 ^{13}CO intensities. There are well-known correlations between ^{13}CO chemical shifts and local conformation with distributions of 178.5 ± 1.3 and 175.7 ± 1.5 ppm for Leu in helical and β-strand conformations, respectively, and corresponding distributions of 175.5 ± 1.2 and 172.6 ± 1.6 ppm for Gly.[20] The peak ^{13}CO chemical shifts for G1, G4, and L99 were 174.7, 177.8, and 178.0 ppm, respectively, and were consistent with helical conformation. These results correlated with the helical conformations observed for L99 in the SHA2 crystal structure and for functionally critical G1 and G4 in the IFP structure in detergent.[21] This NMR filtering approach had previously been applied to chemically synthesized fusion peptides, and the present work demonstrates general applicability to a large expressed membrane protein.[7] Over 50% of the backbone FHA2 COs are in unique sequential pairs, so this particular SSNMR approach should be applicable to mapping the membrane-associated FHA2 conformation including regions which were not part of the previous SHA2 or IFP structures. Detection of residue-specific conformational changes as a function of pH, membrane cholesterol, and mutations is also feasible and will provide data to assess existing structure−function models for HA2-mediated fusion.[4,21] For example, addition of membrane cholesterol has correlated with a helical to β-strand conformational change in the IFP and effects of cholesterol may now be examined in the full FHA2 protein.[7,10] The SSNMR samples had 0.003 < FHA2/lipid < 0.02, and at larger FHA2,lipid, it may be possible to determine internuclear distances and other structural parameters with SSNMR methods. In addition, the FHA2 quantities are such that liquid-state NMR and diffraction methods could be applied to detergent-associated FHA2.

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Supporting Information Available: Detailed descriptions of cell growth, FHA2 expression, isotopic labeling, and purification, LM assay, CD and SSNMR spectroscopies, and membrane reconstitution at pH 7.4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Materials. The FHA2 sequence was [1]GLFGAIAGFIENGWEGMIDGWYGFR HQNSEGTGQAADLKSTQAAIDQIN[50]GKLNRVIEKTNEKFHQIEKEFSEVEGRIQD LEKYVEDTKIDLWSYNAELL[100]VALENQHTIDLTDSEMNKLFEKTRRQLRENAEE MGNGSFKIYHKCDNACI[150]ESIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKD WVEHHHHHH and the FHA2 plasmid was obtained from Dr. Yeon-Kyun Shin at Iowa State University and contained the Lac promoter and kanamycin resistance. The plasmid was transformed into E. coli BL21(DE3) cells. Unless noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). **LB medium** was purchased from Acumedia (Lansing, MI) and 15 mg/L kanamycin was then added to it. **M9 minimal medium** contained per liter 6.8 g Na_2HPO_4, 3.0 g NaH_2PO_4, 0.50 g NaCl, 1.0 g NH_4Cl, 0.25 g $MgSO_4$, 10 g glucose and 15 mg kanamycin. The initial pH of the M9 medium was adjusted to be 7.4. The **feeding buffer** contained 100 mM sodium phosphate at pH 8.0 and 20 g/L glucose. **Buffer A** contained 50 mM sodium phosphate at pH 8.0, 300 mM NaCl, 20 mM imidizole, and 0.5% (w/v) *N*-laurylsarcosine detergent. **Buffer B** contained 50 mM sodium phosphate at pH 8.0, 300 mM NaCl, 250 mM imidizole, and 0.5% (w/v) *N*-laurylsarcosine detergent. **Buffer C** contained 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 7.4 and 0.5% β-octylglucoside (BOG) (Anatrace, Maumee, OH). **Buffer D** contained 5 mM HEPES and 10 mM MES at pH 5.0 and 0.5% BOG. **Buffer E** contained 5 mM HEPES at pH 7.0. **Buffer F** contained 5 mM HEPES and 10 mM MES at pH 7.4. **Buffer G** contained 5 mM HEPES and 10 mM MES at pH 5.0.

Cell growth and protein expression. A 250 mL baffled Erlenmeyer flask containing 50 mL of LB medium was inoculated with a single colony of cells containing the FHA2 plasmid. The culture was grown for ~12 hours at 37 ^{o}C in a shaker (New Brunswick, Edison, New Jersey) operating at 200 rpm. The OD_{600} of the culture reached a maximum value of ~7 and the cell suspension was subsequently diluted to 1 L in fresh LB medium in a 2.8 L baffled fernbach flask. After overnight growth at 37 ^{o}C with a shaking rate of 200 rpm, the OD_{600} of the 1 L culture reached a maximum value of ~7. The cell suspension was centrifuged at 9000*g* at 4 ^{o}C and the resulting cell pellet mass was > 6 g. It was found that using a baffled rather than a regular Erlenmeyer flask was important for achieving the large cell mass presumably because of better aeration with the baffled flask.

The cell pellet was resuspended in 1 L M9 medium and cell growth resumed while shaking at 37˚C and 200 rpm. After 30 minutes and after 60 minutes of shaking, a 50 mL aliquot of feeding buffer was added that helped to maintain the pH and the glucose concentration in the medium and resulted in higher yield of FHA2. The OD_{600} of the cell suspension increased by ~1.0 during the 60 minutes of growth in M9 medium and the cells were in mid-log phase growth at this time. The shaker temperature was reduced to 23˚C and 100 mg of each isotopically labeled amino acid was added to the flask. After fifteen minutes, induction of FHA2 expression began with addition of IPTG to a final concentration of 0.2 mM. The induction period was 3 hours and ended with centrifugation of the cell suspension for 15 minutes at 9000*g* and 4 ^{o}C. The cell pellet mass was >7 g/L fermentation culture and the cell pellet was frozen at –80 ^{o}C for >12 hours to enhance cell lysis.

The above optimized protocol was based on variation of the following parameters: media composition, cell growth temperature, shaking rate, and oxygen and glucose concentrations, cell concentration at the start of induction, IPTG concentration, and temperature and duration of induction period. The isotopic labeling protocol was based on methods previous published by other groups (Marley, J.; Lu, M.; Bracken, C. *J. Biomol. NMR* **2001,** *20,* 71-75 and Cai, M.; Huang, Y.; Sakaguchi, K.; Clore, G. M.; Groneborn, A. M.; Craigie, R. *J. Biomol. NMR* **1998,** *11,* 97-102).

Purification. All buffers were refrigerated prior to use in the purification. The following protocol was optimized to obtain optimal purity and yield from 5 g cells. Purification of larger cell quantities may require further optimization. Cells (5 g) were suspended in 25 mL of buffer A and cell walls were lysed during 4 sonication periods of 1 minute duration with a 1 minute delay between sonication periods. Each period contained 0.8 s on/0.2 s off cycles with 80% amplitude during the on cycle. Cell debris was removed by centrifugation at 48000*g* and 4 ^{o}C for 20 minutes and 0.5 mL of cobalt His-Select resin (Sigma) was added to the centrifugation supernatant. The FHA2 protein was bound to the resin during agitation for 1 hour at ambient temperature. Cobalt rather than nickel resin was used because there was strong binding of the SlyD protein to the nickel resin (Zhang, J. W.; Butland, G.; Greenblatt, J. F.; Emili, A.; Zamble, D. B. *J. Biol. Chem.* **2005,** *280,* 4360-4366). A resin pellet was formed by centrifugation at 1000*g* for 1 minute and the pellet was transferred to a 10 mL disposable column (Biorad, Hercules, CA) and then washed with 3 column volumes (1.5 mL) of buffer A. FHA2 was eluted from the resin with 5 column volumes (2.5 mL) of buffer B and the FHA2 solution volume was subsequently reduced to ~0.5 mL by centrifugation in a Vivaspin concentrator (Sartorius,

Goettingen, Germany) whose membrane had a molecular weight cutoff of 3500 D. Buffer exchange was achieved by dilution of the FHA2 solution in the concentrator with buffer C followed by reconcentration. The dilution/concentration steps were repeated until the nominal buffer C/buffer B ratio (v/v) was >1000. The final FHA2 solution contained ~2 mg FHA2 in ~2 mL buffer C; i.e. [FHA2] ~ 50 μM. The FHA2 purity was >95% as observed in a SDS page gel. Some fraction of the protein may have an N-terminal methionine (Chang Sup Kim, personal communication).

Preparation of large unilamellar vesicles (LUVs). Lipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). The "LM3" mixture represented the approximate composition of host cells of the influenza virus (Worman, H.J.; Brasitus, T.A.; Dudeja, P.K.; Fozzard, H.A.; Field, M. *Biochemistry* **1986,** *25,* 1549-1555) and contained 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3 phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS), sphingomyelin, phosphatidylinositol (PI) and cholesterol in a 10:5:2:2:1:10 mol ratio. One set of LUVs was made from LM3 (5.4 μmol total lipid + 2.7 μmol cholesterol) mixture while another set was made from LM3 mixture (0.6 μmol total lipid + 0.3 μmol cholesterol) and an additional 2 mol% of the lipid *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE) and 2 mol% of the lipid *N*-(lissamine Rhodamine B sulfonyl) phosphatidylethanolamine (*N*-Rh-PE). Lipids and cholesterol were dissolved in chloroform and the chloroform was subsequently removed with nitrogen gas and overnight vacuum pumping. A 1 ml aliquot of buffer E was added to each of the dry lipid/cholesterol mixture and the dispersion was homogenized with ten freeze-thaw cycles. LUVs were prepared by extrusion through a polycarbonate filter with

100 nm diameter pores (Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta.* **1985**, *812*, 55-65).

Lipid mixing assay. One feature of vesicle fusion is mixing of lipids between different vesicles. The NBD and Rh groups are fluorescent and quenching functionalities, respectively, so that lipid mixing between the fluorescently labeled and unlabeled LUVs will increase the average distance between fluorescent and quenching lipids and will be detected as increased fluorescence (Struck, D. K.; Hoekstra, D.; Pagano, R. E. *Biochemistry* **1981**, *20*, 4093-4099). Fluorescence was monitored in mixtures made from ~1.9 mL of either buffer F (pH 7.4) or buffer G (buffer 5.0), 50 μL of unlabeled LUV solution and 50 μL of labeled LUV solution. The resultant solutions had [total lipid] = 150 μM, [total cholesterol] = 75 μM, and [labeled LUV]/[unlabeled LUV] = 0.11. The fluorimeter (Fluoromax 2, HORIBA Jobin Yvon Inc, Edison, NJ) was set with excitation and emission wavelengths of 465 and 530 nm, respectively. For each data set, the "F_0" fluorescence of the vesicle solution was recorded and then the "F_1" fluorescence was detected after addition of an aliquot of FHA2 solution in buffer C. The typical FHA2 stock concentration was ~100 μM and the volume of buffer was adjusted in each trial so that the total solution volume $V_{FHA2} + V_{vesicles} + V_{buffer}$ = 2.0 mL. F_1 was the equilibrium fluorescence of the FHA2/LUV solution as the time for the FHA2-induced fluorescence change was shorter than the ~2 s dead time between addition of the FHA2 aliquot and sealing the fluorimeter enclosure. After measurement of F_1, a 10 μL aliquot of a 20% w/v triton solution was added to the FHA2/LUV solution. The triton solubilized the vesicles and resulted in a large average distance between the fluorescent and quenching lipids and a maximum "F_2" fluorescence. The "percent lipid mixing" was calculated using the

literature convention (Yang, J.; Gabrys, C. M.; Weliky, D. P. *Biochemistry* **2001,** *40,* 8126-8137):

$$\text{Percent lipid mixing} = 100 \times (F_1 - F_0)/(F_2 - F_0)$$

The typical variation in percent lipid mixing was ±2% as determined by comparison of different trials with the same vesicle and FHA2 stocks and concentrations. As a control, fluorescence was also recorded with addition of aliquots of buffer C which did not contain FHA2. There was very little change in fluorescence and $F_1 - F_0$ can therefore be attributed to FHA2- rather than BOG-induced lipid mixing.

Circular dichroism (CD) spectroscopy. Solutions were prepared with either buffer C or buffer D and with [FHA2] = 0.2 mg/mL ~ 10 μM. Spectra were obtained at 4 ^{o}C using a CD instrument (Chirascan, Applied Photophysics, Surrey, United Kingdom), a cuvette with 1 mm pathlength, a 260-200 nm spectral window, wavelength points separated by 0.5 nm, and 0.5 seconds signal averaging per point. Each of the spectra in Fig. 1C in the main text is the difference between the spectrum of the FHA2 sample and a background spectrum of either buffer C or buffer D. A mean residue molar ellipticity $\theta_{222\text{nm}}$ value of –33000 deg-cm^2-dmol^{-1} corresponds to 100% helicity (O'Shea, E. K.; Rutkowski, R.; Kim, P. S. *Science* **1989**, *243*, 538-542).

Solid-state nuclear magnetic resonance (SSNMR) spectroscopy. Data were obtained with a 9.4 T instrument (Varian Infinity Plus, Palo Alto, CA), a triple resonance magic angle spinning (MAS) probe, and a 4.0 mm diameter rotor with ~40 μL sample volume. Typical parameters of the rotational-echo double-resonance (REDOR) pulse sequence were: (1) 8.0 kHz MAS frequency; (2) a 6 μs ^{1}H π/2 pulse; (3) a 1.6 ms cross-polarization period with 63 kHz ^{1}H Rabi frequency and 80 kHz ^{13}C Rabi frequency; (4) a

2 ms dephasing period with alternating 19 μs ^{15}N π pulses and 8 μs ^{13}C π pulses and 88 kHz two-pulse phase modulation (TPPM) ^{1}H decoupling; (5) ^{13}C detection with 88 kHz TPPM ^{1}H decoupling; and (6) 1 sec delay. Data were acquired without (S_0) and with (S_1) the ^{15}N π pulses during the dephasing period and respectively represented the full ^{13}C signal and the ^{13}C signal minus ^{13}Cs directly bonded to ^{15}N nuclei. Because the FHA2 contained a ^{13}CO/^{15}N unique sequential pair, the $S_0 - S_1$ difference was predominantly the filtered signal of this pair (Yang, J.; Parkanzky, P. D.; Bodner, M. L.; Duskin, C. G.; and Weliky, D. P. *J. Magn. Reson.* **2002,** *159,* 101-110). Data were acquired at –50 ^{o}C because of the three-fold higher signal to noise relative to 25 ^{o}C and because chemical shifts and presumably structure did not vary greatly as a function of temperature (Bodner, M. L.; Gabrys, C. M.; Parkanzky, P. D.; Yang, J.; Duskin, C. G.; Weliky, D. P. *Magn. Reson. Chem.* **2004,** *42,* 187-194). Spectra were externally referenced to the methylene carbon of adamantane at 40.5 ppm, which corresponds to the ^{13}C referencing used in liquid-state NMR of soluble proteins (Morcombe, C. R.; Zilm, K. W. *J. Magn. Reson.* **2003,** *162,* 479-486 and Zhang, H. Y.; Neal, S.; Wishart, D. S. *J. Biomol. NMR,* **2003,** *25,* 173-195). Peak chemical shifts were determined with ± 0.3 ppm precision.