Communication

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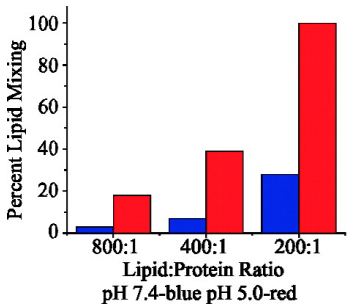

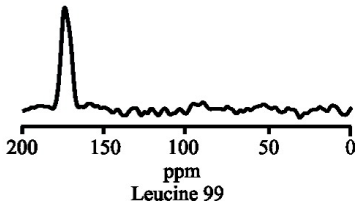

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Solid-State NMR Structural Measurements on the Membrane-Associated Influenza Fusion Protein Ectodomain

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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.

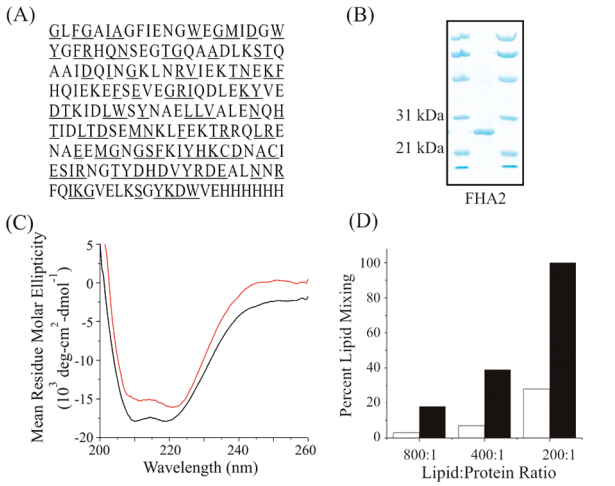

Figure 1. (A) FHA2 amino acid sequence from the influenza X31 strain. Each of the underlined residues is a first residue in a unique sequential pair. (B) SDS-PAGE gel of purified FHA2, MW = 22.5 kD. (C) Circular dichroism spectra at 4 °C of FHA2 in 0.5% BOG detergent at pH 5.0 (red line) and pH 7.4 (black 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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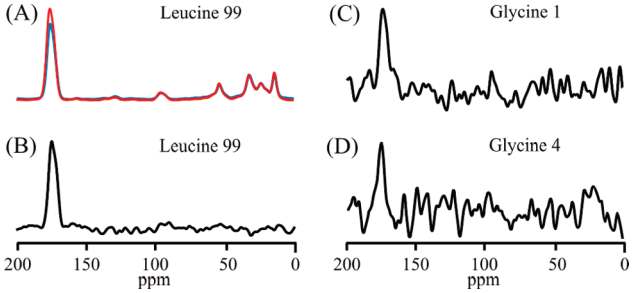

Figure 2. ^{13}C SSNMR spectra of membrane-associated FHA2. (A) REDOR S_0 (red) and S_1 (blue) spectra for a Leu-^{13}CO, Val-^{15}N sample and (B−D) S_0-S_1 spectra, respectively, representing ^{13}CO signals from the L99, G1, and G4 ^{13}COs and having peak chemical shifts of 178.0, 174.7, and 177.8 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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