

1H, 13C, 15N resonance assignments of murine hepatitis virus nonstructural protein 3a

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Abstract Nonstructural protein (nsp) 3 is the largest of 16 nsps translated from the murine hepatitis virus (MHV) genome. The N-terminal most domain of nsp3, nsp3a, has been identified by reverse genetics as a likely binding partner of MHV nucleocapsid protein. Here we report the backbone and side chain resonance assignments of MHV nsp3a (residues 1-114).

Keywords Coronavirus · Murine hepatitis virus · Nonstructural protein · SARS · Severe acute respiratory syndrome

Biological context

Coronaviruses (CoVs) are the causative agents of a number of important human and veterinary diseases including the severe acute respiratory syndrome (SARS), SARS-CoV (Zhong et al. 2003). Murine hepatitis virus (MHV) has served as an excellent model system for studying the biology of the related SARS-CoV. CoVs are highly recombinogenic and are capable of crossing species barriers and infecting “non-native” hosts. Additionally, a number of CoVs have been identified in bats, which are important reservoir hosts for zoonotic viruses (Quan et al. 2010). CoVs harbor a very large (≈30 kb), positive sense, single-stranded RNA genome. This genome serves as a template for replication, transcription (of sub-genomic mRNAs which encode structural and accessory proteins), and translation (of nsps). The nsps are translated from the first two-thirds of the genome and are then proteolytically processed into 16 mature proteins, which are components of the viral replicate. Nsp3 is the largest of all CoV nsps and is by far the best structurally characterized of any CoV nsp. In SARS-CoV, nsp3 contains seven domains denoted nsp3a through nsp3g sequentially from the N-terminus. The three-dimensional structures of all of the SARS-CoV soluble domains are now available (Johnson et al. 2010).

Recent genetic evidence points to an interaction between MHV nsp3a, the N-terminal most domain, and the nucleocapsid protein (Hurst et al. 2010). In an effort to begin to understand the nature of this interaction, we have initiated NMR structure determination of MHV nsp3a (residues 1-114). Here, we report the nearly complete sequence-specific assignments for backbone (1HN, 15N, 13Ca, 13C′) and side chain atoms of MHV nsp3a.

Methods and experiments:

Protein expression and purification

The coding sequence for MHV nsp3a (aa 1-114) was amplified from the pGEX6P-nsp3 plasmid (Dr. Paul Masters, Wadsworth Center New York State Department of Health), digested with NdeI and BamHI and ligated into pET3a expression plasmid (pns3a). The plasmid pns3a was transformed into E. coli BL21(DE3)-pLysS competent cells and plated on LB agar plates containing 100 mg/L ampicillin and 34 mg/L chloramphenicol. Uniformly 15N,13C-labeled proteins were expressed in sterile minimal media containing 1× M9 salts (6 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, pH 7.4), 2 mM MgSO4, 0.1 mM CaCl2, 0.25 mg/L thiamine hydrochloride, 100 mg/L ampicillin, 2.5 g/L 13C glucose.
and 1 g/L \textsuperscript{15}NH\textsubscript{4}Cl. A single colony from the transformation plate was used to inoculate a 200 mL M9 culture containing 100 mg/L ampicillin and grown overnight at 25 °C with shaking. The starter culture was used to inoculate 6 × 1 L LB (100 mg/L ampicillin) cultures such that the 1 L cultures would have an initial OD\textsubscript{600} of 0.1. Large cultures were grown at 37 °C with shaking until the OD\textsubscript{600} reached 0.6–0.8 at which time expression was induced with the addition of 500 μM IPTG. Cells were grown at 25 °C for an additional 16 h prior to harvesting by centrifugation at low speed. Cell pellets were frozen at −80 °C, thawed, and resuspended in 200 mL lysis buffer (25 mM Tris, 2 mM EDTA, 500 mM NaCl, 5 mM DTT, 0.1 g protease inhibitor cocktail (Sigma), pH 8.0) and lysed by sonication. The lysate was clarified by centrifugation and 0.15 % (v/v) PEI (polyethyleneimine) was added dropwise to the supernatant with stirring at 4 °C to precipitate nucleic acids. After stirring for 2 h, the solution was clarified by centrifugation. The supernatant was then subjected to (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitation (50 then 80 % saturation) and the 80 % pellet was resuspended in 30 mL of Buffer A (25 mM Tris, 2 mM EDTA, 5 mM DTT, pH 8.0) and extensively dialyzed against Buffer B (25 mM Tris, 2 mM EDTA, 100 mM NaCl, 5 mM DTT, pH 8.0). The sample was then subjected to purification using a Q-Sepharose anion exchange column (Pharmacia) equilibrated with Buffer B. The dialyzed sample was loaded onto the column and eluted with a linear gradient from 100 mM NaCl to 400 mM NaCl over 24 column volumes. Fractions containing purified nsp3a were pooled and concentrated to a volume of less than 5 mL. The sample was then subjected to size exclusion chromatography on a Superdex75 16/60 column (GE Healthcare) equilibrated with Buffer C (50 mM KP, 100 mM KCl, 5 mM TCEP, pH 6.0). The purity of all final products was estimated to be >95 % by inspection of Coomassie-stained 18 % Tris–glycine SDS–polyacrylamide gel and the integrity verified by ESI–MS.

**NMR spectroscopy**

Samples for NMR were prepared in buffer containing 50 mM KP, 100 mM KCl, 5 mM TCEP, pH 6.0 in a 10 %
\( ^2\text{H}_2\text{O}/90 \% \text{H}_2\text{O} \) mixture or in 100 \% \( ^2\text{H}_2\text{O} \) after lyophilization at protein concentrations \( \sim 200 \) \( \mu \text{M} \). All NMR experiments were acquired on a Varian DDR 600 or 800 MHz spectrometer, each equipped with either a cryogenic or room temperature probe, at the Indiana University METAcyt Biomolecular NMR Laboratory. All data were collected at 25.0 \(^\circ\)C. Proton resonances were referenced to an internal standard, 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). Nitrogen and Carbon resonances were referenced indirectly based on the proton reference frequency using the following values: \( C_0 = 0.251449530^*H_0 \), \( N_0 = 0.101329118^*H_0 \) where \( H_0 \) represents the referenced proton frequency. Data were processed using NMRPipe/nmrDraw and analyzed using Sparky (Delaglio et al. 1995; Goddard and Kneller 2008).

Backbone resonance assignments were made using three-dimensional CBCA(CO)NH, CBCANH, and HNCA experiments. Carbonyl carbon chemical shifts were obtained from a three dimensional HCBCGCDHD spectrum and a three-dimensional H(CCO)NH-TOCSY, 15N-edited TOCSY-HSQC, HCCCH-TOCSY, and HNHA. Aromatic proton resonances were assigned using a two-dimensional HNCO experiment. Backbone torsion angles \( (\phi, \psi) \) were derived from \( ^1\text{H}, ^15\text{N}, \) and \( ^13\text{C} \) chemical shifts using the program TALOS+ (Shen et al. 2009). Aliphatic side chain carbons identified using the following suite of three-dimensional experiments: \( \text{C}(\text{CO})\text{NH}-\text{TOCSY}, \text{H}(\text{CCO})\text{NH}-\text{TOCSY}, \text{15N}-\text{edited TOCSY-HSQC}, \text{HCCCH-TOCSY}, \) and HNHA. Aromatic proton resonances were assigned using a two-dimensional HBCBCGCDHD spectrum and a three-dimensional \( ^13\text{C} \)-edited NOESY-HSQC (Marion et al. 1989) focused on the aromatic region.

**Assignments and data deposition**

The \( ^1\text{H}, ^15\text{N} \) HSQC spectrum of MHV nsp3a acquired at 25.0 \(^\circ\)C and pH 6.0 is shown in Fig. 1. The spectrum is well dispersed and contains 108 resonances of which 106 were confidently assigned (98 \%). The backbone amides of D83 and M105 were not assigned. In total, greater than 92 \% of all side chain protons have been assigned. Chemical shift assignments for MHV nsp3a have been deposited in the BioMagResBank under accession ID 18587.

Based on these backbone resonance assignments, secondary structure prediction of MHV nsp3a was analyzed using chemical shift indexing (CSI) (Wishart and Sykes 1994) and TALOS+ (Shen et al. 2009) and the results are shown in Fig. 2. Both outputs are in good general agreement and collectively reveal that MHV nsp3a harbors four \( \beta \)-strands and three \( \alpha \)-helices arranged from the N-terminus as \( \beta_1-\alpha_1-\beta_2-\alpha_2-\alpha_3-\beta_3-\beta_4 \). The amide group of L27 is significantly downfield shifted and is located in a region of the molecule that is predicted as random coil by both TALOS+ and CSI. This downfield shift could be reporting residual \( \beta \)-strand character here but more likely reflects the influence of a strong ring current effect induced by the side chain of nearby F31 in the N-terminal region of the \( \alpha_1 \) helix immediately following.

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