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Backbone chemical shift assignments and secondary structure analysis of the U1 protein from the Bas-Congo virus

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Abstract

The Bas-Congo virus (BASV) is the first rhabdovirus associated with a human outbreak of acute hemorrhagic fever. The single-stranded, negative-sense RNA genome of BASV contains the five core genes present in all rhabdoviral genomes plus an additional three genes, annotated U1, U2, and U3, with weak (<21%) sequence similarity only to a handful of genes observed in a few other rhabdoviral genomes. The function of the rhabdoviral U proteins is unknown, but, they are hypothesized to play a role in viral infection or replication. To better understand this unique family of proteins, a construct containing residues 27–203 of the 216-residue U1 protein (BASV-U1*) was prepared. By collecting data in 0.5 M urea it was possible to eliminate transient association enough to enable the assignment of most of the observable ¹H^N, ¹H^α, ¹⁵N, ¹³C^α, ¹³C^β, and ¹³C' chemical shifts for BASV-U1* that will provide a foundation to study its solution properties. The analyses of these chemical shifts along with ¹⁵N-edited NOESY data enabled the identification of the elements of secondary structure present in BASV-U1*.

Keywords

Viral hemorrhagic fever; Rhabdovirus; Drug design; Chemical shift index

Biological context

Viral hemorrhagic fever is the name given to a group of potentially fatal illnesses with symptoms that include fever, vomiting, diarrhea, mucosal and gastrointestinal bleeding, hypotension, petechiae, malaise, shock, and edema (Paessler and Walker 2013). The pathogenic mechanisms for these symptoms are diverse, depending on the viral agent and the host's response. Until 2009, only four families of viruses had been associated with producing hemorrhagic symptoms in humans, *Arenaviridae*, *Bunyaviridae*, *Flaviviridae*, and *Filoviridae* (Paessler and Walker 2013), with the later family containing the Ebola virus. In 2009, through the use of deep DNA sequencing, a novel virus associated with acute hemorrhagic fever was identified from blood serum collected from three infected patients in the Democratic Republic of Congo and named the Bas-Congo Virus (BASV) (Grard et al. 2012). Two of these three patients died shortly after displaying symptoms and there was evidence the virus was spread through human-to-human contact. Phylogenetic analysis of the assembled BASV genome originally placed it into the *Rhabdoviridae* family in a deeply rooted branch distantly related to the Tibrogargan group and the *Ephemerovirus* genus (Grard et al. 2012). Today the Tibrogargan group viruses and BASV are grouped together into the *Tibrovirus* genus (Stremlau et al. 2015). Before the identification of BASV the only *Rhabdoviridae* genera known to contain human pathogens were the *Vesiculovirus*, a genus containing the Chandipura virus that is known to cause acute encephalitic syndrome and other viruses that cause vesicular stomatitis, and *Lyssavirus*, a genus that contains the rabies virus (Kuzmin et al. 2009).

Rhabdoviruses are bullet-shaped, enveloped viruses with a single-stranded, negative-sense RNA genome that generally encode for five core proteins as shown in Fig. 1. An unusual feature of the BASV genome is that it encodes for three additional proteins of unknown function, U1, U2, and U3. It has been postulated that these U proteins play accessory roles during infection although supporting functional data is not available (Gubala et al. 2011). The primary sequences for the three U proteins appear to be unique, as all three are only observed in the *Tibrovirus* genus with less than 21% sequence identity between common family members (Grard et al. 2012). Consequently, the structures of the BASV U proteins also promise to be unique and may provide valuable insight into the biological function of these proteins. Towards fulfilling this goal a 176-residue construct for BASV-U1 was prepared with the N-terminal 26 and C-terminal 13 residues removed (BASV-U1*). Solution conditions were eventually optimized enough to enable the assignment of the majority of the backbone $^1\text{H}^{\text{N}}$, $^1\text{H}^{\alpha}$, ^{15}N , $^{13}\text{C}^{\alpha}$, and $^{13}\text{C}'$ chemical shifts along with many of the $^{13}\text{C}^{\beta}$ chemical shifts. Complete assignment of all the backbone and side chain chemical shifts was hindered by transient association, even in the presence of 500 mM urea, that effected magnetization transfer in three-dimensional NMR experiments with a carbon-13 dimension and made the available assignments challenging. The analysis of these chemical shift assignments made it possible to predict the secondary structure for BASV-U1 from the chemical shift index (Hafsa et al. 2015) and lay the foundation for future structural, dynamic, and biochemical studies of this hemorrhagic virus (Borio et al. 2002).

Methods and experiments

Cloning, expression, and purification

Analysis of the full length BASV-U1 amino acid sequence (Genebank Accession Number [JX297815.1](https://www.ncbi.nlm.nih.gov/nuclot/JX297815.1)) using the JPred4 server (<http://www.compbio.dundee.ac.uk/www-jpred/>) (Drozdetskiy et al. 2015) suggested regions near the N- and C-termini were unstructured, and therefore, 26 and 13 residues, respectively, were removed when preparing the synthetic gene flanked by BamHI and HindIII restriction sites (GeneArt). A TGAT sequence was inserted before the HindIII site to generate a double stop and the gene codon optimized with GeneComposer™ to eliminate rare *Escherichia coli* codons, balance the %GC content, and eliminate any serendipitous BamHI and HindIII restriction sites and cryptic Shine Dalgarno sequences. This gene was sub-cloned from its shuttle vector into the BamHI and HindIII restriction sites of the expression vector pEMB32, a modified version of pET28a containing a 6xHistidine-Smt3 tag fused in-frame to the N-terminus of the inserted gene. Following full sequence verification of the open reading frame the construct was then transformed into *E. coli* BL21(DE3) cells. Uniformly ^{15}N -, ^{13}C -labeled BUSV-U1* was obtained by growing the transformed cells (310 K) in 750 mL of minimal medium containing $^{15}\text{NH}_4\text{Cl}$ (1 mg/mL) and D- $^{13}\text{C}_6$ glucose (2.0 mg/mL), NaCl (50 $\mu\text{g/mL}$), MgSO_4 (120 $\mu\text{g/mL}$), CaCl_2 (11 $\mu\text{g/mL}$), FeCl_3 (10 ng/mL) and the antibiotic kanamycin (34 $\mu\text{g/mL}$). When the cell culture reached an OD_{600} reading between 0.8 and 0.9, protein expression was induced with isopropyl β -D-1-thiogalactopyranoside (0.026 $\mu\text{g/mL}$) and the culture transferred to a 298 K incubator. Cells were harvested approximately four to six hours later by mild centrifugation and then stored at 193 K. Triple labeled (^2H , ^{15}N , ^{13}C) samples that were either “fully” or ~50% deuterated were prepared similarly by preparing the medium in either 98% or 50% D_2O , respectively, as previously described (Buchko et al. 2015). Upon thawing the frozen pellet, the cells were resuspended in 32 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0), brought to 0.2 μM in phenylmethylsulfonyl fluoride, and run three times through a French press (SLM Instruments, Rochester, NY). Insoluble cell debris was removed by centrifugation for 60 min at $24,000\times g$ in a JA-20 rotor (Beckman Instruments, Fullerton, CA) with the resulting supernatant filtered through a 0.45 mm pore-size membrane (Corning Incorporated, Corning, NY) and applied onto an ~20 mL Ni-nitrotriacetic acid (NTA) affinity column (QIAGEN, Valencia, CA). The NTA column was washed step-wise (40, 40, 40, 20, and 45 mL) with buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0) containing increasing concentrations of imidazole (5, 10, 20, 50, and 300 mM). The 300 mM imidazole fraction containing BUSV-U1* was dialyzed overnight at 278 K in 4 l of Ulp1 digestion buffer (25 mM Tris-HCl, 200 mM NaCl, 0.5 mM DTT, pH 8.0). The following morning 2 μg of Ulp1 was added per mg of BASV-U1*, incubated for ~12 h at 279 K, and then concentrated to ~10 mg/mL (Amicon Centriprep-10). One mL aliquots of the digestion mixture was then loaded onto a Superdex75 gel-filtration (HiLoad 16/50) column pre-equilibrated with 100 mM NaCl, 20 mM Tris-HCl, pH 7.0 (flow rate = 1 mL/min) to separate the major components: residual uncleaved protein (54 min), BASV-U1* (60 min), and Smt3 (67 min). The fractions containing BASV-U1* were made 500 mM in urea using a 5 $\times$ stock solution in the same buffer and then concentrated to approximately 5–14 mg/mL for NMR studies.

Dynamic light scattering (DLS)

The DLS measurements were obtained at 298 K on an approximately 0.5 mM sample of BASV-U1* in 100 mM NaCl, 20 mM Tris-HCl, pH 7.0, using a ZetaPALS instrument (Brookhaven Instruments, Holtsville, NY) equipped with a 657 nm 35 mW laser. Data were collected in triplicate sets of 10 runs on a 200 μ L sample passed through a 0.2 mm filter using a 90° scattering angle and an acquisition time of 2 min per run. To obtain size distributions, the autocorrelation functions were deconvoluted using the non-negatively constrained least-squares fit (NNLS) algorithm.

Nuclear magnetic resonance spectroscopy

Four-channel Varian spectrometers operating at proton resonance frequencies between 500 and 800 MHz were used to collect NMR data at 293 K on three different samples of double-labeled (^{13}C , ^{15}N) BASV-U1* that were either fully protonated, ~50% deuterated, or ~90% deuterated. The assignment of the $^1\text{H}^{\text{N}}$, $^1\text{H}^{\alpha}$, $^{13}\text{C}'$, $^{13}\text{C}^{\alpha}$, and ^{15}N backbone resonances and the $^{13}\text{C}\beta$ side chain resonances were made from a standard two-dimensional ^1H - ^{15}N HSQC experiment and three-dimensional HNCA, HNCOCACB, HNCACB, CC-TOCSY-NNH, and ^{15}N -edited NOESY experiments using the Varian Biopack suite of pulse programs. The ^{15}N -edited NOESY experiment employed a 90 ms mixing time and the HNCOCACB and HNCACB experiments (using the ~90% deuterated sample) employed a tauCC value of 7 ms to optimize for the detection of β -carbons. An overall rotational correlation time (τ_c) for BASV-U1* was estimated from backbone amide $T_1/T_{1\rho}$ ratios using a modified ^1H - ^{15}N HSQC experiment. Amides involved in α -helical or β -strand hydrogen bond networks were identified by lyophilizing a ^{15}N -labeled NMR sample, re-dissolving in 99.8% D_2O , and quickly collecting a ^1H - ^{15}N HSQC spectrum (deuterium exchange experiment). The NMR data was processed using Felix2007 (MSI, San Diego, CA) and analyzed using Sparky (v3.115). Indirect methods (DSS=0 ppm) were used to reference the ^1H , ^{13}C , and ^{15}N chemical shifts that were deposited into the Biological Magnetic Resonance Bank database (<http://www.bmrb.wisc.edu>) under the BMRB accession number 26,858. Any $^{13}\text{C}\beta$ chemical shifts obtained from data collected on the ~90% deuterated sample were corrected for the deuterium isotope effect (Gardner et al. 1997).

Assignments and data depositions

The elution time of BASV-U1* from a Superdex75 gel filtration column (60 min), the isotropic overall rotational correlation times (τ_c) inferred from ^{15}N spin relaxation times (11.5 ± 0.5 ns at 293 K), and the diameter estimated from dynamic light scattering (3.7 ± 0.2 nm) were values all consistent for a monomeric, ~19.3 kDa protein under our initial conditions (100 mM NaCl, 20 mM Tris-HCl, 1 mM DTT, pH 7). Unfortunately, while the initial ^1H - ^{15}N HSQC spectrum of an ~1 mM ^{15}N -, ^{13}C -labeled sample at 293 K showed evidence for a folded protein, dispersed amide cross peaks in both the proton and nitrogen dimensions, it also harbored signs of transient association, a subset of broad amide cross peaks with variations in size and shape (Yee et al. 2002). The spectral properties were improved by collecting data at 308 K, however, BASV-U1* was unstable and precipitated out of solution over 24–48 h at this temperature and concentration. Upon

extensive experimentation with the solution conditions it was observed that the ^1H - ^{15}N HSQC spectrum for BASV-U1* was improved most in the presence of 500 mM urea (Chae et al. 2004) where the amide cross peaks became more uniform and slightly sharper. Except for the appearance of a handful of additional resonances, the ^1H - ^{15}N HSQC spectrum in the presence of 500 mM urea superimposed well on the ^1H - ^{15}N HSQC spectrum in the absence of urea suggesting the denaturant did not significantly alter the structure of BASV-U1*. Even under these conditions, there was still poor magnetization transfer in some of the three-dimensional NMR experiments and it was necessary to use a fully deuterated sample to complete the $^{13}\text{C}\beta$ assignments.

As shown in Fig. 2a, 119 out of the 165 expected backbone amide resonances for BASV-U1* [178—(12 Pro + S26)] are assigned (72%) to cross peaks in the ^1H - ^{15}N HSQC spectrum. While there are 46 unassigned residues, the orange circles in Fig. 2a show that there are only 19 remaining amide cross peaks not unambiguously assigned in the ^1H - ^{15}N HSQC spectrum for BASV-U1* primarily due to poor magnetization transfer to carbon resonances in the three-dimensional backbone assignment experiments. This leaves 27 amide cross peaks that are “missing” in the ^1H - ^{15}N HSQC spectrum.

The difference in the $^1\text{H}\alpha$, $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ chemical shifts relative to values adopted by random coils is called the Chemical Shift Index (CSI) and can be used to identify elements of secondary structure in proteins (Hafsa et al. 2015). The assigned $^1\text{H}^{\text{N}}$, $^1\text{H}\alpha$, ^{15}N , $^{13}\text{C}^{\text{N}}$, $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ chemical shifts for BASV-U1* were submitted to the CSI 3.0 web server (<http://csi3.wishartlab.com>) and the results are summarized on the primary amino acid sequence of BASV-U1* in Fig. 2b. Six β -strands ($\beta 1 = \text{C36-S43}$; $\beta 2 = \text{N104-T108}$; $\beta 3 = \text{L126-P130}$; $\beta 4 = \text{S143-T150}$; $\beta 5 = \text{K186-Y188}$; $\beta 6 = \text{A194-R196}$) and three α -helices ($\alpha 1 = \text{A53-L64}$; $\alpha 2 = \text{I84-R92}$; $\alpha 3 = \text{F172-L182}$) were predicted. Analysis of the ^{15}N -edited NOESY data supported the presence of the α -helices and β -strands one through four generally in the regions predicted by CSI 3.0 while the analysis was inconclusive for the presence of $\beta 5$ and $\beta 6$. Long range amide to amide and amide to $^1\text{H}\alpha$ NOEs were observed for the residues under- and overlined by solid cyan bars in Fig. 2b. The pattern of NOEs identified the registration of the first four β -strands into one anti-parallel β -sheet, $\beta 2$: $\beta 1$: $\beta 4$: $\beta 3$. The C-terminal β -strands identified by CSI 3.0, $\beta 5$ and $\beta 6$, are separated by only five residues, and hence, likely also form a small anti-parallel β -sheet although it is not known if it integrates with the major β -sheet.

Forty-six amides are unassigned in BASV-U1* due to poor magnetization transfer in 3D NMR experiments with 27 of the amides “missing” resonances. The missing and unassigned amide residues are mapped in dark blue on the primary amino acid sequence of BASV-U1* in Fig. 2b. The majority of these residues are clustered in three regions, between $\alpha 1$ and $\alpha 2$, $\beta 2$ and $\beta 3$, and $\beta 4$ and $\alpha 3$, in regions that could be unstructured loops undergoing motion or chemical exchange in the intermediate timescale (ms to μs) between structured elements (e.g. Buchko et al. 2015). The other unassigned or missing residues are near the N-terminal, which may be flexible, and two glycine residues, which may be in a tight turn between $\alpha 3$ and $\beta 5$. Indirect evidence that the unassigned resonances in Fig. 2a are in unstructured regions of the protein was their rapid disappearance in a deuterium exchange experiment (data not shown). Analysis of the primary amino acid sequence with the secondary structure

prediction server JPred4 (Drozdetskiy et al. 2015) predicts that most of these 46 unassigned residues are between elements of canonical secondary structure.

The three U proteins encoded in the Bas-Congo virus genome are the major difference between this potentially lethal virus that causes hemorrhagic fever and the genomes of closely related rhabdoviruses. Of these three U proteins, the U1 primary amino acid sequence is most unlike any other known protein sequence, suggesting the BASV-U1 structure may also be unique. Analysis of the chemical shifts assignments and ^{15}N -edited NOESY data for BASV-U1* suggests this protein contains one four-strand anti-parallel β -sheet, one small two-strand anti-parallel β -sheet, and three α -helices. Also identified are a large number of unassigned or missing amide resonances that are clustered primarily into three regions between individual elements of secondary structures. These may be unstructured loops undergoing motion or chemical exchange in the intermediate timescale (ms to μs) (Buchko et al. 2016) with important biochemical properties because such dynamics are often associated with binding and catalysis (e.g. Müller et al. 1992). Given that the U proteins have been postulated to play accessory roles during infection (Grard et al. 2012), these dynamic regions may be the areas of BASV-U1 responsible for interacting with host cell proteins. Alternatively, missing amide resonances have also been identified at dimer interfaces (Buchko et al. 2015). Given that the NMR data for BASV-U1* was acquired in 500 mM urea to limit transient association, some of the unassigned and missing amide resonances may represent regions of the protein at the transient interface. Note that transient interactions may still be occurring even in the presence of 500 mM urea. Evidence for this is the poor magnetization transfer still observed with 90% perdeuterated BASV-U1* samples that made the assignments reported here challenging (and incomplete), but also, the observation that three-dimensional ^{13}C -edited NOESY spectra contained few NOEs. While efforts are in progress to identify conditions that will enable the collection of data to determine how the individual elements of secondary structure in BASV-U1* fold, the chemical shift assignments presented here lay the foundation for solving its 3D structure and for future dynamic and biochemical studies of a protein that may play a vital role in the pathology of the Bas-Congo virus.

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Fig. 1.

Schematic comparison of the genome organization of a typical rhabdovirus and BASV. The genome of BASV contains three proteins, U1, U2, and U3, which are approximately 11, 18, and 21% identical, respectively, to corresponding proteins in the two Tibrogargan group viruses. N, 3'-nucleoprotein; P, phosphoprotein; M, matrix-protein; G, glycoprotein; and L, RNA-dependent RNA polymerase

Fig. 2.

a A ^1H - ^{15}N HSQC spectrum of ~50% deuterated, ^{13}C - and ^{15}N -labeled BASV-U1* (~0.8 mM) collected at a proton resonance frequency of 750 MHz, 293 K, in 100 mM NaCl, 500 mM urea, 20 mM Tris, 1 mM DTT, pH 7.0, with the assigned amide cross peaks labeled. An “x” marks cross peaks that are below the contour limit and resonances that are folded into the spectrum are labeled in *black*. Unassigned cross peaks are *circled orange* and amide side chain resonance pairs are connected by a *red dashed line*, **b** Primary amino acid sequence of BASV-U1* highlighting the α -helices (red) and β -strands (cyan) identified by CSI 3.0 analysis of the NMR chemical shifts. The *solid cyan lines* identify residues for which long

range amide-to-amide and amide-to- $^1\text{H}\alpha$ NOEs were identified in a ^{15}N -edited NOESY experiment. Residues with missing or unassigned amide cross peaks are colored *dark blue*

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