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Letter to the Editor: Complete ^{1}H, ^{13}C and ^{15}N backbone assignments for the hepatitis A virus 3C protease

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Biological context

Hepatitis A is the most widespread form of acute hepatitis in the world. The disease, which is characterized by jaundice and debilitating flu-like symptoms, is caused by the hepatitis A virus (HAV). HAV is a small, RNA-containing virus belonging to the picornaviral family. As with all members of this family, HAV matures through proteolytic self-cleavage of its virally encoded polyprotein. This cleavage is carried out by a unique viral protein called the HAV3C protease. Because of its importance in viral maturation, the HAV3C protease has long been considered an attractive target for drug development (Malcolm et al., 1992). X-ray studies of the HAV3C protease have revealed that this 217-residue cysteine protease has a trypsin-like fold with a long shallow groove to accommodate a range of peptide substrates (Allaire et al., 1994). Efforts aimed at co-crystallizing the HAV3C protease with various inhibitors have, so far, met with limited success (Bergmann et al., 1999). To complement these X-ray studies we have undertaken the task of completing the backbone NMR assignments of this protein.

Methods and experiments

HAV3C protease was expressed in *E. coli* MM294 cells containing the pHAV-3CEX plasmid (Malcolm et al., 1992). ^{15}N, ^{13}C/^{15}N and ^{2}H/^{13}C/^{15}N uniformly labeled protein samples were prepared by growing cells on M9 minimal media containing $^{15}NH_4Cl$, ^{13}C glucose and/or 100% D_2O. Selectively ^{15}N labeled HAV3C protease samples were also prepared by growing either prototrophic MM294 cells or auxotrophic CT16 cells (Waugh, 1996) on a defined medium containing the desired ^{15}N amino acid. In this way, selective ^{15}N labeled leucine, isoleucine, lysine, alanine, glycine and phenylalanine HAV3C protease samples were obtained.

The HAV3C protein was expressed by growing the host cells to mid-log phase, followed by induction with isopropyl-β-D-thiogalactopyranoside (0.2%) for 5 h (30 °C). The cells were subsequently harvested, frozen and then lysed via multiple cycles of freezing and thawing. The supernatant was loaded onto a 2.5 × 30 cm CM-sepharose ion exchange column pre-equilibrated with phosphate buffer (pH 5.4). HAV-3C protease was eluted from the column using a 0–500 mM NaCl salt gradient. The protein yields using this protocol typically approached 60 mg/L.

For collection of NMR spectra, protein samples were concentrated to ∼1 mM *via* ultrafiltration. Extensive testing of solution conditions revealed that HAV3C protease was most stable in aqueous 100 mM NaCl, pH 5.4. Subsequently, all protein samples were prepared and collected under these conditions. A small amount of DSS (0.1 mM) was added for internal referencing. NMR spectra were collected at 25 °C on Varian 500 MHz and 600 MHz spectrometers fitted with 5 mm triple-resonance, z-gradient PFG probes or a Varian 800 MHz spectrometer fitted with a 5 mm triple-axis gradient, triple-resonance PFG probe. ^{15}N HSQC and ^{15}N NOESY-HSQC spectra were collected on both the uniformly ^{15}N labeled (Figure 1) and selectively ^{15}N labeled samples. Ad-

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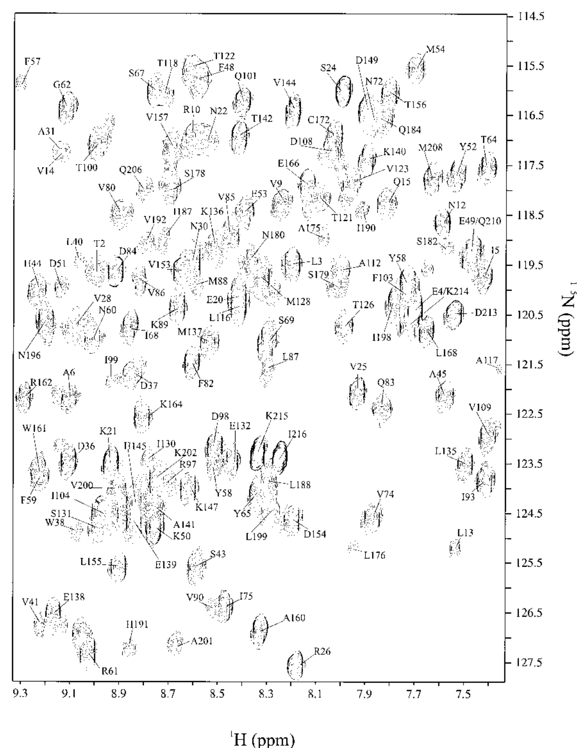

Figure 1. A portion of the ^{15}N-HSQC spectrum of HAV-3C illustrating a number of the assigned backbone ^{15}N resonances.

ditionally, HNCO, HN(CA)CO, HNCA, HN(CO)CA, HN(CA)CB, and HNHA spectra were collected on the uniformly $^{13}C/^{15}N$ and $^{2}H/^{13}C/^{15}N$ labeled samples. All experiments were implemented using pulse sequences provided by Dr. Lewis Kay (Shan et al., 1996) or those supplied in the Varian Protein Pack. Spectra were processed and analyzed using NMRpipe and NMRdraw (Delaglio et al., 1995) and referenced to DSS using indirect methods (Wishart et al., 1995). Note that because a deuterated protein sample was primarily used in the assignment process, the reported ^{13}Cα, ^{13}Cβ and ^{15}N resonances are shifted upfield (by 0.43 ppm, 0.82 ppm and 0.23 ppm, respectively) relative to those expected for a fully protonated sample (Gardner et al., 1997).

Standard heteronuclear assignment techniques were employed to complete the backbone assignments of HAV-3C protease (Fairbrother et al., 1997). Spectra for the residue-specific ^{15}N labeled samples provided a means of confirming or validating a number of tentative assignments. Additional confirmation of assignments was obtained by comparing all observed chemical shifts with calculated chemical shifts derived from the 1.9 Å X-ray structure (1QA7) using an in-house program (Wishart and Nip, 1998).

Extent of assignments and data deposition

The use of deuterated samples and deuterium modified 3D NMR experiments were crucial to facilitating the backbone assignments for this protein. The use of selectively labeled protein samples also helped clarify and confirm a number of tentative assignments. In total, 95% of all ^{1}Hα and ^{1}HN, 98% of all ^{13}Cα, ^{13}Cβ and ^{13}CO as well as 98% of all ^{15}N assignments were obtained. Complete backbone assignments for W28, E56, and Q181 were not obtainable. However, all residues involved in the protease active site were completely assigned. The backbone shifts have been deposited in the BioMagResBank under accession number 4836.

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