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# $^{1}H$, $^{13}C$, $^{15}N$ backbone and side chain resonance assignment of the HNH nuclease from *Streptococcus pyogenes* CRISPR-Cas9

**Helen B. Belato**[#1], **Kyle W. East**[#2], **George P. Lisi**[1,2]

[1]Graduate Program in Molecular Pharmacology and Physiology, Brown University, Providence, RI 02903, USA

[2]Department of Molecular Biology, Cellular Biology, and Biochemistry, Brown University, Providence, RI 02903, USA

[#] These authors contributed equally to this work.

## Abstract

HNH is one of two endonuclease domains of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein Cas9 that perform site-specific cleavage of double-stranded DNA. We engineered a novel construct of this critical nuclease from *Streptococcus pyogenes* Cas9 that not only maintains the wild-type amino acid sequence and fold, but displays enhanced thermostability when compared to the full-length Cas9 enzyme. Here, we report backbone and side chain assignments of the HNH nuclease as a foundational step toward the characterization of protein dynamics and allostery in CRISPR-Cas9.

### Keywords

CRISPR; Cas9; HNH nuclease; NMR assignments

## Biological context

The CRISPR-associated protein 9 (Cas9) system provides an exciting tool for precision editing (Charpentier and Doudna 2013; Charpentier and Marraffini 2014; Wang et al. 2016) and regulation (Dominguez et al. 2016) of genomic DNA with possible applications to the treatment of heritable human diseases (Maeder and Gersbach 2016; Strong and Musunuru 2017; Xiong et al. 2016) and cancer (Martinez-Lage et al. 2018; Tian et al. 2019). The Cas9 endonuclease system is comprised of a large (1368 residue) multidomain and multifunctional polypeptide chain and either two RNA chains (Jiang and Doudna 2017), tracrRNA and crRNA (Deltcheva et al. 2011; Jinek et al. 2012), or a combined RNA oligonucleotide, referred to as single-guide RNA. The Cas9 polypeptide chain houses two endonuclease domains, HNH and RuvC; a large alpha helical recognition domain (REC), and a Protospacer Adjacent Motif (PAM) interacting (PI) domain (Jiang and Doudna 2017).

George P. Lisi, george_lisi@brown.edu.

**Conflict of interest** The authors declare no conflict of interest.

The power of Cas9 lies in the elegant simplicity of its targeting mechanism. Cas9 binds to a target 20-nucleotide DNA sequence by creating a DNA–RNA hybrid with the crRNA. However, Cas endonucleases are inactive without the recognition of a unique PAM sequence directly downstream of the DNA–RNA duplex (Jiang and Doudna 2017). The allosteric network that transmits the signal of PAM recognition to the endonucleases is not well understood, but to better control the Cas9 machinery for use in the treatment of human disease, knowledge of this allosteric network is vital. Molecular dynamics simulations have suggested the presence of an allosteric pathway connecting the PI domain to the alpha helical REC lobe and HNH domain (Oakes et al. 2016; Palermo et al. 2017). Signaling between the HNH and RuvC nucleases is required for concerted cleavage of double-stranded DNA, but the protein motions responsible for propagating this information have not been identified. NMR is a valuable tool in this endeavor, and the utility of NMR in studies of dynamic allostery is well documented (Lisi and Loria 2016a, b).

At over 160 kDa, the full-length Cas9 protein presents an extremely difficult target to study by solution NMR, especially considering its low thermostability that precludes the use of elevated temperatures to enhance spectral quality of large proteins (Arbogast et al. 2015). Thus, we have engineered a construct of the critical HNH nuclease (15.4 kDa), and report NMR resonance assignments for its amide backbone ($H^N$, $N^H$, $C_\alpha$, $C_\beta$, $C_O$) and aliphatic side chains. These assignments, along with those of the Met-Ile-Leu-Val-Ala-Thr methyl groups, provide a basis for the study of the structure and dynamic network within HNH, which will be expanded on a “per domain” basis to map the entire Cas9 allosteric pathway by NMR.

## Methods and experiments

### Sample preparation

A codon-optimized sequence for the HNH domain (residues 775–909) from *S. pyogenes* Cas9 was inserted into the pET28a vector with an N-terminal $His_6$-tag and a TEV protease cleavage site. The plasmid was transformed into BL21(DE3) cells (Novagen). Labeled samples were grown in M9 minimal media supplemented with $^{15}N$ ammonium chloride (1.0 g/L) and $^{13}C$ glucose (2.0 g/L; Cambridge Isotope Labs). Cells were grown at 37 °C until reaching an $OD_{600}$ of 0.8–0.9 and were subsequently induced with 0.5 mM IPTG. HNH was expressed for 18 h at 20 °C. Cells were collected by centrifugation at 5500 rpm for 25 min and resuspended in a buffer of 20 mM HEPES, 300 mM KCl and 5 mM imidazole at pH 8.0. The resuspended cells were lysed by sonication and the lysate was centrifuged at 15,000 rpm for 45 min to remove cellular debris. The $His_6$-tagged HNH domain was purified from the supernatant by Ni–NTA affinity chromatography and eluted with the same buffer containing 220 mM imidazole. The eluent was dialyzed against a buffer of 20 mM HEPES and 80 mM KCl at pH 7.4. The N-terminal $His_6$-tag was cleaved following incubation of HNH with TEV protease at room temperature for 4 h. The tag and TEV protease were removed by Ni–NTA chromatography and HNH was dialyzed against a buffer of 20 mM HEPES, 80 mM KCl, 1 mM EDTA, 1 mM DTT, and 7% $^2H_2O$ at pH 7.4. Even with no changes to its amino acid sequence, the HNH domain has a thermal unfolding temperature that is nearly 20 °C

higher than that of full-length Cas9, and is stable at concentrations of 1.2–1.7 mM for NMR experiments.

### NMR experiments

NMR data for backbone assignment were collected on a Varian Inova 600 MHz spectrometer at 25 °C equipped with pulsed field gradients and a triple resonance probe. The backbone assignments were completed using the following TROSY triple resonance experiments (Pervushin et al. 1997): $^{1}H–^{15}N$ HSQC, HNCA, HN(CO)CA, HN(CA)CB, HN(COCA)CB, HN(CA)CO, and HNCO (Salzmann et al. 1998). NMR data for side chain assignment were collected on a Bruker Avance III 600 MHz spectrometer at 25 °C equipped with pulsed field gradients and a triple resonance cryo-probe. Side chain assignments were completed using the following experiments: $^{1}H–^{13}C$ ctHSQC, $^{15}N$-edited TOCSY (Marion et al. 1989), (H)CCH-TOCSY, HC(C) H-TOCSY (Bax et al. 1990), and HA(CACO)NH (Feng et al. 1996). All spectra were processed in NMRPipe (Delaglio et al. 1995) and analyzed in Sparky (Lee et al. 2015). Three-dimensional correlations and assignments were made in CARA (Keller 2005).

## Assignment and data deposition

The assigned $^{1}H–^{15}N$ TROSY HSQC spectrum for HNH is shown in Fig. 1. 80.7% of the backbone $^{1}H–^{15}N$ resonances have been assigned including 80.7% of $C_{\alpha}$, 77.0% of $C_{\beta}$, and 77.0% of $C_{O}$. Since future work is geared toward the creation of larger Cas9 constructs, we also assigned the aliphatic side chains and Met-Ile-Leu-Val-Ala-Thr methyl resonances, which are useful probes in NMR spectra of proteins > 40 kDa (Ollerenshaw et al. 2003). The assigned methyl region of the $^{1}H–^{13}C$ ctHSQC spectrum for HNH is shown in Fig. 2. 84.8% of the aliphatic sidechain $^{1}H–^{13}C$ resonances have been assigned including 96.8% of Met-Ile-Leu-Val-Ala-Thr methyl resonances. A list of the $^{1}H$, $^{13}C$, and $^{15}N$ chemical shifts has been deposited into the BioMagResBank under accession number 27949.

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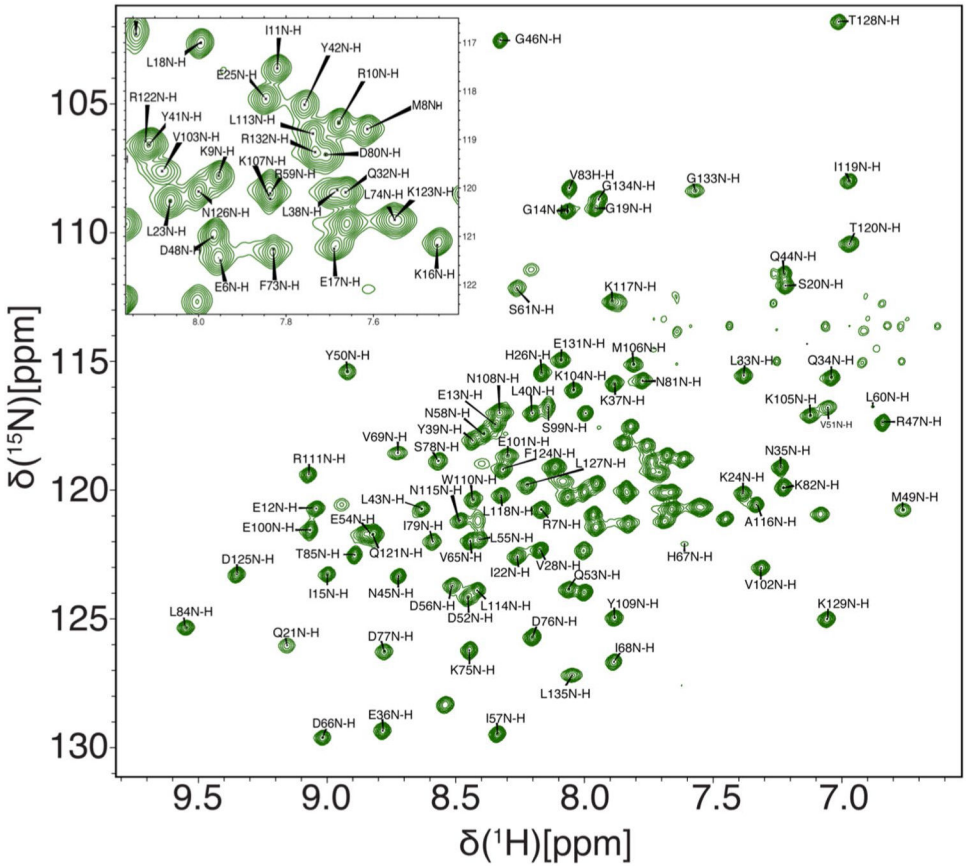


**Fig. 1.**

$^{1}H–^{15}N$ TROSY HSQC spectrum of the HNH domain from *S. pyogenes* Cas9 collected at 25 °C and 600 MHz. The inset shows assigned residues within the crowded center of the spectrum

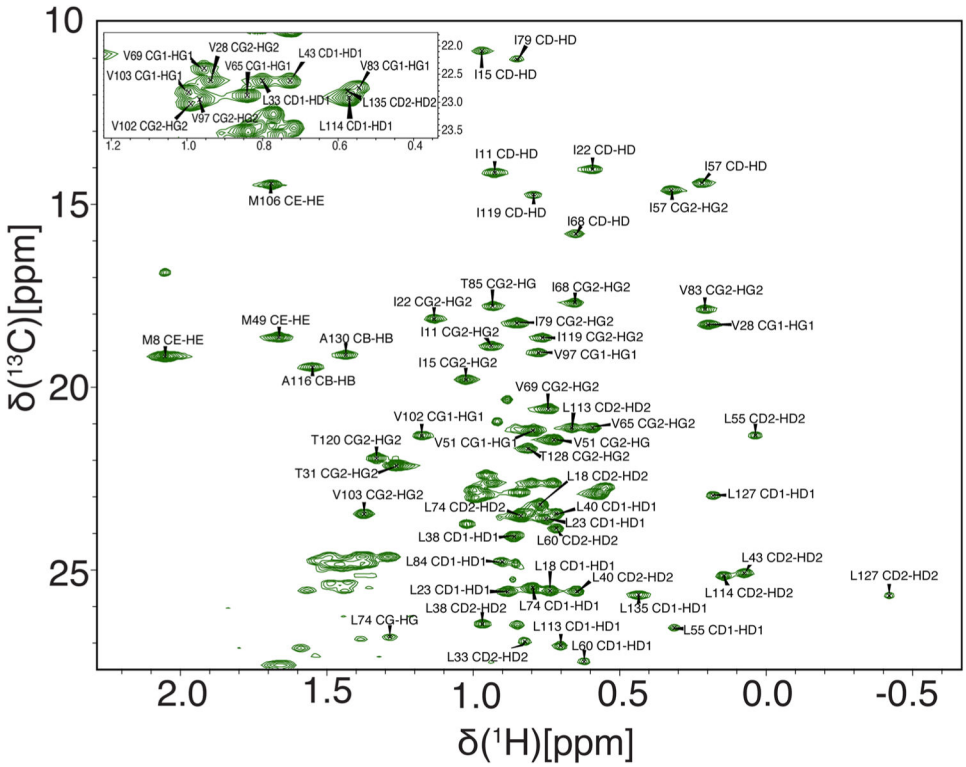


**Fig. 2.**

$^{1}H–^{13}C$ ctHSQC spectrum of HNH collected at 25 °C and 600 MHz. The spectrum depicts the methyl resonances of Met-Ile-Leu-Val-Ala-Thr residues, with the crowded central region of the spectrum shown as an inset