Published in final edited form as:

Biomol NMR Assign. 2019 April ; 13(1): 63–66. doi:10.1007/s12104-018-9852-1.

^1H , ^{13}C , and ^{15}N resonance assignments of *N*-acetylmuramyl-L-alanine amidase (AmiC) N-terminal domain (NTD) from *Neisseria gonorrhoeae*

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Abstract

Gonorrhea infections are becoming more difficult to treat due to the prevalence of strains exhibiting resistance to antibiotics and new therapeutic approaches are needed. *N*-acetylmuramyl-L-alanine amidase (AmiC) from *Neisseria gonorrhoeae* is a hydrolase that functions during cell division by cleaving the bond between the *N*-acetylmuramyl and L-alanine moieties of peptidoglycan. Inhibiting this enzyme offers the prospect of restoring the efficacy of existing antibiotics as treatments against *N. gonorrhoeae*. Of its two domains, the C-terminal domain (CTD) catalyses the hydrolysis reaction and the N-terminal domain (NTD) is believed to target AmiC to its peptidoglycan substrate. Here, we report the ^1H , ^{13}C , and ^{15}N resonance assignments of a 131 amino acid NTD construct of AmiC by heteronuclear NMR spectroscopy. The assignments represent the first for *N. gonorrhoeae* AmiC-NTD, laying the groundwork for detailed examination of its structure and dynamics, and providing a platform for new drug discovery efforts to address antimicrobial-resistant *N. gonorrhoeae*.

Keywords

Neisseria gonorrhoeae; amidase; peptidoglycan metabolism; antibiotic resistance; drug target

Biological Context

Neisseria gonorrhoeae is an obligate human pathogen that causes the sexually transmitted infection gonorrhea. The Centers for Disease Control and Prevention (CDC) estimate 820,000 new cases of gonorrhea occur in the United States per year (Satterwhite et al. 2013). Untreated infections can result in a number of complications, including pelvic inflammatory disease (PID) and ectopic pregnancies in females, and gonococcal arthritis and an increased risk of both contracting and transmitting HIV in both sexes (Piszczyk et al. 2015). Unfortunately, gonorrhea infections have become increasingly harder to treat due to the prevalence of *N. gonorrhoeae* strains exhibiting resistance to antibiotics, including extended-

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Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethical standards The experiments performed comply with the applicable laws of the United States of America and the State of South Carolina

spectrum cephalosporins (ESCs), and development of new anticonococcal agents is therefore needed.

One potential target for new antimicrobial therapeutics is the peptidoglycan hydrolase *N*-acetylmuramyl-L-alanine amidase (AmiC). This enzyme cleaves the peptide moiety of peptidoglycan from the *N*-acetylmuramic acid of the glycan chain. Peptidoglycan hydrolases (or autolysins) are enzymes that break down peptidoglycan, with roles in expansion of the murein sacculus during cell growth and septum formation during cell division (Holtje 1998; Vollmer et al. 2008). Inhibition of autolysins can lower MICs for antibiotics and thus offers a relatively facile route to restore the efficacy of existing β -lactams in the face of growing resistance. Among the five amidases identified in *Escherichia coli*, AmiC has been identified as the primary cell division amidase (Heidrich et al. 2001). By contrast, *N. gonorrhoeae* encodes only a single amidase, AmiC. Although not essential for cell growth, genetic deletion of AmiC impairs the separation of daughter cells (Garcia and Dillard 2006).

The crystal structure of *E. coli* AmiC has been solved (Rocaboy et al. 2013). The protein comprises two domains: a C-terminal domain (CTD) that catalyzes the amidase activity and an N-terminal domain (NTD) that binds peptidoglycan and may act as a targeting domain. Sequence analysis indicates that *N. gonorrhoeae* AmiC has the same overall structure and its domains probably exhibit similar functions. Understanding how *N. gonorrhoeae* AmiC-NTD binds to peptidoglycan at the septum could offer new avenues for development of antimicrobials. By reporting the NMR assignments of an NTD construct of AmiC (residues 29–159), we take the first step toward that goal.

Methods and Experiments

Sample Preparation

DNA corresponding to residues 29–159 of *N. gonorrhoeae* AmiC was cloned in-frame behind an N-terminal His₆-GB1 fusion protein and intervening cleavage site for TEV protease. The construct was transformed into *E. coli* BL21 (DE3) cells, and cells were grown in M9 minimal media containing ¹³C₆-glucose and ¹⁵NH₄Cl as carbon and nitrogen sources, respectively. When the OD_{600 nm} reached approximately 0.8, expression was induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG), followed by overnight incubation and shaking at 18 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris pH 7.8 and 500 mM NaCl) and lysed by sonication. After centrifugation, the soluble lysate was loaded onto a 5 mL Ni²⁺-charged FF affinity column (GE Healthcare), followed by elution with a 0–500 mM imidazole linear gradient in the same buffer. Fractions containing protein were identified by SDS-PAGE and pooled. The His₆-GB1 fusion protein was removed by His₆-TEV protease digestion overnight at 4°C (leaving an N-terminal serine residue as a byproduct of TEV cleavage). The cleaved protein product was separated from uncleaved protein, His₆-TEV protease, and the His₆-GB1 fusion partner by passage over a HisTrap HP column (GE Healthcare). Purified protein was eluted from the column with a 20 mM imidazole step gradient. Proteins samples were then dialyzed and concentrated against a series of buffers used to optimize the number of peaks visible in the NMR spectrum and their intensities. Buffers ranged from pH 6.0 to 7.5 using 20 mM MES or 25 mM NaPO₄, with varying amounts of NaCl or KCl, and each contained 10%

D₂O. The effect of adding 5 mM CaCl₂ or 150 μM NaN₃ was also tested. The final sample selected was 0.5 mM AmiC-NTD in 20 mM NaHPO₄, pH 7.5, 100 mM NaCl, 150 μM NaN₃ with 10% D₂O.

NMR Experiments

After testing sample temperatures of 293, 298, and 303K, NMR spectra were recorded at 303K on a shielded Bruker Avance III US Plus Compact Standard Bore 850 MHz spectrometer equipped with triple resonance inverse detection (TCI) probe and a shielded 600 MHz Bruker Avance II spectrometer equipped with a triple resonance (TXI) probe. Backbone ¹H, ¹⁵N, ¹³C', ¹³Ca and ¹³Cβ resonances were assigned by pairwise comparison of intra- and inter-residue chemical shifts acquired in complementary 3D HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA, HNCO, HN(CA)CO, HBHACONH, and NH-TOCSY experiments. Additional side chain resonances were identified using 3D (H)CCH-TOCSY, H(C)CH-TOCSY and ¹⁵N-¹H HSQC-NOESY experiments. ¹H-¹³C correlations identified from backbone and sidechain experiments were used to assign the 2D CT-HSQC spectrum. Data were processed using NMRPipe (Delaglio et al. 1995) and analyzed with CCPN (Vranken et al. 2005). ¹H chemical shifts were referenced to trimethylsilyl propanoic acid (TSP) while ¹⁵N and ¹³C chemical shifts were indirectly calibrated. Talos+ (Shen et al. 2009) was used to generate secondary structure predictions based on experimentally derived H, N, C', Ca and Cβ chemical shifts.

Assignments and data deposition

The 2D [¹H-¹⁵N]-TROSY-HSQC spectrum of the AmiC-NTD construct is shown in Fig. 1. Under the conditions utilized in this experiment, 97% (108/111) of the observable amide ¹H-¹⁵N correlations were unambiguously assigned. Overall, 91% of both Ca and Cβ resonances were assigned. Nineteen residues were missing or severely broadened in the ¹⁵N-edited 2D-HSQC, with eleven of those (Lys29, Arg32, Ala33, His45, Gln105, Pro108, Pro129, Gly132, Leu157, Asn158, and Gly159) also failing to provide heteronuclear Ca, Cβ or C' assignments. Since all of these unobserved resonances cluster within terminal or predicted loop regions of the protein, their absence is likely the result of peak broadening due to solvent exchange or conformational averaging on an unfavorable timescale. The chemical shift assignments from these experiments have been deposited in the BioMagResBank (www.bmrb.wisc.edu) under accession number 27593.

Analysis of the backbone chemical shifts by Talos+ (Shen et al. 2009) suggests that the AmiC-NTD construct contains four β strands, followed by a single α-helix and then a further four β-strands (Fig. 2). This secondary structure prediction is consistent with the crystal structure of the NTD of *E. coli* AmiC (PDB ID:4BIN), which comprises a sandwich of two β sheets, with only minor differences in secondary structure boundaries between the two proteins.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) Award GM066861 (to C.D.). B.Y. was supported by the NIH training award, GM072643.

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Fig. 1.
2D [^1H - ^{15}N]-TROSY-HSQC of the N-terminal domain (NTD) of AmiC recorded on a shielded Bruker Avance III US Plus Compact Standard Bore 850 MHz spectrometer equipped with triple resonance inverse detection (TCI) probe at pH 7.5 and 303K. An enlarged view of the crowded center region of the spectrum is shown in the top-left corner. Residues corresponding to each backbone amide N-H correlation are labeled. Horizontal lines indicate NH_2 sidechain correlations and asterisks mark the three weak correlations that lack inter-residue connectivity for unambiguous assignment.

Fig. 2. Characterization of the AmiC-NTD construct from *N. gonorrhoeae* based on NMR chemical shifts (S-1 denotes a serine residue that is a byproduct of TEV cleavage site). **A.** Raw chemical shift deviations ($\delta(C\alpha) - \delta(C\beta)$) with respect to corresponding random coil values are plotted vs. residue number. Positive and negative values indicate α -helix and β -strand structure, respectively. **B.** The probability of secondary structure formation as predicted by Talos+. α -helices are represented by blue regions, β -strands by red regions and the random coil index is represented by black circles. **C.** Secondary structure comparison between Talos+ (Shen et al. 2009) predictions (top) and the crystal structure of the NTD of *E. coli* AmiC (PDB ID: 4BIN) (bottom). Amino acid stretches that meet the requirements for secondary structure definition are indicated; α -helices are represented by blue bars and β -strands by red bars.