Published in final edited form as:

Biomol NMR Assign. 2019 April ; 13(1): 139–142. doi:10.1007/s12104-018-9866-8.

^1H , ^{13}C , and ^{15}N backbone resonance assignments for KPC-2, a class A serine- β -lactamase

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Abstract

The ever-increasing occurrence of antibiotic resistance presents a major threat to public health. Specifically, resistance conferred by β -lactamases places the efficacy of currently available antibiotics at risk. *Klebsiella pneumoniae* carbapenemase-2 (KPC-2) is a β -lactamase that enables carbapenem resistance and represents a clear and present danger to global public health. In order to combat bacterial infections harboring KPC-2 expression, inhibitors with improved potency need to be developed. Although the structure of KPC-2 has been solved by X-ray crystallography, NMR provides the unique opportunity to study the structure and dynamics of flexible loop regions in solution. Here we report the ^1H , ^{13}C , and ^{15}N backbone chemical shift assignments for KPC-2 in the apo state as the first step towards the study of KPC-2 dynamics in the presence and absence of ligands to enable the rational design of optimized inhibitors.

Keywords

KPC-2; β -lactamase; carbapenemase; antibiotic resistance; NMR

Biological context

Antibiotic resistance has emerged as a serious public health threat. Bacteria have evolved multiple mechanisms to greatly decrease the efficacy of many commonly used antibiotics

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Disclosure

The corresponding author (RCP) is the recipient of a research contract from Allegra Therapeutics to study KPC-2. The authors RCP and RAB are the co-recipients of a research contract (MISP #57394) from Merck & Co. to study inhibition of KPC-2.

(Blair et al. 2015). A key mechanism utilized by bacteria to become resistant to antibiotics is the expression of a family of proteins called β -lactamases. β -lactamases target antibiotics that contain a β -lactam ring and render them ineffective through hydrolysis (Majiduddin et al. 2002). A strategy for combating antibiotic resistance would be to develop inhibitors that target and inactivate β -lactamases, thereby restoring efficacy to current generation antibiotics. KPC, *Klebsiella pneumoniae* carbapenemase, is a Class A serine- β -lactamase that enables carbapenem resistance. There are at least 18 known variants of KPC, however KPC-2 is among the most widespread globally (Nordmann et al. 2009).

Bacterial infections harboring the *bla*_{KPC-2} gene are notoriously difficult to treat, as KPC-2 can hydrolyze a broad spectrum of substrates, including penicillins, carbapenems, and cephamycins (Papp-Wallace et al. 2010). KPC-2 infections lead to high mortality rates due to the limited treatment options and are therefore a critical concern for global public health (Munoz-Price et al. 2013). Serine- β -lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam are ineffective at treating infections harboring KPC-2 expression (Papp-Wallace et al. 2010; Shapiro 2017), as each contains a β -lactam ring that can be hydrolyzed by KPC-2. The only FDA approved inhibitor effective against KPC-2 is avibactam, a non- β -lactam β -lactamase inhibitor. Avibactam is a member of a small class of β -lactamase inhibitors known as diazabicyclooctanes (Coleman 2011). Avibactam reacts with the catalytic serine residue in the KPC-2 active site and operates under a reversible covalent mechanism (Ehmann et al. 2013). Unlike other Class A β -lactamases, KPC-2 can degrade avibactam through desulfation, however, the desulfation rate is slow enough that avibactam maintains activity against KPC-2 (Ehmann et al. 2013; Krishnan et al. 2015). Due to the typically rapid evolution of β -lactamases, worries abound that new variants of KPC may soon acquire the ability to degrade avibactam at faster rates. In fact, recent studies have shown that a subset of lab-generated mutants exhibit increased resistance to avibactam (Papp-Wallace et al. 2015), further solidifying the importance of the development of new KPC-2 inhibitors.

The Ω -loop, a conserved structural element of the Class A β -lactamases, plays a key role in substrate binding due to its position adjacent to the active site (Palzkill et al. 1994). In KPC-2, the sixteen residue Ω -loop is stabilized by a salt bridge formed between Arg₁₆₄ and Asp₁₇₉. Based on the results of a study with TEM-1 (Raquet et al. 1994) that showed increased flexibility in the Ω -loop after disruption of the salt bridge, it was predicted that future variants of KPC may exhibit similar mutations to accommodate binding of bulkier substrates. Lab-generated KPC-2 mutants with substitutions at Arg₁₆₄ or Asp₁₇₉ were generated to test this hypothesis and some mutants exhibited increased rates of hydrolysis of last resort antibiotics, such as ceftazidime (Levitt et al. 2012; Barnes et al. 2017). Future KPC-2 inhibitors may need to consider targeted interactions with the Ω -loop in order to be effective. However, to enable systematic and rational drug design, additional structural and dynamics information for KPC-2 is greatly needed. Although crystal structures of KPC-2 are available (Ke et al. 2007; Krishnan et al. 2015), these do not allow for a detailed exploration of KPC-2 dynamics in the presence and absence of substrates. For example, when comparing the structure of apo-state KPC-2 (PDB: 2ov5) (Ke et al. 2007) to the avibactam-bound structure (PDB: 4zbe) (Krishnan et al. 2015), there are only minor structural differences localized to loop regions, but no large global structural changes. Furthermore,

crystal packing can perturb the structure of flexible regions making it difficult to investigate the Ω -loop structure. In order to further study the structural and functional nuances of KPC-2, we have turned to NMR. Prior to delving into structural and dynamic studies, the chemical shift assignments must be made. Here we report our backbone resonance assignment for KPC-2 with the goal of laying the groundwork for the β -lactamase field in pursuit of developing potent KPC-2 inhibitors.

Methods and experiments

Protein expression and purification

A plasmid encoding for a truncated portion of the gene encoding for KPC-2 (Ke et al. 2007), representing residues 26–290 of full-length KPC-2, was received as a generous gift from the laboratory of Dr. Focco van den Akker. The KPC-2_{26–290} plasmid was transformed into BL21(DE3) *E. coli* competent cells (New England Biolabs) and transferred to a LB agar plate containing 60 μ g/mL kanamycin. The transformation plate was incubated overnight at 37 °C and selected colonies were used to inoculate a 120 mL LB culture containing 60 μ g/mL kanamycin. Starter culture was grown overnight at 37 °C and was used the next morning to inoculate two 500 mL flasks of M9 minimal media without isotopes. The cells were grown at 37 °C to an OD₆₀₀ of 1.2, spun down at 3,800 RPM at 25 °C for 6 minutes, and non-isotopically labeled media was decanted and removed. In order to incorporate uniform ¹⁵N labeling, the cell pellets were immediately resuspended and divided into two flasks of 500 mL of M9 minimal media supplemented with 2 g/L ¹⁵NH₄Cl and 10 mL of ¹⁵N Celtone complete media (Cambridge Isotope Laboratories, Inc.). For the expression of uniformly ¹⁵N/¹³C-labeled KPC-2, two flasks were supplemented with 2 g/L ¹⁵NH₄Cl, 4 g/L ¹³C-glucose, and 10 mL of ¹⁵N/¹³C Celtone complete media (Cambridge Isotope Laboratories, Inc.). Cells were incubated at 37 °C for 1 hour, followed by induction with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubation overnight at 20 °C. Cells were harvested and resuspended in 180 mM Tris, 450 mM NaCl, 10% glycerol, pH 7.9. Resuspended cells were supplemented with 1 mg/mL lysozyme, 20 μ g/mL DNase1, and 100 μ g/mL 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and immediately frozen in liquid nitrogen. Frozen cell suspensions were stored at –80 °C freezer until purification. This method produced KPC-2 with >99% ¹⁵N incorporation or >99% ¹⁵N/¹³C incorporation as confirmed by MALDI-TOF mass spectrometry.

Cells were thawed at 4 °C with rotation the night before purification. Cell lysate was separated by centrifugation at 11,500 rpm for 45 minutes. Lysate supernatant was dialyzed overnight in buffer A (20 mM CHES, pH 9.0). Lysate was filtered through a 0.45 μ m filter prior to loading onto a HiPrep Q FF 16/10 sepharose column (GE Healthcare). KPC-2 was eluted using a gradient of buffer A and increasing percentage of buffer B (20 mM CHES, 500 mM NaCl, pH 9.0). Fractions determined by SDS-PAGE to contain KPC-2 were concentrated to 1 mL and were loaded onto a HiLoad 16/600 Superdex 75 (S75) size exclusion column (GE Healthcare) previously equilibrated with 20 mM phosphate, 50 mM NaCl, pH 5.9. Column fractions with KPC-2 were identified by SDS-PAGE and pooled to maximize purity. Size exclusion chromatography steps were repeated until KPC-2 was

>95% pure, as judged by SDS-PAGE. Typical batches required five iterations of size exclusion chromatography.

Nuclear magnetic resonance spectroscopy

For NMR data collection, KPC-2 samples were prepared in 20 mM phosphate, 50 mM NaCl, 3% glycerol, pH 5.9 containing a protease inhibitor cocktail (0.2% sodium azide, 1 μ M AEBSF, and 5 mM ethylenediaminetetraacetic acid) and 10% D₂O. All protein samples were transferred to 5 mm Shigemi tubes for data collection. NMR spectra were collected at 35 °C on Bruker 600 MHz and 850 MHz spectrometers equipped with triple resonance ¹H/¹³C/¹⁵N probes. Spectra were processed using NMRPipe (Delaglio et al. 1995) and analyzed in Sparky (Lee et al. 2015). The chemical shifts for KPC-2 backbone atoms were assigned using the following experiments: ¹H/¹⁵N-HSQC, ¹H/¹³C/¹⁵N-HNCO, HN(CA)CO, HN(CO)CA, HNCA, CBCA(CO)NH, HNCACB, and HBHA(CO)NH, ¹⁵N-HBANH, and ¹⁵N-edited NOESY-HSQC.

Assignment and data deposition

The KPC-2 construct used in this study is comprised of 265 residues with a molecular weight of 28 kDa. As seen in the two-dimensional ¹⁵N-HSQC spectra (Fig. 1), there are regions with severe overlap of amide resonances. In order to assign the backbone for KPC-2, multiple sets of complementary 3D triple resonance experiments were collected (see Methods section). Overall, 98.4% of the amide backbone chemical shifts for the non-proline residues were assigned. The amide proton and nitrogen could not be assigned for Leu₂₆, Ser₇₀, Thr₂₃₇, and Tyr₂₄₇. 99.6% of the C α chemical shifts were assigned, with only the C α corresponding to the first residue, Leu₂₆, missing. 98.3% of all C β resonances were assigned, the exceptions being Leu₂₆, Ser₁₀₉, Thr₁₄₉, and Thr₁₈₇. All but five carbonyl carbon chemical shifts were assigned, resulting in 98.1% completeness. The missing carbonyl assignments belong to Thr₉₃, Ser₁₀₆, Ile₁₇₃, Ser₁₈₂, and Trp₂₅₁, residues that were all followed by a proline. Backbone chemical shifts have been deposited into the BioMagResBank (<http://www.bmrb.wisc.edu>) with accession number 27617. KPC-2 secondary structure was predicted using TALOS+ (Shen et al. 2009) using the deposited assigned backbone chemical shifts for the C α , C β , C γ , H α , and N α atoms as input. The TALOS+ predictions (Fig. 2A) identified twelve α -helices and eight β -strands, consistent with the crystal structure of KPC-2 (Fig. 2B).

Acknowledgements

The authors acknowledge financial support from the National Institutes of Health through the National Institute of General Medical Sciences under award number R35GM128595 to RCP and through the National Institute of Allergy and Infectious Diseases under award numbers R01AI100560, R01AI063517, R21AI114508, and R01AI072219 to RAB. This study was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, Award Number 1I01BX001974 to RAB from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development and the Geriatric Research Education and Clinical Center VISN 10 to RAB. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Veterans Affairs. MALDI-TOF data were collected using instrumentation purchased with NSF grant award CHE0839233. The authors acknowledge institutional support from Miami University through the Robert H. and Nancy J. Blayney Professorship to RCP.

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Figure 1. (A) $^1\text{H}/^{15}\text{N}$ -HSQC of KPC-2. (B) Expansion of boxed region (*black dashed line*) in (A) shows the crowded region of the spectrum. Backbone amide resonances are labeled according to amino acid residue type and number. The unlabeled peaks near the bottom of the spectra from 6.8 ppm to 10 ppm correspond to aliased Arg N_e resonances. Other unlabeled resonances, identified in a multiplicity edited HSQC (data not shown), correspond to Asn and Gln side chain NH_2 and Trp N_e resonances. A complete list of resonance assignments can be found in the BMRB repository under accession number 27617.

Figure 2. Secondary structure predicted by KPC-2 resonance assignments. Fraction α -helical (*red*) and β -strand (*blue*) content (A) predicted by TALOS+ (Shen et al. 2009) are shown as histograms. The confidence per residue reported by TALOS+ for secondary structure assignment is shown as a line (*black*). Above the plot (A), cylinders represent KPC-2 helices and arrows represent β -strands. Each secondary structure element is shown colored according to position within the structure of KPC-2 (B) from PDB accession ID 4zbe.