Probing the role of the conserved residue Glu166 in a class A β -lactamase using neutron and X-ray protein crystallography

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The amino-acid sequence of the Toho-1 β -lactamase contains several conserved residues in the active site, including Ser70, Lys73, Ser130 and Glu166, some of which coordinate a catalytic water molecule. This catalytic water molecule is essential in the acylation and deacylation parts of the reaction mechanism through which Toho-1 inactivates specific antibiotics and provides resistance to its expressing bacterial strains. To investigate the function of Glu166 in the acylation part of the catalytic mechanism, neutron and X-ray crystallographic studies were performed on a Glu166Gln mutant. The structure of this class A β -lactamase mutant provides several insights into its previously reported reduced drug-binding kinetic rates. A joint refinement of both X-ray and neutron diffraction data was used to study the effects of the Glu166Gln mutation on the active site of Toho-1. This structure reveals that while the Glu166Gln mutation has a somewhat limited impact on the positions of the conserved amino acids within the active site, it displaces the catalytic water molecule from the active site. These subtle changes offer a structural explanation for the previously observed decreases in the binding of non- β -lactam inhibitors such as the recently developed diazobicyclooctane inhibitor avibactam.

1. Introduction

β -Lactam antibiotics are used to treat a broad spectrum of bacterial infections. Acting on the bacterial cell-membrane-associated penicillin-binding proteins (PBPs), these antibiotics halt peptidoglycan cross-linking to trigger cell lysis and cell death (Drawz & Bonomo, 2010). Transported to and activated within the periplasmic space of resistant organisms, β -lactamase enzymes hydrolyze β -lactam antibiotics (penicillins, cephalosporins and carbapenems; Davies & Davies, 2010) and prevent them from inhibiting their target PBPs. There are four distinct groups of β -lactamases (A–D; Ambler *et al.*, 1991). Class B β -lactamases are metalloenzymes that use a water molecule bound to a zinc ion to hydrolyze the amide bond of the β -lactam ring. Class A, C and D enzymes utilize a catalytic serine to break the β -lactam ring through the formation and release of an acyl-enzyme intermediate. Toho-1 is a 262-amino-acid class A β -lactamase composed of two highly conserved domains (α/β and α) and an active-site cavity that is formed at the domain interface (Ibuka *et al.*, 1999, 2003). Other examples of class A β -lactamases include temoneira (TEM), sulfhydryl reagent variable (SHV) and the emergent extended-spectrum β -lactamase (ESBL) CTX-M enzymes (Bonnet, 2004). ESBLs possess increased hydrolytic activity against monobactams and first-, second- and third-generation extended-spectrum cephalosporins (Ishii *et al.*, 1995; Bonnet,

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2004; Drawz & Bonomo, 2010). Toho-1, with its broad activity against the extended-spectrum cephalosporins, is classified as a CTX-M-type ESBL (Ambler *et al.*, 1991). In class A β -lactamases, several highly conserved amino acids play roles in the catalytic reaction mechanism (Ser70, Lys73, Ser130 and Glu166). Ser70 is the catalytic nucleophile that attacks the β -lactam carbonyl to form a transient acyl-enzyme intermediate that undergoes a base-catalyzed attack via a water molecule to form a tetrahedral intermediate, which collapses into a post-covalent complex from which the hydrolyzed product is released (Drawz & Bonomo, 2010). Glu166 has been proposed to act as a catalytic base (Chen *et al.*, 2005; Tomanicek *et al.*, 2010, 2011, 2013) in the acylation step of the reaction (Fig. 1a). By deprotonating the hydroxyl of Ser70 via a catalytic water molecule with simultaneous proton transfer from Lys73 to Ser70 (Chen *et al.*, 2007; Meroueh *et al.*, 2005), a

pre-covalent complex in which Ser70, Lys73 and Glu166 are all neutral is produced. Lys73 then deprotonates Ser70, triggering it to attack the carbonyl C atom of the β -lactam ring, forming the acylation tetrahedral intermediate (Meroueh *et al.*, 2005). Following the formation of the acylation tetrahedral intermediate, protonation of the β -lactam N atom must occur to break the β -lactam ring and form the acyl-enzyme adduct. In recent studies (Meroueh *et al.*, 2005; Vandavasi *et al.*, 2016, 2017; Langan *et al.*, 2018), it has been proposed that the β -lactam ring is opened by the transfer of a proton from the NZ group of Lys73 to the OG group of Ser130 followed by the simultaneous transfer of a proton from the Ser130 OG group to the β -lactam ring N atom (Meroueh *et al.*, 2005; Langan *et al.*, 2018). The final step in the acylation pathway involves proton transfer from Lys73 to Glu166, which initiates the start of the deacylation reaction.

To help counter β -lactamase-mediated resistance, β -lactam antibiotics are often combined with a β -lactamase inhibitor such as tazobactam, sulbactam or clavulanic acid (Drawz & Bonomo, 2010). Non- β -lactam inhibitors such as the recently developed diazobicyclooctane (DBO) avibactam form a unique covalent carbamyl linkage with the catalytic nucleophile Ser70 (Lahiri *et al.*, 2013) and have a broad spectrum of activity, inhibiting class A, class C and some class D β -lactamases (Ehmann *et al.*, 2013). Avibactam is not broken down by a hydrolysis mechanism, as is the case for β -lactam-based inhibitors. Instead, a decarbamylation reaction occurs in which the recyclization of avibactam forms an intact inhibitor that is released from the enzyme (Fig. 1b). The carbamylation and decarbamylation reactions involve the same set of catalytic residues, and the exact mechanism by which the carbamylation and decarbamylation occurs has been investigated by several groups using CTX-M15 as a model system (King *et al.*, 2015; Lahiri *et al.*, 2013). Differing catalytic mechanisms have been proposed, in one of which Glu166 is protonated in the avibactam-bound form and deprotonated in the native form (Lahiri *et al.*, 2013) and is proposed to act as the general base during acylation.

Figure 1

(a) The acylation reaction initiates by the formation of a pre-covalent enzyme-substrate complex. A general base-catalyzed nucleophilic attack on the β -lactam carbonyl by the serine hydroxyl proceeds through a tetrahedral intermediate, forming a transient acyl-enzyme adduct. In the deacylation part of the reaction, the acyl-enzyme adduct undergoes a general base-catalyzed attack by a hydrolytic water molecule to form a second deacylation tetrahedral intermediate, which then collapses to form an enzyme-product complex. (b) An overview of the reversible avibactam-mediated inhibition of class A β -lactamases. During the inhibition process, the N6-C7 bond is broken and a proton is added to the N6 atom to open the diazobicyclooctane ring.

An alternative mechanism proposed that Glu166 is not catalytically active but rather that Ser130 acts as both an acid and a base during the carbamylation and decarbamylation. In enzyme kinetic measurements conducted on CTX-M15 (King *et al.*, 2015) the mutation Glu166Gln reduced the rate of carbamylation by around 40% compared with the wild type, with the decarbamylation rate being almost equal (King *et al.*, 2015) to that of the wild-type enzyme. These results suggest that while Glu166 is not a catalytically essential amino acid for carbamylation, the Glu166Gln mutation does significantly affect the carbamylation reaction rate.

To probe the role that the Glu166Gln mutation may play in affecting carbamylation rates, we conducted X-ray and neutron diffraction studies of a Glu166Gln mutant. Both the CTX-M15 and Toho-1 (also known as CTX-M44) β -lactamases are class A β -lactamase enzymes and share 85% amino-acid sequence homology. While a Glu166Gln mutant of a class A β -lactamase enzyme had not been successfully crystallized prior to this study, Chen and Herzberg reported X-ray diffraction from a class A β -lactamase Glu166Gln/Asn170Asp double mutant (Chen & Herzberg, 1999). In that structure, which had a resolution of 2.30 Å, the electron density between residues 163 and 171 was discontinuous, with very poor electron density for the Gln166 residue itself. In our structure, electron and neutron density is continuous throughout this region, enabling us to clarify the structural changes caused by the Glu166Gln mutation.

2. Methods

The Glu166Gln/Arg274Asn/Arg276Asn Toho-1 β -lactamase enzyme was overexpressed using an *Escherichia coli*-based expression system and purified as described previously (Tomanicek *et al.*, 2010, 2011, 2013). Crystals of the perdeuterated protein were grown at 20°C using 150 μ l of protein at a concentration of 10 mg ml⁻¹ in a solution consisting of 2.0 M ammonium sulfate, 0.2 M sodium citrate pH 6.1 by the batch method. For neutron and X-ray room-temperature data

collection, the crystal was mounted within a VitroCom fused-quartz capillary with a small plug of mother liquor (Fig. 2).

Neutron diffraction data were collected first by wavelength-resolved time-of-flight Laue diffraction (Langan *et al.*, 2008) on the MaNDi instrument at the Spallation Neutron Source (SNS; Coates *et al.*, 2010, 2015) using neutrons with wavelengths of between 2 and 4 Å. A total of ten Laue diffraction images were collected, with the sample being rotated by 10° about the φ axis between images. The exposure time per image was 8 h, giving a total data-collection time of 80 h. The ten neutron diffraction images were reduced using the *Mantid* suite (Arnold *et al.*, 2014; Sullivan *et al.*, 2018), with wavelength normalization being performed by the *LAUENORM* program from the *LAUEGEN* suite (Campbell *et al.*, 1998; Campbell, 1995). After the neutron data collection had been completed, X-ray data were collected from the same crystal using an in-house Rigaku MicroMax-007 HF X-ray diffractometer. A total of 280 frames of data were collected with a θ rotation of 0.5° and an exposure time of 15 s per frame. X-ray diffraction data were processed using *CrysAlis^{Pro}* (Rigaku) and scaled using *AIMLESS* from the *CCP4* suite (Winn *et al.*, 2011). Both the neutron and the X-ray data were used to refine the structure to convergence using the joint refinement option within *phenix.refine* (Afonine *et al.*, 2010; Liebschner *et al.*, 2019). All model building was performed using the *Coot* molecular-graphics program (Emsley *et al.*, 2010) and figures were created using *Chemtool* and *PyMOL* (Yuan *et al.*, 2016). The X-ray and neutron data-reduction and refinement statistics for the structure are given in Table 1. The Arg274Asn and Arg276Asn mutations prevent crystal twinning and increase the diffraction resolution (Nitanai *et al.*, 2010; Shimamura *et al.*, 2009), while the Glu166Gln mutation was added to study its effects on the structure of the active site. All structural data have been deposited in the Protein Data Bank (PDB) with accession code 6u58.

3. Results and discussion

Neutron protein crystallography (NPX) complements even high-resolution X-ray crystallography as it can provide the locations of D atoms experimentally rather than by inference (Schaffner *et al.*, 2017; Tomanicek *et al.*, 2013). As X-rays are scattered by electrons, H atoms scatter X-rays weakly, even at high resolution, and are usually not detected. Neutrons are scattered by the atomic nucleus, and those scattered from the hydrogen isotope deuterium (²H) are scattered at the same level as the heavier elements in proteins such as carbon, nitrogen and oxygen (Coates *et al.*, 2014). Thus, the positions of D atoms can be determined directly, even at moderate resolutions, using NPX.

Perdeuteration is the complete isotopic substitution of all H atoms with deuterium and provides two powerful benefits in NPX studies. Firstly, it significantly increases the signal-to-noise ratio of the diffraction data by substantially reducing the primary source of background in an NPX experiment: incoherent scattering. The incoherent scattering length of deuterium is 40 times smaller than that of hydrogen; thus, the

Figure 2
A Toho-1 β -lactamase crystal with a volume of 1.8 mm³ that was used for neutron and X-ray room-temperature data collection is shown mounted within a fused-quartz VitroCom capillary with an inner diameter of 1.5 mm.

Table 1

Data-collection and refinement statistics for the joint neutron/X-ray Glu166Gln Toho-1 structure.

	Neutron	X-ray
Space group	<i>P</i> 3 ₂ 21	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	73.54, 73.54, 99.72	
α , β , γ (°)	90, 90, 120	
Resolution range (Å)	14.42–1.89 (1.96–1.89)	30.34–1.90 (1.94–1.90)
Total No. of reflections	111496	133121
No. of unique reflections	24170	25146
Completeness (%)	95.68 (85.61)	100.00 (100.00)
Multiplicity	4.61 (3.11)	5.30 (5.20)
$\langle I/\sigma(I) \rangle$	8.40 (2.70)	11.4 (2.30)
$CC_{1/2}$	94.60 (46.90)	99.50 (63.50)
$R_{p.i.m.}$ (%)	9.30 (20.50)	7.00 (44.70)
R factor (%)	24.49	14.62
R_{free} (%)	26.22	17.52
R.m.s.d., bond lengths (Å)	0.013	
R.m.s.d., bond angles (°)	1.23	
Ramachandran plot		
Favored regions (%)	97.68	
Allowed regions (%)	1.93	
Outliers (%)	0.39	

experimental background is significantly lower for crystals produced from perdeuterated protein. Secondly, it enables the direct visualization of almost every atom within the protein structure, as deuterium, in common with most biological elements, has a positive coherent scattering factor, compared with the negative scattering factor of hydrogen. Perdeuteration provides information not only on the protonation states of polar atoms such as nitrogen and oxygen but also the exact locations of all nonpolar side-chain groups such as CH₂ and CH₃ groups. Thus, by combining neutron and X-ray diffraction data taken from the same perdeuterated crystal, we aimed to elucidate the subtle structural changes that accompany the Glu166Gln mutation. The overall structure of the Arg274Asn/Arg276Asn/Glu166Gln mutant is very similar to that of the Arg274Asn/Arg276Asn mutant structure, with an r.m.s.d. for backbone C atoms of 0.16 Å, while the r.m.s.d. for backbone C atoms between our Arg274Asn/Arg276Asn/Glu166Gln mutant and the wild type is only 0.31 Å.

However, the Glu166Gln mutation has more subtle effects on the structure of the ω loop, which is composed of amino-acid residues 163–178. In the room-temperature structure Gln166 adopts a slightly different conformation to that of Glu166 in the non-mutated room-temperature structure (Tomanicek *et al.*, 2011; Figs. 3 and 4). The neutron density shows that Lys73 is protonated and in the ND₃⁺ form, while the DG atom on Ser70 hydrogen-bonds to a new water molecule (WAT2). WAT2 also forms a hydrogen bond to a sulfate molecule within the active site. The neutron density allows us to correctly orient the side chain of Gln166 (Fig. 3), where the NE2 atom of the Gln166 side chain is located just 2.16 Å from the position of the absent catalytic water molecule (WAT1), as shown in Fig. 4.

The introduction of the Glu166Gln mutation shifts the side-chain position of the ND2 atom of Asn170, increasing the hydrogen-bonding distance between the ND2 atom of Asn170 and the OE1 atom of Glu166 from 2.77 Å in the wild type to 3.27 Å in the Glu166Gln mutant. Previous structural and

biochemical studies on the mutation of Asn170 in the TEM-1 β -lactamase have indicated that the position of the catalytic water molecule is altered upon the mutation of Asn170 in comparison with the wild-type structure (Brown *et al.*, 2009). In the Glu166 and Gln166 structures most of the room-temperature water positions within the active site are conserved, with one notable exception. The catalytic water molecule that is normally found hydrogen-bonded to the OE1 atom of Glu166 and the OG atom of Ser70 is absent from the active site (Fig. 4).

We compared the hydrogen-bonding pattern found in our previous non-mutated structure (Tomanicek *et al.*, 2011) with that found in the Glu166Gln mutant detailed in this paper (Fig. 5).

Figure 3

A view of the active site of the Toho-1 Glu166Gln mutant; the structure is shown in stick form and is colored by element. The $2F_o - F_c$ electron-density map at a value of 2σ is shown as a magenta mesh, while the $2F_o - F_c$ neutron density map at a value of 1.3σ is shown as a blue mesh.

Figure 4

A view of the active site of the Toho-1 Glu166Gln mutant; the structure is shown in stick form and is colored by element. The superposed structure of the non-mutated Toho-1 structure (PDB entry 2xqz) is shown as orange sticks, with an orange sphere indicating the position of the catalytic water molecule (WAT1) that is only present in the non-mutated structure.

Figure 5

Hydrogen-bonding patterns in the active site of Toho-1. (a) The hydrogen-bonding pattern observed in the non-mutated structure. (b) The hydrogen-bonding pattern observed in the Glu166Gln structure. All distances are given in ångströms between heavy atoms. The catalytic water molecule (WAT1) only appears in the non-mutated structure.

In the non-mutated structure, Ser70 hydrogen-bonds to the catalytic water molecule (WAT1) that is absent in the Glu166Gln mutant structure; upon the Glu166Gln mutation the DG atom of Ser70 changes its orientation and hydrogen-bonds to a different water molecule (WAT2). The hydrogen-bonding distance between Ser70 and Lys73 decreases to 2.79 Å in the Glu166Gln mutant compared with 3.20 Å in the non-mutated structure. The position of Asn170 alters slightly upon the Glu166Gln mutation, moving towards WAT2, which in turn causes Gln166 to move towards Asn170. The catalytic residues Ser70, Lys73 and Ser130 remain in similar positions to those found in the non-mutated structure, making it likely that the mutation of Glu166 to Gln166 and the removal of the catalytic water molecule from the active site affects its stability and may therefore influence substrate binding. This is likely to cause the 40% reduction in the rate of avibactam carbamylation compared with the wild-type enzyme that was reported previously (King *et al.*, 2015). This observation is in agreement with several X-ray and neutron studies (Nichols *et al.*, 2015; Lewandowski *et al.*, 2018; Tomanicek *et al.*, 2013; Langan *et al.*, 2018; Vandavasi *et al.*, 2016; Chen *et al.*, 2007; Meroueh *et al.*, 2005), which have shown that that Glu166 and the catalytic water molecule are vital in the deprotonation of the catalytic nucleophile Ser70.

4. Conclusions

The coordination, positioning and presence of the hydrolytic water molecule is an essential component for the efficient hydrolysis of β -lactam antibiotics by class A β -lactamases and in the carbamylation of avibactam. By mutating Glu166 to Gln

in a class A β -lactamase, we have observed that this single mutation ejects the catalytic water molecule from the active site, affecting its stability, which may influence the binding of avibactam.

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