The role of conserved surface hydrophobic residues in the carbapenemase activity of the class D β -lactamases

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Carbapenem-hydrolyzing class D β -lactamases (CHDLs) produce resistance to the last-resort carbapenem antibiotics and render these drugs ineffective for the treatment of life-threatening infections. Here, it is shown that among the clinically important CHDLs, OXA-143 produces the highest levels of resistance to carbapenems and has the highest catalytic efficiency against these substrates. Structural data demonstrate that acylated carbapenems entirely fill the active site of CHDLs, leaving no space for water molecules, including the deacylating water. Since the entrance to the active site is obstructed by the acylated antibiotic, the deacylating water molecule must take a different route for entry. It is shown that in OXA-143 the movement of a conserved hydrophobic valine residue on the surface opens a channel to the active site of the enzyme, which would not only allow the exchange of water molecules between the active site and the milieu, but would also create extra space for a water molecule to position itself in the vicinity of the scissile bond of the acyl-enzyme intermediate to perform deacylation. Structural analysis of the OXA-23 carbapenemase shows that in this enzyme movement of the conserved leucine residue, juxtaposed to the valine on the molecular surface, creates a similar channel to the active site. These data strongly suggest that all CHDLs may employ a mechanism whereupon the movement of highly conserved valine or leucine residues would allow a water molecule to access the active site to promote deacylation. It is further demonstrated that the 6 α -hydroxyethyl group of the bound carbapenem plays an important role in the stabilization of this channel. The recognition of a universal deacylation mechanism for CHDLs suggests a direction for the future development of inhibitors and novel antibiotics for these enzymes of utmost clinical importance.

1. Introduction

The use of antibiotics for the treatment of infectious diseases has saved millions of lives over the last 75 years. Currently, more than a dozen classes of antibiotic have been introduced into the clinic, with β -lactams (penicillins, cephalosporins, monobactams and carbapenems) being the most widely used. The production of β -lactamases, enzymes that are capable of deactivating β -lactams, constitutes the major mechanism of resistance to these clinically important antibiotics in Gram-negative bacteria. Four classes of β -lactamases have been described: the zinc-containing class B metalloenzymes and serine-dependent enzymes of classes A, C and D (Poole, 2004; Hall & Barlow, 2005).

Amongst the various β -lactamases, carbapenemases are of the greatest concern as they render bacteria resistant to the last-resort carbapenem antibiotics, which are now used extensively for the treatment of diverse life-threatening

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infections caused by bacteria that are resistant to other classes of antimicrobial agents (Hawkey & Livermore, 2012; Papp-Wallace *et al.*, 2011). Of particular importance is the recent dissemination of carbapenem-hydrolyzing class D β -lactamases (CHDLs), which now play a prominent role in conferring resistance to carbapenem antibiotics in clinically important bacteria, including *Acinetobacter baumannii* and Enterobacteriaceae, transforming these bacteria into deadly pathogens (Fair & Tor, 2014; Peleg *et al.*, 2008; Perez *et al.*, 2007; Roca *et al.*, 2012; Spellberg & Bonomo, 2014). The class D enzymes, which were initially referred to as oxacillinases (or OXA enzymes) owing to their ability to efficiently hydrolyze oxacillin in preference to the classical penicillins (Walther-Rasmussen & Høiby, 2006), now number almost 500 members (<http://www.lahey.org/Studies/other.asp#table1>). Based upon sequence identity, the class D enzymes can be divided into a number of subfamilies with 80–90% sequence identity amongst their members. Seven of these subfamilies, OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, OXA-143 and OXA-235, comprise over 200 enzymes which are classified as CHDLs. All of these subfamilies of CHDLs, with the exception of the OXA-48-like enzymes, were isolated from *A. baumannii*.

The wide dissemination and clinical importance of CHDLs has rejuvenated interest in elucidating the mechanism of their carbapenemase activity. Similar to two other classes (A and C) of serine-dependent β -lactamases, the class D enzymes turn over carbapenems and other β -lactam antibiotics *via* a two-step mechanism. The first step, acylation, is common to all

three classes. It involves the activation of a catalytic serine residue by a water molecule, followed by attack of this serine on the β -lactam ring (Fig. 1*a*) to form an acyl-enzyme intermediate (Fig. 1*b*). The second step of catalysis, deacylation, involves the activation of a water molecule (the deacylating water) which subsequently attacks the acyl bond (Fig. 1*b*) and releases inactive product. In the class A and C enzymes, conserved glutamate (Lamotte-Brasseur *et al.*, 1991) and tyrosine residues (Dubus *et al.*, 1996), respectively, activate the deacylating water, whereas the class D enzymes utilize a carboxylated lysine residue for this purpose (Golemi *et al.*, 2001).

The poor catalytic activity of many β -lactamases against carbapenems, compared with penicillins or cephalosporins, stems from differences in their structures. Although the overall structure of carbapenems resembles those of other β -lactam antibiotics, with a β -lactam ring fused to a second heterocycle, they differ from other β -lactams in three important ways. Firstly, the fused ring is a pyrroline as opposed to a thiazolidine in the penicillins (Fig. 1*a*) or a thiazine in the cephalosporins (Fig. 1*c*). The presence of a double bond in the carbapenem pyrroline ring (Fig. 1*d*) gives rise to a flatter ring and allows tautomerization following cleavage of the β -lactam upon acylation, whereby the C2 atom is either sp^2 -hybridized (the Δ^2 tautomer) or sp^3 -hybridized (the Δ^1 tautomer with either *R* or *S* stereochemistry). Secondly, the side chain at the C6 position of the β -lactam ring is a relatively compact α -hydroxyethyl group, compared with some of the larger aromatic side chains in the penicillins and cephalosporins (Figs. 1*a* and 1*c*). Thirdly, the stereochemistry at the C6 atom is *S* in the carbapenems compared with *R* in the penicillins and cephalosporins.

Based on these characteristic features and structural analysis of CHDLs, several mechanisms have been proposed for the hydrolysis of carbapenem antibiotics by class D β -lactamases. The first mechanism was suggested following elucidation of the structure of the *A. baumannii* CHDL OXA-24/40. This enzyme harbors a hydrophobic bridge-like or tunnel-like structure over the active-site cleft (Santillana *et al.*, 2007) which was not present in previously characterized class D β -lactamases. It was suggested that this bridge prevents pyrroline Δ^2 to Δ^1 tautomerization and maintains the carbapenem substrate in the correct conformation and orientation in the active site, thus promoting more efficient

Figure 1

β -Lactam antibiotics. (a) Chemical structure of ampicillin showing the atom numbering for the thiazolidine and β -lactam rings. The scissile N4–C7 bond is broken during the acylation step of the reaction in the serine-dependent β -lactamase enzymes. (b) Chemical structure of the acyl-enzyme intermediate formed upon reaction of ampicillin with a serine-dependent β -lactamase. The acyl bond is broken during the deacylation step to yield free inactive product. (c) Chemical structure of cephalothin as a representative of the cephalosporin antibiotics. (d) Chemical structure of meropenem as a representative of the carbapenem antibiotics. The difference in stereochemistry at the C6 atom relative to the equivalent C atom in ampicillin and cephalothin is indicated.

deacylation (Schneider *et al.*, 2011). An alternative mechanism was proposed for OXA-48, an enzyme widely disseminated in Enterobacteriaceae which, unlike OXA-24, does not contain the hydrophobic bridge. Based on molecular-dynamics simulation experiments, it was suggested that this enzyme utilizes a mechanism whereby rotation of the 6 α -hydroxyethyl group of the carbapenem antibiotic meropenem facilitates the repositioning of a distant active-site water molecule closer to the scissile bond (Docquier *et al.*, 2009). Finally, a third mechanism was advanced based upon structural characterization of OXA-23 (Smith *et al.*, 2013), another *A. baumannii* enzyme containing a hydrophobic bridge. Analysis of the meropenem complex of this enzyme demonstrated that, unlike in OXA-24, the pyrroline of the acylated carbapenem is in a Δ^1 tautomeric conformation. Owing to the obstruction by the bulky 6 α -hydroxyethyl moiety of the bound carbapenem, a water molecule from the milieu cannot enter through the active-site entrance to a position where it would promote deacylation. It was demonstrated that binding of the carbapenem triggers the movement of a conserved leucine residue (Leu166) located on the surface of the enzyme, which opens a channel for a water molecule from the milieu to reach the vicinity of the catalytic lysine to perform deacylation (Smith *et al.*, 2013).

Thus, three different mechanisms for the carbapenemase activity of class D β -lactamases have been presented over the last decade, derived from the analysis of three different class D enzyme structures. All three mechanisms suggest that improved deacylation plays the major role in this process. However, given the high degree of structural and functional similarity between various CHDLs, it is highly unlikely that they would utilize diverse mechanisms for turnover of carbapenem antibiotics. To further address this issue, we performed detailed microbiological, kinetic and structural characterization of the *A. baumannii* carbapenemase OXA-143, a close relative of OXA-24/40. These studies, complemented by molecular docking and modeling, indicate that a conserved surface hydrophobic residue (Val130) plays a key role in the deacylation step of this clinically important CHDL and that this may underpin a more universal mechanism for carbapenemase activity in the class D β -lactamases.

2. Materials and methods

2.1. Cloning and protein purification

The *oxa143* gene was cloned between the unique NdeI and HindIII restriction sites of pHF016 and pNT221 vectors (Antunes *et al.*, 2014; Frase *et al.*, 2009). The resulting plasmids were transformed into *Escherichia coli* JM83 and *A. baumannii* ATCC 17978, respectively. The gene for OXA-143 (accession No. ACX70402) was codon-optimized to achieve a high level of expression in *E. coli*. The custom-synthesized gene (GenScript) encoding the mature enzyme (lacking the first 20 amino-acid residues) was cloned between the unique NdeI and HindIII restriction-endonuclease sites of the pET24a(+) expression vector. The resulting pNT73 plasmid was transformed into *E. coli* BL21 (DE3) cells. For

enzyme production, 300 ml of bacterial culture in LB broth supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin was grown until an optical density of 0.6 at 600 nm was reached, and expression of the mature OXA-143 was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Following induction, the cells were grown at 22°C overnight and harvested by centrifugation (4000g, 15 min). The pellet was resuspended in 30 ml 25 mM HEPES buffer pH 7.0 and the bacteria were disrupted by sonication at 4°C. The disrupted cells were pelleted by centrifugation (20 000g, 30 min) and the supernatant was loaded onto a DEAE anion-exchange column (Bio-Rad) at 4°C. The majority of OXA-143 was collected in the unbound fraction, and the enzyme was further separated using a cation-exchange column (Macro-Prep CM Support, Bio-Rad) with a 0–1 M NaCl gradient. All collected fractions were tested for the ability to hydrolyze the chromogenic substrate nitrocefin and analyzed for purity by 12% PAGE. Purified OXA-143 was collected and the protein concentration was measured using a BCA kit (Pierce) and calculated by measuring the optical density at 280 nm using an extinction coefficient of 43 430 $\text{M}^{-1} \text{cm}^{-1}$.

2.2. Antibiotic-susceptibility profile and enzyme kinetics

Antibiotic-susceptibility testing was performed in triplicate in Mueller–Hinton Broth II using 96-well microtiter plates according to the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2009). Enzyme kinetics were determined with homogeneously pure OXA-143 as described previously (Antunes *et al.*, 2014). Briefly, reactions containing various concentrations of β -lactam antibiotics, 50 mM sodium bicarbonate and 0.2 mg ml $^{-1}$ bovine serum albumin in 100 mM phosphate buffer pH 7.0 were initiated by addition of the enzyme. Data were collected at 20°C using a Cary 60 spectrophotometer (Agilent). The initial linear phase of each reaction was used to calculate steady-state velocities, which were fitted nonlinearly using the Michaelis–Menten equation in the Prism 6 software (GraphPad). For determination of the dissociation constants (K_s) the reactions were as described above except that the β -lactam antibiotics were treated as inhibitors and nitrocefin was used at three different concentrations as a reporter substrate. Data were generated using the Dixon plot (Dixon, 1953). All kinetic experiments were performed at least in triplicate.

2.3. Crystallization and diffraction data collection and processing

The OXA-143 protein was concentrated to approximately 15 mg ml $^{-1}$ and crystallization trials were undertaken using Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2 (Hampton Research). Initial crystal hits were observed in multiple conditions, and these were tested for diffraction quality on SSRL beamline BL7-1. The final conditions (0.1 M Tris–HCl pH 8.5, 0.2 M MgCl $_2$, 30% PEG 4000) yielded crystals that diffracted to approximately 1.15 Å resolution and belonged to space group $P2_1$, with unit-cell parameters

Table 1

OXA-143 data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Unit-cell parameters (Å, °)	$a = 40.96$, $b = 62.33$, $c = 87.09$, $\beta = 91.7$
Space group	$P2_1$
Resolution range (Å)	87.05–1.15 (1.18–1.15)
No. of reflections (observed/unique)	499245/150376
R_{merge}	0.058 (0.623)
$\langle I/\sigma(I) \rangle$	11.45 (1.9)
Completeness (%)	96.1 (87.8)
$CC_{1/2}$ †	99.9 (73.4)
Multiplicity	3.4 (3.0)
Wilson B factor (Å ²)	9.0
Refinement statistics	
$R_{\text{work}}/R_{\text{free}}$ ‡ (%)	13.61/16.03
Total atoms	
Protein (chain A/chain B)	2002/1955
Solvent	588
B factors (Å ²)	
Protein (chain A/chain B)	12.4/18.2
Solvent	30.9
R.m.s. deviation from ideality	
Bond distances (Å)	0.005
Bond angles (°)	0.822
Ramachandran plot§	
Residues in most favored regions (%)	98.20
Outliers	0
PDB code	5iy2

† Percentage of correlation between intensities from random half-sets of data (Karplus & Diederichs, 2012). ‡ R_{free} was calculated with 5% of the reflections. § Calculated using *MolProbity* (Chen *et al.*, 2010).

$a = 40.96$, $b = 63.33$, $c = 87.09$ Å, $\beta = 91.7^\circ$. The Matthews coefficient (Matthews, 1968), calculated to be $2.1 \text{ Å}^3 \text{ Da}^{-1}$, suggested a solvent content of 53% with two OXA-143 molecules in the asymmetric unit.

A data set to 1.15 Å resolution was collected on SSRL beamline BL12-2 using X-rays at 12 658 eV (0.9795 Å). A total of 1200 fine φ -sliced (0.2° rotation) images were measured using a PILATUS 6M pixel-array detector running in shutterless mode. The images were processed using *XDS* (Kabsch, 2010) and were scaled and merged with *AIMLESS* (Evans, 2006). Data-collection statistics are given in Table 1.

2.4. Structure determination and refinement

The structures of five CHDLs (OXA-23, OXA-24/40, OXA-48, OXA-51 and OXA-58) are known and available in the Protein Data Bank (<http://www.rcsb.org>; Berman *et al.*, 2000). The OXA-143 sequence was aligned with the sequences of these five CHDLs using the online *Clustal Omega* server (<http://www.ebi.ac.uk/Tools/msa/clustalo>), with the highest similarity existing between OXA-143 and OXA-24/40 (almost 90% sequence identity; Supplementary Table S1). The *CCP4* program *CHAINS*AW was used to convert OXA-24/40 (PDB entry 3znt; P. Power, E. Sauvage, R. Herman, M. Galleni, G. Gutkind, P. Charlier & F. Kerff, unpublished work) into a pseudo-OXA-143 model by pruning nonconserved residues between the two enzymes using the *CCP4* program *CHAINS*AW (Stein, 2008), such that the sequences were consistent. This pseudo-OXA-143 model was subsequently used to solve the OXA-143 structure using *MOLREP* (Vagin

Table 2

Antimicrobial susceptibility profile produced by the β -lactamase OXA-143.

Antimicrobial	MIC ($\mu\text{g ml}^{-1}$)					
	<i>E. coli</i> JM83			<i>A. baumannii</i> ATCC 17978		
	Control†	OXA-143	Fold change	Control†	OXA-143	Fold change
Ampicillin	2	1024	512	64	4096	64
Amoxicillin	4	2048	512	32	>2048	>64
Oxacillin	256	1024	4	512	2048	4
Ticarcillin	2	2048	1024	8	4096	512
Piperacillin	2	128	64	16	1024	64
Cephalothin	4	8	2	256	512	2
Cefoxitin	2	2	—	32	64	2
Ceftazidime	0.25	0.25	—	2	2	—
Cefotaxime	0.031	0.031	—	8	8	—
Aztreonam	0.062	0.062	—	32	32	—
Imipenem	0.125	1	8	0.125	128	1024
Meropenem	0.031	1	32	0.25	256	1024
Ertapenem	0.004	4	1024	2	1024	512
Doripenem	0.031	1	32	0.125	256	2048

† Parental *E. coli* JM83 and *A. baumannii* ATCC 17978 strains without a β -lactamase gene.

& Teplyakov, 2010), and the resultant structure was initially refined with *REFMAC* (Murshudov *et al.*, 2011). Refinement was later switched to *phenix.refine* (Afonine *et al.*, 2012). The structure was manually rebuilt and water molecules were added using *Coot* (Emsley & Cowtan, 2004). The atomic displacement parameters (ADPs) for all atoms, including water molecules, were refined anisotropically. The final R_{work} and R_{free} values were 13.61 and 16.03%, respectively (see Table 1 for the final statistics).

2.5. Computational methods

Figures were generated with *PyMOL* (DeLano, 2002). Solvent-accessible surfaces were also calculated with *PyMOL*, using a probe radius of 1.4 Å (equivalent to the radius of a single water molecule). Ligand docking to OXA-143 was performed using *ICM-Pro* 3.8-4 (Molsoft; Abagyan & Totrov, 1994; Abagyan *et al.*, 1994). Three different OXA-143 models were prepared: (i) OXA-143^{open+water}, with Val130 in the open conformation and the deacylating water present; (ii) OXA-143^{open}, with the open valine conformation and no deacylating water; and (iii) OXA-143^{closed}, with the closed valine conformation. All water molecules were removed from the models, with the exception of the deacylating water in the OXA-143^{open+water} model. These models were then loaded into *ICM-pro* and converted to ICM objects, with optimization of H-atom placement. The OXA-23–meropenem structure (PDB entry 4j4; Smith *et al.*, 2013) was used to define an initial position for the substrate-binding site in the receptors, prior to the calculation of receptor maps within *ICM-Pro*. The β -lactam substrates were subsequently docked to all three OXA-143 models using the *ICM-Pro* covalent docking procedure. The ligand-docking runs were performed multiple times, and the binding modes for the three receptor models with the two substrates were extracted from *ICM-Pro*.

3. Results and discussion

3.1. Antibiotic-susceptibility profile and enzyme kinetics

Expression of the β -lactamase OXA-143 in *E. coli* JM83 resulted in a significant (64-fold to 1024-fold) increase in the resistance of the microorganism to the majority of penicillins used in this study, with the exception of oxacillin, for which the minimal inhibitory concentration (MIC) value increased only fourfold (Table 2). The enzyme only marginally (twofold) elevated resistance to the narrow-spectrum cephalosporin cephalothin and failed to produce any increase in MICs for the cephamycin cefoxitin, the extended-spectrum cephalosporins cefotaxime and ceftazidime, and the monobactam aztreonam (Table 2). Of the four carbapenem antibiotics tested, OXA-143 elevated the MIC of imipenem eightfold and the MICs of meropenem and doripenem 32-fold, while resistance to ertapenem increased the most significantly at 1024-fold.

Expression of OXA-143 in *A. baumannii* ATCC 17978 resulted in higher MICs for all antibiotics tested (Table 2). When compared with the background parental strain, the most dramatic differences were observed in the MICs of the carbapenems, which were elevated 512-fold to 2048-fold to the clinically significant levels of 128–1024 $\mu\text{g ml}^{-1}$. In fact, of the three other major carbapenemases of *A. baumannii* (OXA-23, OXA-24/40 and OXA-58), OXA-143 produced the highest increase in the MICs of carbapenems (Antunes *et al.*, 2014).

In good agreement with the MIC data, OXA-143 had the highest catalytic efficiency (k_{cat}/K_m) against penicillins (in the range 1.8×10^5 to $1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$), with the lowest value observed for oxacillin (Table 3). Cefepime is a very poor substrate for the enzyme, while the other extended-spectrum cephalosporins, cefoxitin, cefotaxime and ceftazidime, along with the monobactam aztreonam, are not substrates at all (Table 3), which explains why the expression of OXA-143

Table 3
Steady-state kinetic parameters for the β -lactamase OXA-143.

Ceftazidime, cefotaxime, cefoxitin and aztreonam are not substrates of OXA-143. The K_s values in parentheses are given in nM.

Antibiotic	k_{cat} (s^{-1})	K_m (K_s) (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
Ampicillin	1400 ± 100	800 ± 100	$(1.7 \pm 0.3) \times 10^6$
Piperacillin	120 ± 10	140 ± 20	$(8.6 \pm 1.4) \times 10^5$
Ticarcillin	>40	>150	$(2.7 \pm 0.2) \times 10^5$
Oxacillin	>160	>1000	$(1.8 \pm 0.2) \times 10^5$
Cephalothin	>2	>200	$(9.0 \pm 0.2) \times 10^3$
Cefepime	>0.073	>100	$(2.2 \pm 0.2) \times 10^2$
Imipenem	1.3 ± 0.1	0.5 ± 0.1 (320 ± 40)	$(2.6 \pm 0.6) \times 10^6$
Meropenem	0.23 ± 0.01	<0.5 (40 ± 5)	$>4.6 \times 10^5$
Doripenem	0.20 ± 0.01	<0.5 (28 ± 3)	$>4.0 \times 10^5$
Ertapenem	0.17 ± 0.01	<0.5 (12 ± 2)	$>3.4 \times 10^5$

does not result in an increase in the MICs of these antibiotics. Despite the relatively low turnover numbers of OXA-143 with carbapenems ($0.17\text{--}1.3 \text{ s}^{-1}$), the enzyme has a very high apparent affinity for these substrates, which ultimately translates into catalytic efficiencies of $>3.4 \times 10^5$ to $2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Table 3), which are the highest amongst all major CHDLs of *A. baumannii* (Antunes *et al.*, 2014). As the K_m values for carbapenems were very low and we were able to determine only their upper limit, we evaluated K_s values for these substrates, which we found ranged from mid-nanomolar (imipenem) to low-nanomolar (meropenem, doripenem and ertapenem) (Table 3).

3.2. Overall structure of OXA-143

The OXA-143 structure comprises 240 amino-acid residues (36–275) separated into two structural domains (domain 1, residues 36–75 and 207–275; domain 2, residues 76–206;

Figure 2
The OXA-143 structure. (a) Ribbon representation of OXA-143 showing the two structural domains (domain 1, dark green; domain 2, bright green) and the secondary-structure identification. The locations of the active site and of the catalytic serine (Ser81) and lysine (Lys84) are indicated. Three external loops adjacent to the active site, the Ω -loop (yellow), the short $\alpha 4$ – $\alpha 5$ loop (magenta) and the flexible $\beta 4$ – $\beta 5$ loop (cyan), are also indicated. (b) Close-up view of the active-site cleft showing the residues which are important for the catalytic activity of the enzyme. Domain 1 (dark gray ribbons) is on the left and domain 2 (light gray ribbons) is on the right.

Fig. 2*a*). The first 16 residues at the N-terminus of the mature enzyme are not observed in the electron density. The two domains pack tightly against each other such that an extensive interface ($>3000 \text{ \AA}^2$) is buried between them. Superposition of OXA-143 onto the known OXA enzyme structures gives r.m.s.d.s for matching C^α positions ranging from 0.7 $\text{\AA}$ for OXA-24/40 to 1.5 $\text{\AA}$ for OXA-1 (Supplementary Table S1). Not surprisingly, the four enzymes from *A. baumannii* show a significantly greater structural similarity to OXA-143 than those from other bacterial species, as they have the highest amino-acid sequence identities.

The active site of OXA-143 is located in a cleft at the edge of the interface between the structural domains, and includes the important catalytic residues Ser81, Lys84, Trp167, Ser219 and Arg261 (Fig. 2*b*). It is known from the substrate-bound structures of some of the OXA enzymes that the Ser81 residue forms the initial covalent bond in the acylation step to produce the acyl-enzyme intermediate (Golemi *et al.*, 2000; Schneider *et al.*, 2009, 2011; Smith *et al.*, 2013). Two residues from the N-terminal domain, Ser219 and Arg261, interact with the carboxylate group of the substrate and serve to orient the substrate correctly in the active site. The side chain of Lys84 is carboxylated in the OXA-143 structure, and this was confirmed by the calculation of initial $F_o - F_c$ electron-density maps with the carboxylate group absent (Fig. 3*a*). The carboxylate is held in place *via* a bifurcated hydrogen-bonding interaction with the $N^{\epsilon 1}$ atom of the conserved Trp167 (Fig. 3*a*) on the Ω -loop between helix $\alpha 6$ and strand $\beta 6$ (Fig. 2*a*). This Ω -loop is in the same topological location as the Ω -loop identified in the class A enzymes (Banerjee *et al.*, 1998). Carboxylation of the lysine is a key component of the deacylation step in the OXA enzymes, whereby a water molecule positioned between the lysine and the catalytic Ser81

is deprotonated by the carboxylate moiety. This activated water molecule then attacks the acyl bond, leading to hydrolysis and release of the deactivated substrate.

The carboxylated Lys84 is sandwiched between the Ω -loop and a short highly conserved loop between helices $\alpha 4$ and $\alpha 5$ (Fig. 2*a*). Two juxtaposed highly conserved hydrophobic residues, Leu168 on the Ω -loop and Val130 on the $\alpha 4$ – $\alpha 5$ loop, flank the catalytic lysine (Fig. 3*b*). The side chain of Val130 exists in two conformational states (Figs. 3*a* and 3*b*) in both molecules of OXA-143 in the asymmetric unit. In one conformation the $C^{\gamma 1}$ and $C^{\gamma 2}$ atoms are both directed away from Ser81, hereafter called the *open* Val130 conformation. In the second conformation the $C^{\gamma 2}$ atom projects towards the serine, and this is referred to as the *closed* Val130 conformation. The open Val130 conformation creates a small pocket bounded by Ser81, Lys84, Val130 and Leu168, which allows a partially occupied water molecule to be present, hydrogen-bonded to one of the O atoms of the carboxylated Lys84 and to the O^γ atom of Ser81. The water molecule is approximately 3.6 $\text{\AA}$ from the closest atom of Val130 (C^β) when the side chain is in the open conformation, which is beyond the van der Waals close-contact distance between an O atom and a C atom. The relocation of the $C^{\gamma 2}$ atom of Val130 in the closed conformation effectively blocks this water pocket, since the $C^{\gamma 2}$ atom and the water would be only 2.3 $\text{\AA}$ apart, a highly unfavorable close contact (Fig. 3*b*). The occupancy of this water molecule was linked to the occupancy of the open Val130 conformation during refinement with *phenix.refine* (Afonine *et al.*, 2012) and gave rise to a self-consistent average occupancy for the pair of 67%. This was subsequently validated by removing the water molecule from the structure, resetting the occupancies of the two valine conformers to 50% and re-refining their partial occupancies. In the absence of the

water molecule, the valine side-chain occupancies refined to a 67:33 ratio favoring the open conformation.

Two OXA-143 models were subsequently generated, each containing only one of the two observed Val130 conformers. Calculation of the solvent-accessible molecular surface of OXA-143 shows that when Val130 is in the closed conformation there is a continuous hydrophobic surface (Fig. 4*a*) created between the valine side chain and the side chain of Leu168, and the carboxylated Lys84 is shielded from the external environment. Conversely, when Val130 is in the open conformation a significant hole is opened in the molecular surface both in the apoenzyme and the acyl-enzyme intermediate, such

Figure 3

The OXA-143 active site. (a) OMIT $2F_o - F_c$ density for the Val130 side chain (green mesh) on the $\alpha 4$ – $\alpha 5$ loop (magenta) shows that both conformations of the valine are present. Residual $F_o - F_c$ electron density (pink mesh), calculated following molecular replacement, shows the presence of a carboxylate moiety attached to the N^ϵ atom of Lys84. Final $2F_o - F_c$ density (blue mesh) is shown for the refined carboxylated lysine side chain, the catalytic serine (Ser81) and the Trp167 side chain on the Ω -loop (yellow). (b) Close-up view of the immediate environment around the catalytic Lys84 residue. The two conformations of the Val130 residue on the $\alpha 4$ – $\alpha 5$ loop (magenta) are shown. In the open conformation (green), the closest C atom (C^β) is 3.6 $\text{\AA}$ away from a partially occupied water molecule (Wat; magenta sphere). In the closed conformation (red) of the valine, the closest C atom ($C^{\gamma 2}$) is only 2.3 $\text{\AA}$ away from the water molecule. The location of the juxtaposed Leu168 residue on helix $\alpha 7$ of the Ω -loop (yellow) is also indicated.

that the lysine is exposed (Fig. 4*b*). The formation of a surface channel leading directly from the external solvent into the active site near the lysine allows an unimpeded exchange of water between the milieu and the active site of OXA-143, and creates a space to accommodate the deacylating water.

3.3. Modeling of carbapenem-bound OXA-143

To understand how the open and closed conformations of Val130 in OXA-143 influence the binding of carbapenem substrates, and to validate the flexibility of Val130 in the presence of these last-resort antibiotics, docking studies with meropenem and doripenem in three protein–receptor models derived from the apo OXA-143 structure were performed. The three receptors are designated OXA-143^{open+water} (open Val130 conformation with water), OXA-143^{open} (open Val130 conformation without water) and OXA-143^{closed} (closed Val130 conformation). The docking results are given in Supplementary Table S2. Both carbapenems show the best docking in the OXA-143^{open} receptor, as shown for meropenem and doripenem in Supplementary Figs. S1(*a*) and S1(*b*), respectively. Docking into the OXA-143^{closed} receptor shows a slightly decreased binding affinity (Supplementary Table S2). In the presence of the deacylating water (receptor OXA-143^{open+water}), however, the docking scores for meropenem and doripenem are significantly lower, indicating a further decrease in binding affinity. Comparison of the binding energies (Supplementary Table S2) shows that the lower scores are driven primarily by large decreases in the van der Waals interaction energies, along with modest decreases in the energetics of the hydrogen-bonding interactions (Supplementary Table S2). Regarding the binding of carbapenem substrates, in the OXA-143^{closed} receptor the pyrroline rings and the 6 α -hydroxyethyl groups of the docked meropenem and doripenem are in almost identical positions. Rotation of the Val130 side chain from the closed to the open conformation (OXA-143^{open} receptor) creates a larger space between this residue and the juxtaposed Leu168, and the bound carbapenems move approximately 0.7 Å towards the valine (Supplementary Fig. S2). The 6 α -hydroxyethyl group now effectively blocks the pocket which originally housed the closed valine residue, such that movement from the open to the closed conformation would result in a serious steric clash with the substrate (Supplementary Fig. S2). This suggests that once the acyl-enzyme intermediate has been formed (and any resident water molecule has been expelled) the valine residue is locked into an open conformation by the 6 α -hydroxyethyl moiety

and the water channel is maintained in an open configuration.

When the water molecule is present (the OXA-143^{open+water} receptor), the hydroxyethyl group of the carbapenem is pushed out into the active site by approximately 1.7 Å from the position in the OXA-143^{open} receptor. This movement is facilitated by a pivoting of the pyrroline ring by about 15–20°, with the acyl bond remaining intact. This results in a highly unfavored steric clash between the 6 α -hydroxyethyl group and the water molecule, as shown by a large decrease in the van der Waals interaction energy for both substrates (Supplementary Table S2) of between 10 and 15 kcal mol^{−1}.

Our docking experiments show that the simultaneous presence of both the acylated substrate and the water molecule that we observe in the active site of the apo OXA-143 structure with the open Val130 conformation is energetically unfavored, and acylation of the carbapenem substrate would ultimately lead to the expulsion of this water from its position and movement of the substrate further into the binding pocket. Thus, the water molecule that we observe in OXA-143 with the open Val130 conformation, hydrogen-bonded to the carboxylated lysine and the catalytic serine, cannot act as the deacylating water molecule: either it has to be pushed away by the bound antibiotic to a new position where it can perform deacylation, or another water molecule must come from the external milieu to fulfil this role.

3.4. Formation of an alternative channel into the active site of class D carbapenemases

The very close similarity between OXA-143 and OXA-24/40 (90% sequence identity; Supplementary Table S1) suggests that the mechanisms underlying carbapenem acylation and deacylation in these two enzymes might be similar. Comparison of the docked poses for the two carbapenem substrates in the OXA-143^{open} receptor shows that they closely resemble the observed substrate-binding mode in the crystal structure of OXA-24/40 (Schneider *et al.*, 2011).

Figure 4

Solvent-accessible surface representation of the OXA-143 active site. (*a*) The surface of OXA-143 (white) calculated with Val130 in the closed conformation, showing a continuous hydrophobic surface between Val130 and Leu168, which buries the carboxylated Lys84 residue. (*b*) The surface of OXA-143 calculated with Val130 in the open conformation, showing a hole which opens into the active site. The side chain of the carboxylated Lys84 (green ball-and-stick representation) can be seen at the bottom of the hole. The surface of doripenem based upon its docking into the enzyme with Val130 in the open conformation is shown in semi-transparent green, with the doripenem in green ball-and-stick representation.

Superposition of the doripenem–OXA-143^{open} model onto the OXA-24/40–doripenem structure (PDB entry 3pae; Schneider *et al.*, 2011) is shown in Fig. 5(a). One feature which is immediately obvious from this superposition is that the equivalent valine residue in OXA-24/40 (Val130 in OXA-24/40 numbering) is also in an open conformation. Examination of the apo OXA-24/40 structure (PDB entry 2jc7; Santillana *et al.*, 2007) shows, however, that a closed Val130 conformation is favored in the absence of substrate. This suggests that mobility of the valine residue also plays a key role in the deacylation mechanism of OXA-24/40, not only by allowing carbapenem substrates to bind deeper into the active site in a more energetically favored position but also by facilitating the opening of a transient channel to the surface in the same way as we have outlined for OXA-143. Moreover, both our modeling experiments with OXA-143 and the structure of OXA-24/40 show that the 6 α -hydroxyethyl group of the bound carbapenem would not allow Val130 to flip back to a closed configuration because of the ensuing steric hindrance, thus maintaining the channel in an open configuration.

The formation of a channel into the active site has also been previously described for OXA-23, wherein a similar hydrophobic surface adjacent to the carboxylated lysine is formed between the juxtaposed residues Val128 and Leu166 (OXA-23 residue numbering) in the apo form of the enzyme (Smith *et al.*, 2013). In the OXA-23–meropenem complex it is the leucine residue (Leu166 in OXA-23 numbering) which moves; it is forced to adopt an alternate rotamer conformation by steric contact from the 6 α -hydroxyethyl group of the incoming meropenem substrate. The Val128 residue remains in the closed conformation, which results in the 6 α -hydroxyethyl group of the carbapenem substrate sitting slightly further out of the deacylation pocket in an almost identical position to the modeled carbapenem substrates in the OXA-143^{closed}

receptor. Superposition of the meropenem–OXA-143^{closed} model onto the OXA-23–meropenem structure (PDB entry 4jff4) is shown in Fig. 5(b).

Analysis of the structure of the CHDL OXA-23 demonstrated that the hydroxyethyl moiety of the acylated meropenem would prevent the entry of a deacylating water into the vicinity of the carboxylated lysine through the entrance to the active site. It was also shown that the channel formed by the movement of the conserved hydrophobic Leu166 residue would allow a water molecule from the milieu to bypass the 6 α -hydroxyethyl group and enter near the lysine to perform deacylation (Smith *et al.*, 2013). Our current data with OXA-143 show that in this CHDL a transient water channel, similar to that in OXA-23, is formed into the active site of this enzyme, although in OXA-143 this channel is opened not by the conserved leucine but by the juxtaposed conserved valine residue. This is supported by our observation that the equivalent valine residue is able to move upon the binding of carbapenem to OXA-24/40, a close relative of OXA-143.

3.5. Proposed deacylation mechanism for the *A. baumannii* CHDLs

Analysis of the structures of OXA-143, OXA-24/40 and OXA-23 suggest that these three subfamilies of CHDLs may operate *via* a single mechanism. Formation of acyl-enzyme intermediates of these β -lactamases with carbapenem antibiotics leaves no space in the active site for a water molecule, which would prevent the second step of catalysis: deacylation. However, the movement of either a conserved leucine or valine residue juxtaposed on the surface of the enzymes would open a channel into the active site. A water molecule from the milieu would move into this channel, where it would be activated by the carboxylated lysine for deacylation. Our data from OXA-143, coupled with analyses of OXA-24/40 and OXA-23, indicate that there could be some variation in the way that the channel is formed. In OXA-143 the channel forms even in the absence of the acylated antibiotic and is transient in nature, where the Val130 residue is continuously testing both the open and closed conformations in the apo-enzyme. Our modeling experiments demonstrate that formation of the acyl-enzyme intermediate of OXA-143 with carbapenems would lock the valine residue into an open conformation, and that the partially occupied water molecule that is present only when Val130 is open must necessarily be expelled. In OXA-24/40 the channel is closed in the

Figure 5

Comparison of docked OXA-143 models with CHDL crystal structures. (a) The active site of the doripenem–OXA-143^{open} receptor (gray secondary structure and sticks) with the open Val130 colored green. The docked doripenem is shown as pale green sticks. For comparison, the OXA-24/40–doripenem complex (PDB entry 3pae) was superimposed based upon residues in the active site. For this complex, only residues Val130 and Leu168, along with the doripenem substrate, are shown in magenta. (b) The active site of the meropenem–OXA-143^{closed} receptor (gray secondary structure and sticks) with the closed Val130 colored red. The docked meropenem is shown as pink sticks. For comparison, the OXA-23–meropenem complex (PDB entry 4jff4) was superimposed based upon residues in the active site. For this complex, only residues Val128 and Leu166 (labeled in italics), along with the meropenem substrate, are shown in cyan.

apoenzyme structure, but formation of the acyl-enzyme intermediate with doripenem results in transition of the conserved valine into the open conformation. Similar to OXA-24/40, the channel is closed in the apoenzyme structure of OXA-23. In the acyl-enzyme intermediate of this enzyme with meropenem it is not the valine but the conserved juxtaposed leucine residue (Leu166 in OXA-23 numbering) which moves into the open conformation to form the channel into the active site. The 6 α -hydroxyethyl group of the acylated carbapenems not only forces the transition of the conserved valine or leucine residue into the opened conformation, but also maintains the open channel by not allowing the residues to flip back.

Although the accepted mechanism of carbapenem hydrolysis comprises two distinct steps, acylation and deacylation, the process should be envisaged as a continuum. The structural details gained from apo OXA-143 and the docked models, in conjunction with the apo structures and carbapenem complexes of OXA-24/40 and OXA-23, serve as static snapshots at different stages along the reaction trajectory; namely, the pre-acylation state with the channel closed (OXA-24/40 and OXA-23) or either opened or closed (OXA-143), and the post-acylation state where the channel is locked in an open configuration in all three enzymes by the intervening 6 α -hydroxyethyl group of carbapenems. The stereochemistry at the C6 atom of the β -lactam ring of the carbapenems invariably causes the hydroxyethyl group at this position to project towards the carboxylated lysine following acylation (in penicillins and cephalosporins, the C6 substituent projects outward). Although this would be expected to impede deacylation in the class D enzymes, the CHDLs have overcome this by gaining the ability to open the transient channel behind the 6 α -hydroxyethyl group to house the deacylating water molecule.

4. Conclusion

Carbapenem-hydrolyzing class D β -lactamases are enzymes of the utmost importance, as they are capable of inactivating clinically important carbapenem antibiotics such as imipenem, meropenem, doripenem and ertapenem. The worldwide spread of CHDLs in *A. baumannii* and Enterobacteriaceae has severely compromised the utility of these last-resort β -lactams for the treatment of various life-threatening infections. Over many years of extensive research, three different mechanisms by which CHDLs exert their carbapenemase activity have been postulated. However, the similarity of the CHDL structures and functions argues against multiple mechanisms of carbapenemase activity for these closely related enzymes. Our structural and modeling studies of OXA-143, an efficient CHDL that produces high levels of resistance to carbapenem antibiotics, and the comparative analysis of related structures of OXA-24/40 and OXA-23 CHDLs in complex with carbapenems, have provided important insights into how these enzymes have developed their carbapenemase activity.

Given the high levels of sequence conservation of the leucine and valine residues responsible for opening the transient water channel in the class D carbapenemases (85 and 89%, respectively, among 211 known CHDL sequences; the remaining enzymes harbor other small aliphatic residues at these positions), it is highly likely that all CHDLs will utilize the above-described mechanism of deacylation to exert their carbapenemase activity. Because the transient channel still allows the deacylating water to access the carboxylated lysine and the acyl bond, essentially bypassing the blockage caused by the 6 α -hydroxyethyl group, one could envision modified carbapenem compounds with bulkier side chains at the C6 position which, while they may still trigger the opening of the channel, would significantly further restrict the space available and permanently impede water access.

5. Related literature

The following references are cited in the Supporting Information for this article: Bondi (1964).

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