The First Structural and Mechanistic Insights for Class D β-Lactamases: Evidence for a Novel Catalytic Process for Turnover of β-Lactam Antibiotics

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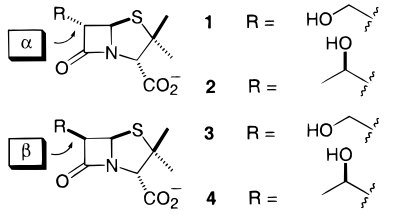
β-Lactamases hydrolyze β-lactam antibiotics, and as such serve as the primary cause of bacterial resistance to these antibiotics. Four classes of β-lactamases, classes A, B, C, and D, are known. Classes A, C, and D follow the active-site-serine mechanism in their chemistries, and class B enzymes are zinc-dependent.[1,2] Class D β-lactamases are the smallest (27 kDa) among active-site-serine β-lactamases, and they are the least studied. To date, more than 20 of these enzymes have been identified. Class D β-lactamases lack any overall amino acid sequence homology to class A and C enzymes. These enzymes have become important clinically with the recent discoveries of new variants such as Oxa-11 and Oxa-14 to Oxa-20, which show an extended-spectrum profile.[3] The genes for most of these enzymes are borne on integrons or on plasmids,[4] which facilitate their dissemination among various organisms.

The current knowledge about the catalytic mechanism of the class D β-lactamases is extremely limited and there is no known crystal structure for any of these enzymes. We have undertaken to study the Oxa-10 β-lactamase from *Pseudomonas aeruginosa*, a human pathogen. We present herein the X-ray structure of this enzyme, which is the first such structure for any class D β-lactamase. Crystals of Oxa-10 diffracted to at least 2.4 Å resolution. They belong to space group $P2_1$ with cell parameters of $a = 66.4$ Å, $b = 89.4$ Å, $c = 101.5$ Å, $\beta = 95.4°$.[5] The structural and kinetic results indicate this to be a novel enzyme with a distinct catalytic machinery.

The comparison of the structure of the Oxa-10 β-lactamase with those of the class C enzyme from *Enterobacter cloacae* P99 and of the class A TEM-1 enzyme from *Escherichia coli* conclusively indicates that the class D and class A enzymes share an overall common fold, although the distribution of secondary structure elements in space varies considerably. A remarkable feature is the nearly perfect superimposition of all atoms from residues providing the catalytic machinery for acylation (Ser-67, Lys-70, Ser-115, Lys-205 in Oxa-10 and Ser-70, Lys-73, Ser-130, Lys-234 in TEM-1) (rmsd = 0.5 Å).

The first difference occurs in the connection between helices αC and αD (Figure 1A; residues 98 and 99 shown in green). Instead of the classical long loop between helix αH and strand β3 that lines the active site in class A enzymes, a three-residue long strand (β3a) is found in Oxa-10. This feature, which has never been observed in any β-lactamase, provides an extension of the substrate-binding site. This groove is defined by residues from strands β3a, β3, and β4 and the C-terminal helix, and may either be a reminiscent vestige of the substrate-binding site of an ancestor to class D β-lactamases or it indicates that class D enzymes may have another function that remains to be characterized. The last highly significant feature in the Oxa-10 enzyme is the completely new fold of the Ω loop between residues 141 and 159. The loop is reduced in size compared to that in class A enzymes, and very importantly, it bears no acidic amino acid that would act as a general base in promotion of the hydrolytic water for deacylation of the acyl-enzyme intermediate, such as is the case in class A β-lactamases.

The striking identical spatial positions of the serines and lysines in the active sites of class A and D enzymes underscore the statement that these residues, involved in enzyme acylation, were handed down from the ancestral penicillin-binding proteins.[2] The similarity of the acylation machineries between class A and D enzymes is strengthened by the presence of an oxyanion hole provided by the main chain nitrogen atoms of Ser-67 and Phe-208. The identical spatial location of all of these atoms enabled us in model building of the acyl-enzyme species of Oxa-10 (not shown), based on the knowledge of such complexes in the X-ray data for the TEM-1 β-lactamase.[6]



This model indicates that the approach of a water molecule to the ester of the acyl-enzyme species is hindered from the β-face. The approach of the hydrolytic water in the class A enzymes is from the α-face. This would appear to be the case for the class D Oxa-10 enzyme as well, since the α-face is open and exposed to the milieu.

We have used the 6α-hydroxyalkylpenicillanates, such as **1** and **2**, as effective inhibitors of class A β-lactamases. These compounds acylate the active site serine and resist deacylation in class A enzymes by the virtue of the fact that the hydroxyalkyl moiety makes a critical hydrogen bond to the hydrolytic water. This interaction both sterically prevents the approach of the hydrolytic water and attenuates its basicity.[6,7] In a further application of this principle, we have shown that a judicious

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(5) The final model comprises 243 residues per molecule and four molecules per asymmetric unit. Six Au(CN)-2 species, five sulfate ions, and 461 water molecules were included in refinement. The final *R* factor was 0.187 (R_{free} is 0.254) for all reflections between 20 and 2.4 Å; rms deviations from ideal geometry are: bond length 0.008 Å, angle 1.9°, plane distance 0.007 Å. Ultracentrifugation analysis of the protein indicated that the Oxa-10 β-lactamase is a dimeric protein in solution in agreement with the similar observation of the crystal packing.

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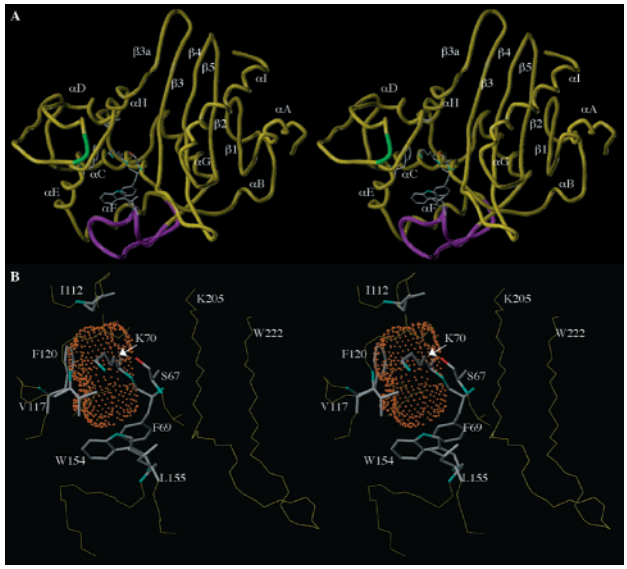

Figure 1. (A) Stereo view of the main-chain tracing of the 2.4 Å X-ray crystal structure of the Oxa-10 β-lactamase. The region in green corresponds to the residues 98 and 99, in magenta corresponds to the Ω-loop. (B) Stereo view of the active site region is depicted. The hydrophobic pocket is shown as a dot surface in orange. Backbone of the enzyme is shown in yellow. In both panels, amino acid residues in the active site are shown as capped sticks, and color-coded according to the atom type (carbon in white, oxygen in red, and nitrogen in cyan).

hydrogen bond formation by the inhibitor to the active site of serine protease chymotrypsin may even prevent enzyme acylation upon formation of the noncovalent preacylation complex.[8] We have used the penicillanate derivatives **1**−**4** for probing the mechanism of the Oxa-10 β-lactamase. Compounds **3** and **4** are substrates for the enzyme.[9] On the other hand, compounds **1** and **2** are not turned over, but serve as competitive inhibitors for the Oxa-10 β-lactamase, with the K_i values of 1.9 ± 0.1 and 1.2 ± 0.1 mM, respectively. Compounds **1** and **2** did not acylate the enzyme, suggesting that binding of these two inhibitors in the active site impairs the machinery for enzyme acylation.[10] In light of these observations with compounds **1**−**4**, we conclude that the hydrolytic water molecule approaches the ester of the acyl-enzyme intermediate from the α-side (since **3** and **4** are turned over), as further supported by the X-ray structure.

It is important to note that there is no residue besides Lys-70 that can serve as a general base on the α-side to promote the hydrolytic water for deacylation of the acyl-enzyme intermediate. Hence, clearly the mechanisms of deacylation for all three active-site-serine β-lactamases, classes A, C, and D, appear to be distinct. Class A enzymes have a general base (Glu-166) that activates the hydrolytic water from the α-face.[2,6,11] Class C enzymes promote the hydrolytic water from the β-face[12] and there would appear to be a substrate-assisted component to the catalytic process.[2,13] The results reported here indicate that class D β-lactamases also promote the hydrolytic water molecule from the α-face, but the process of water activation is different. The conceivable scenario for promotion of the hydrolytic water involves Lys-70. The side chain nitrogen of Lys-70 in Oxa-10 is ensconced in a rather large hydrophobic pocket (Figure 1B), in contrast with the crowded and polar environment of the corresponding Lys-73 in class A enzymes. This hydrophobic environment is made up of the side chains of Phe-69, Val-117, Phe-120, Trp-154, and Leu-155,[14] and gives room for significant conformational flexibility for Lys-70 (Figure 1B). On acylation of the active site, the substrate would cap the top of the hydrophobic pocket and this environment would be completely shielded from solvent in the acyl-enzyme complex. The low dielectric constant for this pocket may mandate that Lys-70 exist in the free base form. Hence, Lys-70 is likely to be involved in both activation of Ser-67 for enzyme acylation by the substrate[2,15] and promotion of the hydrolytic water for deacylation. Therefore, it would appear that the class D β-lactamases exhibit symmetry in the two catalytic steps, a situation not seen for other β-lactamases. In summary, the mechanism of class D Oxa-10 β-lactamase is distinct among known β-lactamases. Since class B β-lactamases are zinc-dependent and operate by a different mechanism yet,[16] it is clear that nature has devised at least four distinct approaches for destruction of β-lactams in manifestation of resistance to these antibiotics.

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Supporting Information Available: Experimental procedures are provided (PDF). The crystallographic coordinates are deposited in the Brookhaven Protein Data Bank. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Our kinetic analysis of the Oxa-10 β-lactamase with several substrates indicates that the turnover competence of the enzyme depends strongly on the substituents that are found at the C6β and C7β of typical penicillins and cephalosporins, respectively. The absence of these functionalities makes the molecule a poor substrate. These findings are reflected in turnover data for compounds **3** and **4**. They acylate the enzyme and then undergo deacylation, the latter process being the slower one. The k_{cat}/K_m values for **3** and **4** are 1960 ± 80 M^{-1} s^{-1} and 6050 ± 406 M^{-1} s^{-1}, respectively.

(10) A model of the preacylation complex of **1** bound to the enzyme active site indicated that the hydroxymethyl moiety would form a hydrogen bond with the Lys-70 side chain. Such an interaction would impair the ability of Lys-70 to activate Ser-67 for the enzyme acylation event.

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