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Structures of the Michaelis Complex (1.2 Å) and the Covalent Acyl Intermediate (2.0 Å) of Cefamandole Bound in the Active Sites of the *Mycobacterium tuberculosis* β-Lactamase K73A and E166A Mutants[†,‡]

Lee W. Tremblay, Hua Xu, and John S. Blanchard*

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, United States

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ABSTRACT: The genome of *Mycobacterium tuberculosis* (TB) contains a gene that encodes a highly active β-lactamase, BlaC, that imparts TB with resistance to β-lactam chemotherapy. The structure of covalent BlaC−β-lactam complexes suggests that active site residues K73 and E166 are essential for acylation and deacylation, respectively. We have prepared the K73A and E166A mutant forms of BlaC and have determined the structures of the Michaelis complex of cefamandole and the covalently bound acyl intermediate of cefamandole at resolutions of 1.2 and 2.0 Å, respectively. These structures provide insight into the details of the catalytic mechanism.

β-Lactams make up of the most important classes of antibacterial chemotherapeutics in clinical use today. These compounds disrupt bacterial cell wall biosynthesis by irreversibly inhibiting the D,D-transpeptidases that are responsible for cross-linking the pentapeptide components of polymerized peptidoglycan precursors via specific peptide linkages. The cross-linked peptidoglycan stabilizes the bacterium against high internal osmotic pressure (*1*−*3*). β-Lactams have proven to be ineffective in the treatment of infections by *Mycobacterium tuuberculosis* (TB), a pathogen infecting nearly one in three people across the globe (*4*). *M. tuberculosis* harbors a genomically encoded extended-spectrum β-lactamase (ESBL), BlaC (*5*). BlaC has been shown to rapidly hydrolyze penicillin, cephalosporin, and carbapenem classes of β-lactams (*6*). BlaC and other β-lactamases bind β-lactams and form a covalent acyl intermediate, like the D,D-transpeptidases, but have evolved an activity that results in the hydrolysis of the covalently bound product in the active site (*7*). As a result, β-lactamase catalysis effectively destroys the antibiotic, thwarting β-lactam chemotherapy.

β-Lactamases have been categorized according to two different schemes. The Ambler system classifies these enzymes according to sequence homology as Ambler classes A−D (*8*), while more recently, Bush, Jacoby, and Medeiros have devised groups 1−4 based on substrate/inhibitor profiles (*9*). BlaC is a class A, group 2 ESBL. The class A β-lactamases are structurally conserved with the penicillin binding protein (PBP) domain of the D,D-transpeptidases and are likely the result of divergent evolution (*10*). In recent years, there has been a dramatic increase in the number of group 2 ESBL enzymes isolated from antibiotic resistant strains of bacteria resulting from the broad utilization of β-lactams (*9*, *11*). In contrast to the more recently identified β-lactamase isozymes, the genomically encoded *blaC* gene product likely has ancient evolutionary origins dating back to the original divergence of the class A and class D β-lactamases (*12*). Episodic positive selective pressure has been placed throughout history on these two classes of β-lactamases, causing significant deviations from the mutational molecular clock by which the phylogeny would normally be deduced, yet it has been established that the serine-type β-lactamases have been evolving since a period before the divergence of Gram-negative and Gram-positive bacteria, implying they have had an active protective role for more than 2 billion years (*9*, *12*).

The class A β-lactamases have been investigated for the past 70 years (*13*), and a common mechanism of catalysis has emerged. The catalytic mechanism of class A β-lactamases occurs in three steps: binding of the β-lactam to generate the Michaelis complex, attack by the conserved serine nucleophile (Ser70 in BlaC) to form a ring-opened covalent acylated enzyme intermediate, and activation of a conserved active site water molecule for hydrolysis of the covalent acyl intermediate from the enzyme allowing the hydrolyzed product to exit the active site. A number of previous studies have identified the active site of various β-lactamases bound with dead end inhibitors or mutant forms of the enzymes that have been able to "trap" the acyl intermediate for observation (*6*, *14*−*21*). In this study, we have employed mutational techniques to obtain high-resolution crystallographic data of the BlaC active site bound with the second-generation cephalosporin, cefamandole, in both the Michaelis complex and the covalent acyl intermediate, providing us with structural data relevant to the mechanism of the serine-type β-lactamase, BlaC.

The enzymatic mechanism of BlaC relies on two highly conserved active site residues, Lys73 and Glu166, which are involved in the activation of the acylating nucleophile (Ser70) and the activation of the active site water molecule for deacylation (*22*, *23*), respectively. Studies of the Glu166Ala mutant of BlaC have revealed that this residue is essential for deacylation, via hydrolysis by a hydrogen-bonded water molecule, of covalently acylated BlaC. It has been assumed that the ε-amino group of Lys73 in BlaC, by forming a hydrogen bond to the Ser70 hydroxyl group, increases the nucleophilicity of the side chain hydroxyl, permitting attack at the β-lactam carbonyl carbon. We have prepared and crystallized the K73A and E166A mutants of BlaC and have determined the structures of the Michaelis complex of cefamandole (Figure 1A) and the covalently bound acyl intermediate of cefamandole (Figure 1B) at resolutions of 1.2 and 2.0 Å, respectively (Table 1 of the Supporting Information).

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[‡]Coordinates for the three-dimensional structures have been deposited with as Protein Data Bank entries 3NY4 and 3N8S.

*To whom correspondence should be addressed: Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461. Telephone: (718) 430-3096. Fax: (718) 430-8565. E-mail: blanchar@aecom.yu.edu.

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BlaC catalyzes the near diffusion-limited hydrolysis of many penicillins and cephalosporins, yet prior observations have associated the rate-limiting step with the second deacylation half-reaction (*6*, *24*). In the case of cefamandole, the turnover number of the wild-type BlaC enzyme is 3500 min^{-1} with a k_{cat}/K_m value of 2×10^7 M^{-1} min^{-1} (*6*). The K73A mutant exhibits a 2×10^5-fold decrease in cefamandole activity with a k_{cat}/K_m value of 84 M^{-1} min^{-1}, and a more than 10-fold increase in the K_m value, making accurate measurements of k_{cat} impossible because of the high absorbance. The slow reaction rate of BlaC K73A allowed us to trap the Michaelis complex of cefamandole and determine the three-dimensional structure of the BlaC K73A−cefamandole complex before the attack of the Ser70 nucleophile. This represents unambiguous evidence of the role of Lys73 as the primary activator of the Ser70 nucleophile in the first acylation half-reaction.

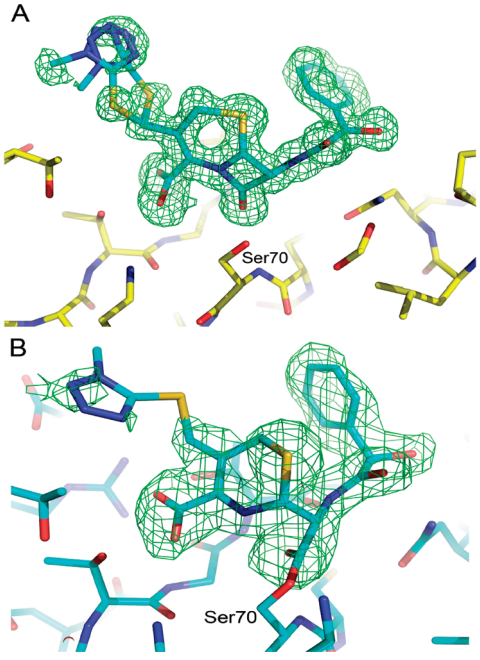
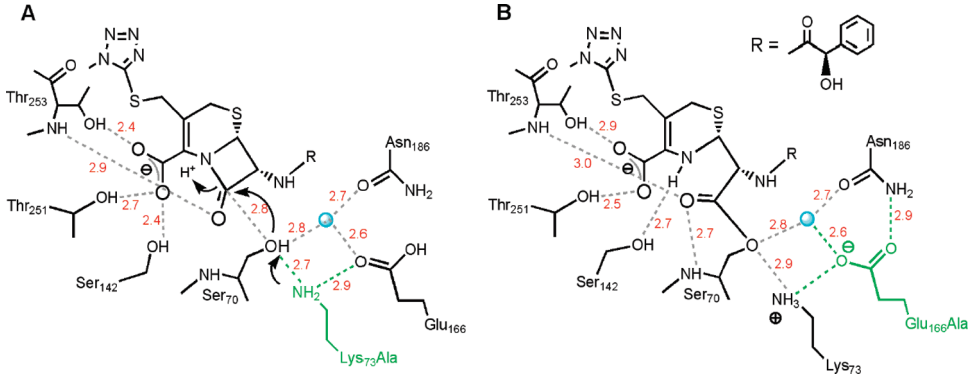


FIGURE 1: (A) $F_o - F_c$ omit density (1.2 Å) of the cefamandole (blue carbons) Michaelis complex bound in the active site of BlaC K73A (yellow carbons). (B) $F_o - F_c$ omit density (2.0 Å) of the cefamandole−BlaC E166A covalent adduct (all blue carbons). These figures were produced with Pymol and contoured at 2σ.

In the Michaelis complex, the cefamandole carboxylate group is hydrogen bonded by the side chain hydroxyl groups of Ser142, Thr253, and Thr251 at 2.4, 2.4, and 2.7 Å, respectively (Scheme 1A). In addition, there is a 2.9 Å hydrogen bond between the Thr253 backbone amide nitrogen and β-lactam carbonyl oxygen, one of two interactions that stabilize the developing negative charge at the β-lactam carbonyl oxygen. These initial contacts with the β-lactam carboxylate act as a hinge around which the β-lactam ring carbonyl oxygen is swung into contact with the oxyanion hole, composed of the backbone amide nitrogens of both Thr253 and Ser70. Hydrogen bonding interactions also exist between the side chain hydroxyl of Ser70 and K73 (inferred from native and E166A structures), along with the catalytic water molecule and Glu166. The cefamandaole side chain exists in two conformations as evidenced by the clear electron density of the thioether sulfur.

When the cefamandole Michaelis complex structure is compared with that of the acylated adduct captured using BlaC E166A (Scheme 1B), multiple changes suggest the order of catalytic events. First, the β-lactam ring carbonyl oxygen is drawn deeper into the oxyanion hole, allowing attack at the β-lactam carbonyl carbon. While maintaining an initial contact distance of 2.9−3.0 Å with the Thr253 backbone amide nitrogen, the β-lactam carbonyl oxygen shifts (from 3.1 to 2.7 Å) closer to the Ser70 backbone amide. The effect of being drawn further into the oxyanion hole is that the position of the β-lactam ring carbonyl carbon moves from a distance of 2.8 Å from the Ser70 hydroxyl oxygen atom to form a covalent bond (1.3 Å) with the hydroxyl group, generating the ester bond and opening the β-lactam ring. The Ser70 hydroxyl side chain position appears unchanged between the wild-type apo Michaelis complex and acylated covalent intermediate, indicating that the β-lactam ring moves into contact with a rigid Ser70 side chain. In concert with these movements, the cefamandole carboxylate shifts from the initial binding positions of 2.4 to 2.9 Å with the Thr253 hydroxyl and from 2.7 to 2.5 Å with the Thr251 hydroxyl, while the hydrogen bond with the Ser142 side chain hydroxyl is broken. In related structures of carbapenem β-lactams covalently bound to BlaC, β-lactam ring opening is accompanied by a tautomerization of the α,β-unsaturated eneamine to the corresponding imine, with protonation at the side chain (*15*). In this case, the 1,3-thiazine ring generated upon cefamandole ring opening retains the carbon−carbon double bond of the eneamine. This may be due to the observation that as the 1,3-thiazine ring nitrogen is

Scheme 1: Model of the Active Site of (A) the Cefamandole−BlaC K73A Michaelis Complex and (B) the Acyl Adduct of Cefamandole with the BlaC E166A Mutant[a]



[a]Implied residues and bonds from alternate BlaC structures are colored green.

protonated, the Ser142 hydroxyl exchanges its hydrogen bond from the carboxyl group and flips nearly 70° to hydrogen bond to the newly formed secondary amine. This active site rearrangement, along with decreases in the hydrogen bonding distance between the cefamandole R group secondary hydroxyl and the carboxamide side chain of Asn186, helps to stabilize the covalently bound cefamandole intermediate (*25*).

Essential to the deacylating second half-reaction is the conserved active site water. In the first half-reaction, Lys73 gains a proton upon deprotonation of the Ser70 hydroxyl. This likely causes a change in the p*K* value of the neighboring Glu166 side chain, generating the general base required for the activation of the water molecule and deacylation. This water is also tightly associated with Asn186, which together with Glu166 serves to orient the water molecule and activate it as a nucleophile. Prior to the formation of the acyl intermediate, the activated water is positioned 3.4 Å from the β-lactam ring carbonyl carbon. After the attack by Ser70 and the formation of the covalent ester bond, the carbonyl carbon is an estimated 2.1 Å from the activated water (as determined by superposition of the wild-type apo BlaC catalytic water position). The estimated water distances of 2.6 Å from E166 and 2.1 Å from the carbon would allow for rapid and efficient deprotonation and "hydroxide" attack. The partial positive charge on the ester carbonyl, caused by the influence of the oxyanion hole residues, and the alignment of the water/hydroxide molecule would allow for efficient attack. Finally, the protonated ε-amino group of Lys73 in the proximity of the Ser70 hydroxyl side chain could assist in the decomposition of the tetrahedral intermediate and deacylation.

For the first time, the Michaelis complex of a β-lactam with a β-lactamase has been directly visualized using X-ray crystallography. The Michaelis complex structure of cefamandole in conjunction with the ring-opened acylated intermediate of cefamandole bound within the active site has given us insights into the BlaC active site and interactions that allow for the rapid acylation and deacylation reactions with this substrate. This information is currently being incorporated into the design of inhibitors.

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SUPPORTING INFORMATION AVAILABLE

Materials, methods, and a table of crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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