

Structure of the Covalent Adduct Formed between *Mycobacterium tuberculosis* β -Lactamase and Clavulanate^{†,‡}

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Received January 19, 2008; Revised Manuscript Received March 31, 2008

ABSTRACT: The intrinsic resistance of *Mycobacterium tuberculosis* to the β -lactam class of antibiotics arises from a chromosomally encoded, extended spectrum, class A β -lactamase, BlaC. Herein, we report the X-ray crystallographic structure of BlaC inhibited with clavulanate at a resolution of 1.7 Å with an *R*-factor value of 0.180 and *R*-free value of 0.212 for the *m/z* +154 clavulanate-derived fragment observed in the active site. Structural evidence reveals the presence of hydrogen bonds to the C1 carbonyl along with a coplanar arrangement of C1, C2, C3, and N4, which favors enolization to generate a *trans*- α,β -eneamine, stabilizing the +154 adduct from hydrolysis. The irreversible inhibition of BlaC suggests that treatment of *M. tuberculosis* with a combination of a β -lactam antibiotic and clavulanate may lead to rapid bactericidal activity.

In recent years, multidrug-resistant (MDR) as well as extremely drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (TB) have evolved, threatening our ability to clinically treat this deadly human pathogen (1). Hence, it is imperative that new drugs and treatment paradigms be made available to curb this crisis. Historically, one of the most effective therapeutic classes of antibacterials have been the β -lactam class of antibiotics, all of which contain the structural β -lactam ring motif (see Figure 1). This class of antibacterial compounds inhibits the bacterial D,D-transpeptidases (2), which catalyze the final step of peptidoglycan cross-linking. This cross-linking activity is essential for cell-wall maturation and cell survival.

Resistance to this class of antibiotics in *M. tuberculosis* arises from a chromosomally encoded Ambler class A β -lactamase, BlaC (3), which catalyzes the hydrolysis of the β -lactam antibiotic ring. The catalytic mechanism of BlaC involves (1) the activation of an active-site nucleophile, Ser70 by Lys73 and/or Glu166, (2) attack on the β -lactam ring carbonyl with the formation of a covalent acyl–enzyme complex, and (3) hydrolysis of the ester bond via a conserved active-site water and Glu166, forming the free enzyme and the ring-opened product (4–8). Because of the intrinsic resistance as a result of the constitutive production of the β -lactamase, β -lactams have never been systematically applied with success to the treatment of TB infections. Current treatment instead relies on a regiment of four compounds (isoniazid, rifampicin, ethambutol, and pyrazinamide) co-administered over a 6-month period.

However, β -lactams in combination with β -lactamase inhibitors have been cited as being effective in vitro (9, 10) and in human TB infections (11). In support of these previous

findings, recent data have shown that the Food and Drug Administration (FDA)-approved β -lactamase inhibitor, clavulanate, uniquely and irreversibly inhibits the genomically encoded BlaC (12) present in TB. Because MDR strains of TB are defined as being resistant to the two rapidly bactericidal agents, isoniazid and rifampicin, this suggests a potential new treatment paradigm for these strains using a combination of FDA-approved β -lactam antibiotics and clavulanate.

EXPERIMENTAL PROCEDURES

Cloning and Purification of BlaC. The *blaC* gene was amplified from genomic *M. tuberculosis* H37Rv DNA and cloned into pET28 using *Nde*I and *Hind*III. BlaC was expressed as a N-terminally truncated form, lacking the first 40 amino acids, as previously described (11). The plasmid was sequenced and transformed into *Escherichia coli* BL21 (DE3) and cultured in LB broth at 37 °C. When the culture OD₆₀₀ reached 0.6, the cultures were cooled and induction was performed by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 16 °C for 12 h. Cells were harvested, resuspended in 25 mM Tris-HCl containing 300 mM NaCl at pH 7.5, and disrupted by sonication. After centrifugation, the soluble extract was loaded onto a Ni-NTA agarose column (Qiagen) and eluted with 200 mM imidazole in 25 mM Tris-HCl containing 300 mM NaCl at pH 7.5. The eluted fractions were dialyzed against 25 mM Tris-HCl containing 300 mM NaCl at pH 7.5 to remove the imidazole, and thrombin was added to cleave the His₆ N-terminal tag. Size-exclusion chromatography was performed using a Superdex 200 Hi-Load 26-60 column (Amersham Pharmacia Biotech) using 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) containing 50 mM NaCl at pH 6.4 as buffer.

Crystallization. BlaC was crystallized in the hanging drop vapor diffusion configuration over well conditions of 0.1 M Tris at pH 8.0 and 2 M NH₄H₂PO₄. The final pH of the well solution was 4.1. Protein at a concentration of 15 mg/mL

[†] This work was supported by (NIH) Grant AI33696 (to J.S.B.).

[‡] Coordinates are available from the Protein Database (accession number 3CG5).

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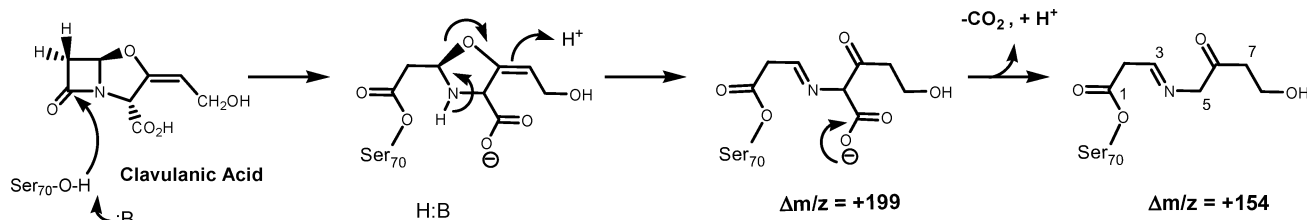


FIGURE 1: β -Lactam ring motif is also visible in the β -lactamase inhibitor clavulanic acid. After nucleophilic attack by Ser70 with the opening of the β -lactam ring, the oxazolidine ring opens to generate the +199 adduct. This is rapidly decarboxylated to generate the final +154 adduct.

was mixed 1:1 with the well solution and incubated at 18 °C. Initial crystals grew within a week but were small, sparse, and amorphous. New wells were sealed and allowed to equilibrate overnight. The next day, the drops were microseeded, which resulted in efficient crystal growth as well as improved morphology. Iterative seeding resulted in diffraction-quality crystals. These crystals were soaked in a solution containing clavulanate mixed with cryo conditions of 20% glycerol and 80% well solution. The data presented here are from an approximate 10 min soaking period in the presence of ~50 mM clavulanate and 20% glycerol, at which point, the crystal was flash-frozen in liquid nitrogen. The addition of nitrocefin to BlaC crystals resulted in the rapid formation of bright red crystals, suggesting that catalytic activity was retained in these crystals, while the addition of nitrocefin to BlaC crystals pretreated with clavulanate for 10 min resulted in no change in the color of the crystals, confirming previous solution studies (Figure S1 in the Supporting Information).

Data Collection and Refinement. A 1.7 Å data set was collected at Brookhaven National Laboratory on beamline X12C. The data were processed using HKL2000 (13). AMoRe (14), within the CCP4 software suite (15), along with the structure of the unliganded *M. tuberculosis* β -lactamase (16) (accession code 2GDN) were employed for molecular replacement. Iterative rounds of structural refinement and model building were performed in Refmac5 (17–20) and Coot (21). Table 1 lists the data collection statistics and the final refinement statistics.

RESULTS

Crystals of BlaC were soaked between 10 and 90 min in the presence of ~50 mM clavulanate prior to vitrification and data collection. The crystallographic data demonstrate the accumulation of the same +154 adduct in the active site for soaking times between 10 and 90 min. The crystallographically determined X-ray structure of the +154 clavulanate adduct inhibited form of BlaC was solved at a resolution of 1.7 Å with a *R* factor of 0.180 and a *R*-free value of 0.212.

A covalent adduct (bond length of 1.36 Å) to the active-site nucleophile (Ambler number Ser70) is an obvious feature in the density (Figure 2) displayed in the $F_o - F_c$ omit map contoured at 2.5σ . The Ser70 side-chain ester oxygen (see Figure 3) hydrogen bonds with the ϵ -nitrogen of Lys87 (2.9 Å) and the side-chain hydroxyl of Ser142 (2.9 Å) and is 3.0 Å from the conserved active-site water 441. The carbonyl oxygen of the ester (numbered 1 in Figure 2) hydrogen bonds with the backbone amides of Thr253 (2.9 Å) and Ser70 (2.8 Å), which represent the oxyanion hole. In addition there is an interaction at the C6 carbonyl oxygen, which is 2.4 Å from water 516 in the structure.

Table 1: Data Collection and Refinement Statistics^a

Data Collection	
resolution (Å)	41.4–1.7 (1.74–1.70)
completeness (%)	96.0 (88)
redundancy	3.3 (3.0)
<i>I</i> / σ (<i>I</i>)	18.0 (2.0)
<i>R</i> _{merge}	0.05 (0.40)
space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
unit cell (Å)	<i>a</i> = 49.67, <i>b</i> = 67.66, <i>c</i> = 75.03, $\alpha = \beta = \gamma = 90.00^\circ$
Refinement Statistics	
<i>R</i> _{work} (%)	0.180
<i>R</i> _{free} (%)	0.212
number of atoms	2290
protein	2004
adducts	11
solvent	260
phosphate ions	3
average <i>B</i> factors (Å ²)	13.99
protein	12.38
adduct	21.30
phosphate ions	27.87
solvent	24.75
rms deviations	
bonds (Å)	0.014
angles (deg)	1.508
PDB accession code	3CG5

^a High-resolution shell data statistics are in parentheses. The model and the data along with additional structural statistics are available from the Protein Data Base using the accession code 3CG5.

The +154 clavulanate adduct accumulates as demonstrated by X-ray crystallography and mass spectrometry (12). The imine generated during the opening of the oxazolidine ring (Figure 1) must therefore either be stabilized against hydrolysis or tautomerize. The acidic C2 protons can enolize with the generation of the *trans*-C2,C3 enamine (Figure 3). This conjugated α,β -unsaturated system is likely to be the major tautomer in the active site, because the clavulanate adduct is stable to hydrolysis for greater than 12 h. The crystallographic data reveal that the conjugated α,β -unsaturated *trans*-enamine is favored over the imine, as was also reported for the SHV-1 clavulanate-inhibited complex (22). As seen in Figure 3, the coplanar arrangement of C1, C2, C3, and N4 suggests that this tautomer is the stable covalently bound form of the clavulanate product. In this manner, the +154 tautomeric adduct is stabilized against hydrolysis by Glu166 and the conserved active-site water, irreversibly inhibiting BlaC catalysis.

DISCUSSION

Clavulanate is known to exhibit suicide inhibition of other Ambler class A β -lactamases (23), and BlaC was shown to be irreversibly inhibited in this manner (12). After nucleo-

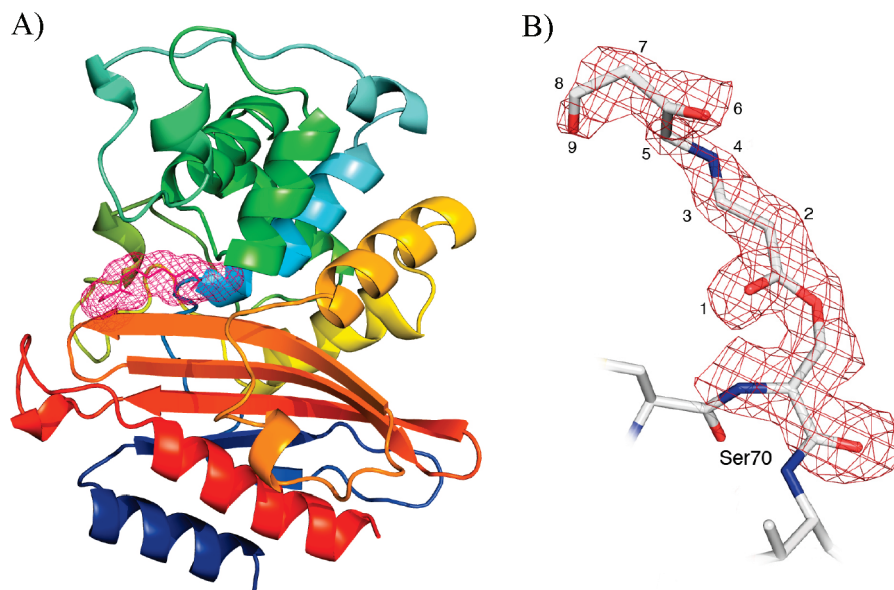


FIGURE 2: (A) Overall structure of BlaC displayed in rainbow from the N term (blue) to the C term (red), with the +154 covalent adduct displayed in a surface mesh. (B) $F_o - F_c$ omit density (red) contoured at 2.5σ surrounding the +154 covalently bound adduct formed at the Ambler active-site residue serine 70. All structure figures were produced using Pymol.

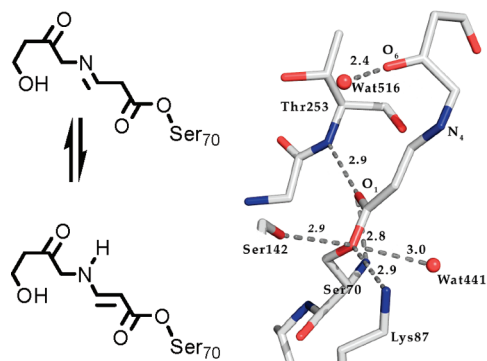


FIGURE 3: (Left) Imine–eneamine tautomerization of the clavulanate-derived +154 Ser70 ester. (Right) View showing the hydrogen-bonding network and the coplanar arrangement of C1, C2, C3, and N4, suggesting the presence of the *trans*- α,β -unsaturated eneamine.

philic attack by Ser70 and β -lactam ring opening, the oxazolidine ring is opened to generate the C3–N4 imine, C6 keto adduct (Figure 1). This is rapidly decarboxylated, and no evidence of this adduct was observed by mass spectrometry (12). From the mass spectrometry results, the predominate clavulanate-derived fragment is the +154 adduct, but both +136 and +70 adducts are also observed in a time-dependent manner.

An observation that links the enzymatic behavior of the solution and crystallized forms of BlaC results from using the BlaC substrate nitrocefin. Nitrocefin is a pale yellow compound in solution, which turns red upon β -lactam ring hydrolysis by BlaC. When BlaC crystals are soaked in solutions containing nitrocefin, the crystals rapidly turn deep red and the solution gradually turns pinkish as the product diffuses out of the crystals. When BlaC crystals are pretreated for 10 min with clavulanate and then soaked in solutions containing nitrocefin, no change in the color of the crystals is observed (Figure S1 in the Supporting Information). These observations demonstrate that the enzyme in the crystals has an active site that is diffusively accessible, allowing for turnover. Thus, the solution inhibition by clavulanate ob-

served by mass spectrometry can be directly compared to the structure observed crystallographically.

In addition to the +154 adducts, +136 and +70 adducts were demonstrated to be formed even at long incubation times by mass spectrometry. On the basis of this, we used the FTICR mass spectral data to approximate the probable percent occupancy (68% for the +154, 11% for the +136, and 21% for the +70) that would represent each adduct within the active site. The three models were assigned occupancies of 100% to N₄ for the +154 and +136 adducts and the terminal oxygen of the +70 adduct. The density beyond this atom position was then modeled at 79% (68 plus 11%) for the +154 and +136 adduct models to C₈ and as 68% for the +154 terminal O₉ oxygen. Figure 4 shows the electron density corresponding to the three refined covalent adducts (model coordinates are available upon request).

Initially, BlaC is covalently bound as the +154 adduct but can be subsequently dehydrated to generate the +136 adduct. A third minor +70 adduct is formed by the addition of a water to the adduct imine bond to form the enzyme–malon-aldehyde complex.

Our crystallographic data show no evidence of a Ser130 adduct, which was previously demonstrated for the TEM-2 β -lactamase (24). The crystallographic data supports, with accuracy, the conformation of the full +154 adduct chain bound in the TB β -lactamase active site, and by extension, when all observations are considered, the electron density is also consistent with a superposition of a population of the three adducts within the active site.

It is worthwhile to discuss briefly previous structural observations of clavulanate bound in the active site of the *Staphylococcus aureus* PC1 β -lactamase (25) and in the E166A SHV-1 β -lactamase mutant (22) (accession codes 1BLC and 2A49, respectively). The adduct position in the active site of the *S. aureus* β -lactamase is very different from what we observe for the +154 TB-bound adduct. In the PC1-clavulanate structure, two complexes were observed: a *cis*-eneamine that had not undergone decarboxylation and the decarboxylated *trans*-eneamine. When the structure of the

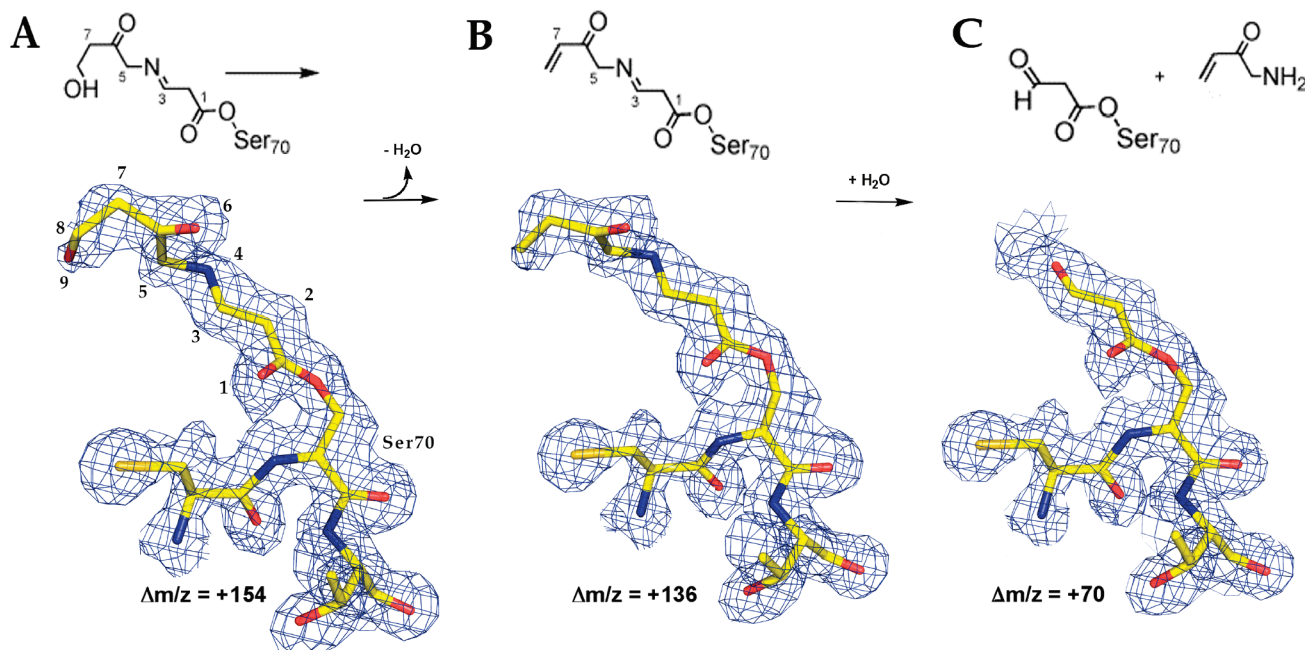


FIGURE 4: (A) +154, (B) +136, and (C) +70 covalent adducts with BlaC active-site Ser70 displayed in their most likely conformations when fitted to the observed electron density. Above are the associated chemistries for the formation of each of the adducts. The +154 and +136 are irreversibly bound to the enzyme. The formation of the +70 enzyme–malonaldehyde adduct is thought to be a hydrolyzable adduct.

clavulanate-derived fragment in the active site of the mutant SHV-1 β -lactamase (22) is superimposed with our structure, both adducts maintain a similar planar conformation along the chain to the N_4 nitrogen, after which the two structures diverge. The data for the SHV-1 appears to show significant density (contoured at 1σ) only to the N_4 nitrogen, as reflected in the high B factors of 40.49 for N_4 , 44.41 for C_5 , 53.85 for O_6 , 55.97 for C_7 , 53.34 for C_8 , and 60.05 for O_9 . It appears that the +154 clavulanate adduct is not as well-stabilized in the SHV-1 active site as compared to BlaC. Further, in the case of SHV-1, it was shown that the S130G mutation imparted resistance to clavulanate inhibition, suggesting that rearrangement of the fragment within the SHV-1 active site occurs (26).

Our structure for the clavulanate adduct bound to BlaC can also be compared to the unliganded BlaC structure (PDB entry 2GDN) that was used as the molecular replacement solution. Superimposing this model over the adduct structure, one observes that Wat329 (from 2GDN) is in the position occupied by O_1 , while Wat521 occupies the position of N_4 and Wat519 occupies the position of O_9 in the +154 structure (Figure S2 in the Supporting Information). These are thus low-energy, favorable binding positions within the active site of TB BlaC. Using this knowledge may prove useful in the construction of new inhibitors, which can structurally and electrostatically mimic these favorable binding positions.

The structural data reported herein are consistent with the mass spectral results, revealing a covalently bound, hydrolytically stable *trans*- α,β -eneamine adduct, and provide an explanation for the unique ability of the suicide inhibitor clavulanate to irreversibly inhibit the *M. tuberculosis* *blaC*-encoded β -lactamase. The ability of clavulanate to inhibit *M. tuberculosis* growth, in combination with poor substrate β -lactams, is currently under investigation.

SUPPORTING INFORMATION AVAILABLE

Two figures showing crystals incubated with nitrocefin in both the absence and presence of clavulanate (Figure S1) and the overlay of the clavulanate-derived +154 covalent adduct with the water molecules observed in the non-inhibited enzyme (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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BI8001055