HIGH RESOLUTION CRYSTAL STRUCTURES OF THE TRANS-ENAMINE INTERMEDIATES FORMED BY SULBACTAM AND CLAVULANIC ACID AND E166A SHV-1 β-LACTAMASE

Pius S. Padayatti1, Marion S. Helfand3, Monica A. Totir2, Marianne P. Carey1, Paul R. Carey1,2, Robert A. Bonomo3, and Focco van den Akker1.

From the 1Department of Biochemistry, 2Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, and 3Research Division, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106.

Running title: Inhibitors bound to E166A SHV-1 β-lactamase

Antibiotic resistance mediated by constantly evolving β-lactamases is a serious threat to human health. The mechanism of inhibition of these enzymes by therapeutic β-lactamase inhibitors is probed using a novel approach involving Raman microscopy and X-ray crystallography. We present here the high resolution crystal structures of the β-lactamase inhibitors sulbactam and clavulanic acid bound to the deacylation deficient E166A variant of SHV-1 β-lactamase. Our previous Raman measurements have identified the trans-enamine species for both inhibitors and were used to guide the soaking time and concentration to achieve full occupancy of the active sites. The two inhibitor bound X-ray structures reveal a linear trans-enamine intermediate covalently attached to the active site S70 residue. This intermediate is thought to play a key role in the transient inhibition of class A β-lactamases. Both the Raman and X-ray data indicate that the clavulanic acid intermediate is decarboxylated. Compared to our previously determined tazobactam bound inhibitor structure, our new inhibitor bound structures reveal an increased disorder in the tail region of the inhibitors as well as in the enamine skeleton. The X-ray crystallographic observations correlate with the broadening of the O-C=C-N (enamine) symmetric stretch Raman band near 1595 cm⁻¹. Band broadening in the sulbactam and clavulanic acid intermediates reflects a heterogeneous conformational population which results from variations of torsional angles in the O-(C=O)-C=C=NH-C skeleton. These observations lead us to conclude that the conformational stability of the trans-enamine form is critical for their transient inhibitory efficacy.

Bacterial infections are often treated using β-lactam antibiotics that inhibit the final stages of peptidoglycan synthesis, leading to bacterial cell death. Unfortunately, resistance to β-lactams has emerged as a result of the production of β-lactamases. Bacteria have become resistant to many of these antibiotics, in large part due to bacterial β-lactamase production and mutation of these enzymes (EC 3.5.2.6) (1-3). These enzymes have progressively evolved to hydrolyze each new generation of penicillins and cephalosporins. The antibiotic resistance conferred by β-lactamases can often be circumvented via an effective therapeutic strategy involving the combination of penicillin with a β-lactamase inhibitor. Currently, three β-lactamase inhibitors are available for clinical use: tazobactam, sulbactam, and clavulanic acid (Figure 1a)(4). Their mechanism of action is thought to be as follows: after forming a Michaelis-Menten complex, the inhibitors proceed to a covalent acyclic linear molecule that inhibits the enzyme both in a transient and irreversible manner (5). However, β-lactamases evolve to counteract the inhibitors by acquiring point mutations that result in resistance to inhibition while the enzymes maintain sufficient activity to degrade β-lactam antibiotics (5-6). In order to counter the effects of drug resistance, detailed molecular information is needed on the mechanism of inhibition by the β-lactamase...
inhibitors as well as on β-lactamase’s ability to become resistant to these inhibitors.

Of the four classes of β-lactamases, class A enzymes are clinically the most prevalent with SHV-1 and TEM-1 as their major members (1). As stated above, the three β-lactamase inhibitors work by acting as very slow substrates thereby occupying the active site long enough such that the antibiotics are not degraded before their antibacterial action has been completed. For inhibitor development, it is therefore crucial to understand the catalytic mechanism of β-lactamases such that the length of time the inhibitor spends in the active site can be optimized to enhance its inhibitory efficacy. The mechanism of inhibition of β-lactamases has been of intense study (7-17).

The initial step of the catalytic mechanism of inactivation of SHV-1 is ligand binding (18) followed by nucleophilic attack on the carbonyl carbon atom by the hydroxyl group of Ser70, resulting in a covalent acyl intermediate and the opening of the β-lactam ring (Figure 1b). This step is followed by opening of the second ring. The latter stage results in the linearization of the inhibitor as an imine intermediate. After isomerization, the intermediate becomes a cis-enamine, that in turn can isomerizes to the more stable trans-enamine state. After several hours, the enzyme becomes irreversibly inhibited via covalent modification at position S130 as was previously observed crystallographically (19).

Prior to our work, crystallographic snapshots of reaction intermediates involving clavulanic acid and tazobactam bound to wt β-lactamases (19-20) and an inhibitor resistant mutant have been obtained (21). Additionally, decylation deficient E166A β-lactamase mutants have been used to maximize the possibility of trapping its acyl-enzyme intermediates for crystallographic analysis (22-24). These important structures were obtained by soaking or co-crystallization experiments carried out in the absence of prior information of the type and amount of intermediate that is present within a crystal at any given time. This information would have aided in choosing the right time point for freezing the crystal in order to obtain stoichiometric complexes. We recently demonstrated that this important aspect of identifying and tracking intermediates inside protein crystals can be achieved using Raman crystallography (25-26). This new approach pinpointed the maximal presence of a tazobactam trans-enamine intermediate in the decylation deficient E166A variant of SHV-1 at about 20-30 minutes after soaking had started (25). Using these conditions, we were able to obtain the 1.63 Å resolution crystal structure of the stoichiometric complex of tazobactam in the trans-enamine intermediate form bound to this SHV-1 E166A decylation deficient variant. We now extend our crystallographic studies to the other two inhibitors, sulbactam and clavulanic acid and present here their 1.34 Å and 1.43 Å resolution crystal structures, respectively. Our inhibitor structures, observed in the trans-enamine form, will aid in the molecular understanding of this ‘waiting room’ intermediate that is important for effective β-lactamase inhibition (8).

Material and Methods

Crystals for Raman and X-ray crystallographic analysis of the decylation deficient E166A mutant of SHV-1 were obtained as described previously (25-26). Crystals were obtained by hanging drop vapor diffusion in which drops of 10 µL volume were prepared using 4 µL protein solution, 1 µL 5.6 mM Cymal-6 (Hampton Research), and 5 µL 20% w/v PEG 6000 0.1M HEPES pH 7.0. The reservoir solution contained 20% w/v PEG-6000 in 0.1M HEPES pH 7.0 buffer. Wells were sealed and stored at room temperature and crystals grew to full size in 2-3 days. Our inhibitor soaking experiments for sulbactam and clavulanate were guided by our previous Raman crystallographic measurements using the decylation deficient E166A variant of SHV-1 β-lactamase (25). In particular, the Raman intensity plots in Figure 2a are used to follow the population of the trans-enamine species. The plots were generated by measuring the integrated intensity of the enamine mode near 1595 cm⁻¹ and ratioing this value to the integrated intensity of the amide I band in the unsubtracted protein spectrum as an internal standard. This procedure was carried out for each point on the plot and has the advantage of correcting for minor changes in
crystal position or laser focus that occur during the time plot. The present data are generally similar to those we have published (25) where we plotted peaks heights without reference to an internal standard. If we make the reasonable assumption that the Raman scattering cross sections are very similar for the three enamine species, since the enamine skeletons are all the same, we observe that the populations of the clavulanic acid and especially the sulbactam-based intermediates are considerably reduced compared to the population produced by tazobactam.

Further analysis of our previously published Raman difference spectra indicated that all three inhibitors, free in solution, have a medium intensity Raman mode near 1400 cm\(^{-1}\) (25). This is due to the symmetric stretching vibration of the -COO\(^-\) group. For the bound ligands, this feature shifts to about 1375 cm\(^{-1}\) in sulbactam and tazobactam reflecting non-covalent interactions of the -COO\(^-\) with active site residues. For clavulanic acid bound to the enzyme, there is no peak in this region, suggesting that the -COO\(^-\) group is no longer present (25), an observation reported for a clavulanic acid bound structure of a different β-lactamase (20). Finally, our previous Raman study also indicated that the peak width for the characteristic trans-enamine \(~1595\) cm\(^{-1}\) peak varies amongst the three inhibitors (25) with tazobactam’s peak being the narrowest (Figure 2b). These spectral peak width features are likely indicative of the degree of order around or near the trans-enamine bonds as will be discussed later.

**Sulbactam and clavulanic acid soaking**

The Raman measurements led us to change our soaking procedure to increase the presence of the trans-enamine for sulbactam. Instead of the 5mM, as we used to obtain our previous stoichiometric tazobactam complex structure (26), we choose a concentration that was close to as high as possible (50mM) for both sulbactam and clavulanic acid. This also allowed us to decrease the soaking time somewhat in an attempt to avoid the side-reactions (such as decarboxylation and other fragmentations) yet still approach full active site occupancy, which we achieved. Therefore, 20 μl drops containing 30% PEG 6000 in 0.1M HEPES pH 7.0 with 0.56mM Cymal-6 and 50mM sulbactam were used for soaking E166A β-lactamase crystal for 10 minutes. Note that the time it takes such compounds to completely penetrate SHV-1 β-lactamase crystals is less than 1 minute as has been determined previously using Raman (25). Crystals soaked with sulbactam were cryoprotected using mother liquor containing 25% MPD for \(~1\) minute before they were submerged in liquid nitrogen using mounted cryoloops (Hampton Research Inc.). Synchrotron data were collected at Berkeley (MB-CAT 4.2.2) to a diffraction limit of 1.34 Å resolution (Table I).

A similar approach was taken for soaking clavulanic acid into E166A SHV-1 crystals: crystals were soaked for 5 minutes with 50mM clavulanic acid (1.43 Å resolution, Table I). After the 5 minute time point, crystals were cryoprotected in mother liquor containing 25% MPD. The data for both complexes were processed with d*TREK (27) and the reflection files further processed using CCP4 (28). Crystallographic refinement was carried out in CNS (29) and model building was done with the program O (30). Data statistics are shown in Table I.

**Results**

**Sulbactam:E166A complex**

The sulbactam bound structure has been refined at 1.34 Å resolution to give an R-factor of 17.5% and R\(_{\text{free}}\) of 19.5%. The final model includes residues 26-292 in the Ambler numbering convention with 4 side chains with alternative conformations, 264 waters, 2 Cymal-6 molecules (1 complete and the other contains only the hydrocarbon tail), part of a HEPES molecule (as was also observed in other structures of SHV (21;31)), and the sulbactam trans-enamine intermediate. The program PROCHECK (32) found that there were 92% of the φ-ψ angles were in the most favored core region of the Ramachandran plot and the remainder of the residues were in the allowed region.

The sulbactam intermediate was observed in the trans-enamine form; the C5-C6 dihedral angle of 188° is even closer to the ideal 180° trans conformation than the 168° angle observed for the tazobactam intermediate (26). The omit electron density for the sulbactam atoms immediately after guest on July 9, 2020http://www.jbc.org/Downloaded from http://www.end.org/
adjacent to the bonded S70 Oγ atom (e.g. C7, O8, C6, C5, N4) is quite strong and then weakens for the more distant atoms (Figure 3). The temperature factor of the tazobactam C7 atom bound to S70 is around 15 Å², compared to 28 Å² for the middle of the linearized sulbactam (N4 atom) and above 50 Å² for the atoms at the tail end (Table II). Although the density is much weaker in its tail, sulbactam seems to be making an internal hydrogen bond between its N4 nitrogen and one of the sulfone oxygens similar to tazobactam (Figure 3a)(26). Both sulbactam and tazobactam have the O8 atom situated in the oxyanion hole formed by backbone nitrogen atoms of S70 and A237 (Figure 4a and (26)). In addition, one of the -COO- atoms of sulbactam is positioned within hydrogen bonding distance of the amine nitrogen of N132 (Figure 3a). Occupancy and B-factor refinement indicated that the occupancy of the sulbactam intermediate is 90% or higher.

The active site has undergone little change compared to tazobactam soaked E166A crystals. The only noticeable difference is that the side chain of N170 is in two alternate conformations, an ‘outward’ conformation similar to that observed in our tazobactam soaked structure, and an ‘inward’ conformation similar to wt SHV-1 (33). Both side chain conformations for N170 in the sulbactam structure show weak density for the side chain amide groups. Therefore, the precise conformation of these groups could not be fully resolved.

Clavulanate:E166A complex

The clavulanic acid structure has been refined at 1.43 Å resolution to an R-factor of 19.4% and an R_free of 22.6%. The final model contains residues 26-292, of which 4 have side chains with alternative conformations. In addition, there are 316 waters, and as seen in the sulbactam crystal, 1 full and 1 partial Cymal-6 molecule, part of a HEPES molecule, and the trans-enamine intermediate of a decarboxylated clavulanic acid. The trans-enamine torsion angle for clavulanic acid of 181° is very close to ideal. The omit density for clavulanic acid shows a similar trend as sulbactam with strong density near atoms C7, O8, C6, and C5, and then the density tapers off between the bond N4-C3 with only weak density for the remaining atoms (Figure 3b). Consistent with the conclusion from the Raman data, the omit density for the clavulanic acid intermediate suggests that the carboxyl group attached to the C3 position had been lost during its reaction within the β-lactamase crystal (see reaction scheme in Figure 1). This decarboxylated intermediate species of clavulanic acid has been previously observed by mass spectroscopy in TEM-2 (16) and by X-ray crystallography in the *Staphylococcus aureus* PC1 β-lactamase structure (20). Although we have modeled the decarboxylated clavulanic acid such that it contains the same number of atoms as when bound to PC1, albeit in a different conformation, we do realize that the weak density in clavulanic acid’s tail could also be due to partial presence of shorter (hydrolyzed) fragments of clavulanic acid intermediates that appear after longer time periods (34), although these would likely yield markedly different Raman spectra. The temperature factors range from 15 Å², for the covalent attached C7, to about 40 Å² for its N4 atom, to a maximum of about 55 Å² for the tail atoms of clavulanic acid (Table II). Clavulanic acid makes relatively few interactions except for its N4 atom which forms a water mediated interaction with the nitrogen atom of the N132 side chain (check), and its O8 atom participating in the usual oxyanion hole interactions (Figure 3b). Similar to sulbactam, the B-factor and occupancy refinement also suggested the occupancy of the clavulanic acid intermediate is greater than 90%.

The active site of the clavulanic acid bound structure is quite unchanged compared to the sulbactam and tazobactam bound E166A structures. A minor change is that the N170 residue, compared to the tazobactam bound structure, is observed in the ‘inward’ conformation similarly to the *wt* SHV-1 (33) and to one of the two conformations in sulbactam.

Discussion

By using Raman crystallography and a deacylation deficient variant of SHV-1, E166A, we are able to capture the critical intermediates formed in the inactivation process (22). The structure of sulbactam bound to E166A SHV is the first time this inhibitor has been observed bound to a β-lactamase. Of the three clinically approved β-lactamase inhibitors, sulbactam is unique since it is the only one which, by itself, also has some level of intrinsic antibiotic activity (35).
Agreement between X-ray and Raman crystallographic measurements

Raman crystallography has correctly observed a trans-enamine species for all three inhibitors that we have characterized by X-ray crystallography ((25-26) and this report). In addition, Raman crystallographic measurements of the intermediates formed in the active site of the deacylation deficient E166A variant of SHV-1 revealed intriguing differences between tazobactam, sulbactam, and clavulanic acid ((25) and Figure 2). The population levels and peak widths of the observed trans-enamine intermediates are quite distinct for the three inhibitors (Figure 2). The latter suggest that there is evidence for conformational heterogeneity which comes from molecules possessing different torsional angles in the enamine skeleton. A band shape analysis of each intermediate’s intense double bond stretching feature leads to the conclusion that there is more conformational heterogeneity within the sulbactam and clavulanic acid species. Figure 2b shows the band shapes for tazobactam’s, sulbactam’s, and clavulanic acid’s enamine symmetric stretch. The sulbactam and clavulanic acid peaks are considerably broader with bandwidths at half height of 25 cm$^{-1}$ and 32 cm$^{-1}$ compared to 18 cm$^{-1}$ for tazobactam. In Raman spectroscopy broadened band shapes often result from conformational heterogeneity (36). Thus, in the present instance, the broader widths of the sulbactam and clavulanic acid-derived features (Figure 2b) are ascribed to the existence of a range of close-lying conformations. Broadly, these have the parent trans-enamine structure found in the tazobactam complex, however, the torsional angles in the –NH-CH=CH-C(=O) fragment are slightly different from those for the tazo-derived fragment accounting, in part, for the differences in the position of the stretching feature. For this fragment itself, dynamic excursions about the observed mean torsional angles can lead to line broadening of the main feature near 1595 cm$^{-1}$. Thus a wider range of these excursions for sulbactam and clavulanic acid could account to for their broader band profiles. A second possibility relates to the ‘tails’ extending beyond N4. Calculations (25) confirm that extensive vibrational coupling exists between motions in the –NH-CH=CH-C(=O) fragment and adjacent bonds. Thus, static or dynamic disorder in the tail can also lead to broadening of the band near 1595 cm$^{-1}$. The time scale of the Raman effect is sub-picosecond thus we cannot distinguish static and dynamic disorder in the present studies. These Raman measurements and analysis are in remarkable agreement with our observations presented here on the X-ray structure of the sulbactam and clavulanic acid complexes. The omit densities for both inhibitors reveal that there is significantly less electron density in the enamine “tail” beyond the N4 atom which is consistent with increased conformational heterogeneity in the inhibitors’ tail region with clavulanic acid being even more disordered compared to sulbactam. In addition the X-ray data indicate an increase in thermal motion within the enamine at N4 (as reflected by its B-factors, Table II) for sulbactam and clavulanic acid. Thus it appears that conformational heterogeneity both within the main enamine fragment and the tail contribute to increased line width for sulbactam and clavulanic acid. The correlation between these two factors and the Raman data is shown in Table II. The observed trend of trans-enamine band width as detected using Raman measurements of tazobactam<sulbactam<clavulanic acid (Figure 2b) is mirrored by both the B-factor of the tail and the B-factor at position N4 (Table II). This shows that the level of agreement between Raman and X-ray crystallography reaches even beyond identifying the trans-enamine intermediate and that Raman can assess fine molecular details of bond rigidity and local disorder.

In addition to the peak width, the intermediate population levels, as measured by Raman band intensities, varied. When soaking at 5mM concentrations, tazobactam seems to form the highest population of the trans-enamine intermediate (Figure 2a). This information led us to adapt our soaking strategies by increasing the concentrations to 50mM for both sulbactam and clavulanate to successfully achieve full occupancies. Finally, the omit density for the clavulanic acid intermediate (Figure 3b) suggests that the carboxylate group is no longer present similar to previous studies as mentioned above. This is in agreement with the analysis of the Raman difference spectra of the clavulanic acid soaked
crystals (25) that confirms the absence of a $-\text{COO}^-$
symmetric stretching band.

**Sulbactam**

The binding mode of sulbactam to $\beta$-lactamase has previously been probed by molecular docking methods using a homology model of the active site of TEM-1 (37). The modeled structures include the Michaelis-Menten complex, the early acyl intermediate with only the $\beta$-lactam ring broken, and the linear imine intermediate. The modeling results indicated a role for residue R244, and a bound structurally positioned water molecule, in interacting with the carboxyl moiety of sulbactam which is also in agreement with the importance noted for this residue in biochemical studies of SHV-1, OHIO, and TEM-1 $\beta$-lactamases (38-41). However, our crystallographically observed structure of the trans-enamine sulbactam intermediate does not show this direct interaction nor indirect interaction (i.e. via the interacting water molecule). The critical trans-enamine (“waiting room”) intermediate was not modeled in the TEM-1 studies thus restraining the inhibitor torsion angles such that the carboxyl moiety is not able to reach the R244 side chain as is possible for the earlier (more flexible) intermediates.

**Clavulanic acid**

Previously, a clavulanic acid bound structure of *S. aureus* PC1 $\beta$-lactamase was determined (20). After soaking the crystals for 1 hour with very high concentrations, clavulanic acid was found to be in two distinct conformations (Figure 5): the cis-enamine and in the decarboxylated trans-enamine conformation. Our Raman guided crystallographic studies revealed a single stoichiometric species for clavulanic acid: a decarboxylated trans-enamine intermediate. However, the dihedral angle of the bond preceding the trans-enamine (along C7-C6) is in a different conformation compared to the PC1 structure resulting in a somewhat different conformation for the inhibitor (Figure 5). The cis-enamine intermediate of clavulanate forms a salt-bridge between its carboxylate moiety and the K234 sidechain of PC1 $\beta$-lactamase (20). This suggests that clavulanic acid can adopt different conformations in the active sites of different class A $\beta$-lactamases probably reflecting differences in soaking time and/or active site differences (as is evident in Figure 5).

Both sulbactam and clavulanic acid are covalently bound to S70 in a linearized trans-enamine intermediate form, with the clavulanic acid decarboxylated. Superpositioning of the three inhibitors, each having different tails, reveals that all of them adopt a similar trans-enamine intermediate with slightly different angles extending from the covalent bond with S70 and have their O8 atom ‘anchored’ in the oxy-anion hole (Figure 5). This latter is a very common observation in many $\beta$-lactamase:inhibitor/substrate complexes yet an exception to this has recently been found in an inhibitor resistant variant of SHV-1 $\beta$-lactamase as the S130G mutation allowed the O8 atom of tazobactam to no longer occupy the oxyanion hole thus favoring the cis-enamine intermediate form (21).

The present sulbactam and clavulanic acid intermediate structures and our previous tazobactam E166A analysis depicting various degrees of trans-enamine bond rigidity and inhibitor tail flexibility could provide a useful starting point for designing improved inhibitors. Our structures of the three inhibitors also reveal subtle yet significant differences in the position of the trans-enamine bond unit with clavulanic acid and sulbactam being more similar to each other compared to tazobactam: tazobactam’s N4 atom has shifted by about 1.3 Å due to a rotation around the covalent inhibitor-protein bond (Figure 6). Potential design strategies for new inhibitors should aim to stabilize the trans-enamine intermediate and prevent the inhibitor-enzyme complex from undergoing conformational changes to free up the active site. Tazobactam’s ability to form a well ordered highly populated trans-enamine intermediate may, in part, be due to the fact that tazobactam has a greater number of favorable active site-ligand contacts when in the trans-enamine configuration (26) compared to sulbactam and clavulanic acid including those made by the triazolyl moiety that is absent in the latter inhibitors. The ability to form these active site interactions is probably also the reason that tazobactam’s trans-enamine skeleton has shifted compared to the clavulanic acid and sulbactam.
enamine moieties (Figure 6). The possibility of rotation of this trans-enamine bond unit shift should be taken into account when designing new β-lactamase inhibitors. Our future efforts involving Raman and X-ray crystallography will also target inhibitor resistant variants of SHV-1 in an effort to delineate the molecular basis of inhibitor resistance, an urgent medical problem (42-43).

Reference List

1. Helfand, M. S. and Bonomo, R. A. (2003) *Curr.Drug Targets.Infect.Disord.* 3, 9-23
2. Bush, K. (2002) *Curr.Opin.Investig.Drugs* 3, 1284-1290
3. Nathan, C. (2004) *Nature* 431, 899-902
4. Lee, N., Yuen, K. Y., and Kumana, C. R. (2003) *Drugs* 63, 1511-1524
5. Therrien, C. and Levesque, R. C. (2000) *FEMS Microbiol.Rev.* 24, 251-262
6. Bradford, P. A. (2001) *Clin.Microbiol.Rev.* 14, 933-51, table
7. Charnas, R. L., Fisher, J., and Knowles, J. R. (1978) *Biochemistry* 17, 2185-2189
8. Brenner, D. G. and Knowles, J. R. (1981) *Biochemistry* 20, 3680-3687
9. Brenner, D. G. and Knowles, J. R. (1984) *Biochemistry* 23, 5839-5846
10. Brenner, D. G. and Knowles, J. R. (1984) *Biochemistry* 23, 5833-5839
11. Charnas, R. L. and Knowles, J. R. (1981) *Biochemistry* 20, 3214-3219
12. Fisher, J., Charnas, R. L., and Knowles, J. R. (1978) *Biochemistry* 17, 2180-2184
13. Fisher, J., Charnas, R. L., Bradley, S. M., and Knowles, J. R. (1981) *Biochemistry* 20, 2726-2731
14. Fisher, J., Belasco, J. G., Khosla, S., and Knowles, J. R. (1980) *Biochemistry* 19, 2895-2901
15. Fisher, J., Belasco, J. G., Charnas, R. L., Khosla, S., and Knowles, J. R. (1980) *Philos.Trans.R.Soc.Lond B Biol.Sci.* 289, 309-319
16. Brown, R. P., Aplin, R. T., and Schofield, C. J. (1996) *Biochemistry* 35, 12421-12432
17. Bonomo, R. A., Liu, J., Chen, Y., Ng, L., Hujer, A. M., and Anderson, V. E. (2001) *Biochim.Biophys.Acta* 1547, 196-205
18. Hokenson, M. J., Cope, G. A., Lewis, E. R., Oberg, K. A., and Fink, A. L. (2000) *Biochemistry* **39**, 6538-6545

19. Kuzin, A. P., Nukaga, M., Nukaga, Y., Hujer, A., Bonomo, R. A., and Knox, J. R. (2001) *Biochemistry* **40**, 1861-1866

20. Chen, C. C. and Herzberg, O. (1992) *J.Mol.Biol.* **224**, 1103-1113

21. Sun, T., Bethel, C. R., Bonomo, R. A., and Knox, J. R. (2004) *Biochemistry* **43**, 14111-14117

22. Strynadka, N. C., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., and James, M. N. (1992) *Nature* **359**, 700-705

23. Ibuka, A., Taguchi, A., Ishiguro, M., Fushinobu, S., Ishii, Y., Kamitori, S., Okuyama, K., Yamaguchi, K., Konno, M., and Matsuzawa, H. (1999) *J.Mol.Biol.* **285**, 2079-2087

24. Chen, C. C. and Herzberg, O. (2001) *Biochemistry* **40**, 2351-2358

25. Helfand, M. S., Totir, M. A., Carey, M. P., Hujer, A. M., Bonomo, R. A., and Carey, P. R. (2003) *Biochemistry* **42**, 13386-13392

26. Padayatti, P. S., Helfand, M. S., Totir, M. A., Carey, M. P., Hujer, H. M., Carey, P. R., Bonomo, R. A., and van den Akker, F. (2004) *Biochemistry* **43**, 843-848

27. Pflugrath, J. W. (1999) *Acta Crystallogr.D.Biol.Crystallogr.* **55 ( Pt 10)**, 1718-1725

28. (1994) *Acta Crystallogr.D.* **55**, 760-763

29. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr.D* **54**, 905-921

30. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard (1991) *Acta Crystallogr.A* **47 ( Pt 2)**, 110-119

31. Nukaga, M., Mayama, K., Hujer, A. M., Bonomo, R. A., and Knox, J. R. (2003) *J.Mol.Biol.* **328**, 289-301

32. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (2001) *J.Appl.Cryst.* **26**, 283-291

33. Kuzin, A. P., Nukaga, M., Nukaga, Y., Hujer, A. M., Bonomo, R. A., and Knox, J. R. (1999) *Biochemistry* **38**, 5720-5727

34. Sulton, D., Pagan-Roderiguez, D., Zhou, X., Liu, Y., Hujer, A. M., Bethel, C. R., Helfand, M. S., Thomson, J. M., Anderson, V. E., Buynak, J. D., Ng, L. M., and Bonomo, R. A. (2005) *J.Biol.Chem.* [Epub ahead of print].
35. Williams, J. D. (1997) *Clin. Infect. Dis.* **24**, 494-497

36. Carey, P. R., Froese, A., and Schneider, H. (1973) *Biochemistry* **12**, 2198-2208

37. Imtiaz, U., Billings, E. M., Knox, J. R., and Mobashery, S. (1994) *Biochemistry* **33**, 5728-5738

38. Giakkoupi, P., Tzelepi, E., Legakis, N. J., and Tzouvelekis, L. S. (1998) *FEMS Microbiol. Lett.* **160**, 49-54

39. Lin, S., Thomas, M., Mark, S., Anderson, V., and Bonomo, R. A. (1999) *Biochim. Biophys. Acta* **1432**, 125-136

40. Zafaralla, G., Manavathu, E. K., Lerner, S. A., and Mobashery, S. (1992) *Biochemistry* **31**, 3847-3852

41. Bonomo, R. A., Dawes, C. G., Knox, J. R., and Shlaes, D. M. (1995) *Biochim. Biophys. Acta* **1247**, 121-125

42. Bonomo, R. A. and Rice, L. B. (1999) *Front Biosci.* **4**, e34-e41

43. Georgopapadakou, N. H. (2004) *Expert. Opin. Investig. Drugs* **13**, 1307-1318

Footnotes

* We would like to thank Vivien Yee, Pamela Hall, and the MB-CAT 4.2.2 beamline personnel at Berkeley, for help with data collection. We would like to acknowledge the American Heart Association (FVDA), NIH (FVDA, PRC, RAB), VA Merit Review Grant (RAB), VA Career Development Grant (MSH), and Steris Foundation (FVDA, RAB) for funding. Coordinates and structure factors for the sulbactam and clavulanic acid complexes have been deposited with the PDB (PDB identifiers 2A3U and 2A49).
Figure legends

**Fig. 1.** *A*, The three β-lactamase inhibitors. *B*, Schematic diagram of the mechanisms of β-lactamase inhibition. Clavulanic acid is shown as an example but inhibition by sulbactam and tazobactam is relatively similar.

**Fig. 2.** *A*, Time dependence of the main trans-enamine peak for the three inhibitors when soaked in crystals of the E166A variant of SHV-1. The Raman intensity is an indication of the population levels of the trans-enamine within the E166A crystal. The data points at each time point are the integrated intensity of the enamine mode near 1595 cm⁻¹ which is standardized to the integrated intensity of the amide I band in the parent protein spectrum, recorded prior to subtraction. *B*, Raman difference spectra of the E166A β-lactamase crystal with 5 mM tazobactam, clavulanic acid, and sulbactam added the mother liquor. The vertical bar represents a 5000 photon event. The region shown is a small part of the Raman difference spectra highlighting the region near the trans-enamine peak (~1595cm⁻¹).

**Fig. 3.** *A*, Electron density of sulbactam. Omit Fobs-Fcalc electron density of the active site of the E166A SHV-1 depicting the covalently bound trans-enamine intermediate of sulbactam. The trans-enamine bond is between atoms C6-C5. Contour levels: red 3.0 σ, magenta 2.0 σ, and green 1.5 σ. *B*, Electron density of clavulanic acid. Omit Fobs-Fcalc electron density depicting a linearized decarboxylated clavulanic acid covalently bound to S70. The trans-enamine bond is between the C5 and C6 atoms. Contour levels are identical as in Figure 3A. The omit density suggests that the carboxyl group attached to C3 is no longer present.

**Fig. 4.** *A*, Sulbactam bound in the active site. Stereo ball-and-stick diagram of sulbactam bound to the active site of the E166A variant of SHV-1 β-lactamase. Intra- and intermolecular hydrogen bonds are depicted by dotted lines. *B*, Clavulanic acid bound in the active site. Stereo diagram showing clavulanic acid in a linearized trans-enamine intermediate covalently attached to S70 of the E166A variant of SHV-1 β-lactamase. Intra- and intermolecular hydrogen bonds are shown as dotted lines.

**Fig. 5.** Superposition of clavulanic acid intermediates. Stereo figure depicting the superpositioning of three covalently bound clavulanic acid structure. Shown are the decarboxylated trans-enamine clavulanic acid from our work (black), the cis-enamine intermediate form observed in PC1 *S. aureus* β-lactamase (magenta), and the decarboxylated trans-enamine form as also observed in PC1 β-lactamase (yellow)(20). The superposed protein coordinates of the SHV-1 E166A clavulanic acid structure (thick and grey ball-and-stick) and PC1 β-lactamase (thick black ball-and-stick) are shown.

**Fig. 6.** Superposition of the three inhibitors. Superposition of tazobactam (black thin lines), sulbactam (blue), and clavulanic acid (green) when bound to the deacylation variant E166A of SHV-1 β-lactamase.
**Table I.** Data collection and crystallographic refinement statistics for the sulbactam and clavulanic acid bound structures of the E166A variant of SHV-1. Values in parenthesis are for higher resolution shells.

|                     | Sulbactam                  | Clavulanic acid             |
|---------------------|----------------------------|-----------------------------|
| **Space group**     | P2$_1$2$_1$2$_1$           | P2$_1$2$_1$2$_1$            |
| **Unit cell dimension (Å)** | a=49.51 b=55.31 c=83.73 | a=49.63 b=55.20 c=84.71     |
| **Wavelength (Å)**  | 1.0                        | 1.0                         |
| **Resolution (Å)**  | 27.68-1.34 (1.39-1.34)     | 14.84-1.43 (1.48-1.43)      |
| **Redundancy**      | 3.52                       | 3.62                        |
| **Total no. of reflections** | 184046                    | 141734                      |
| **Number of unique reflections** | 52285                     | 39126                       |
| $<|\sigma I|>$         | 10.4 (2.9)                 | 23.8 (2.1)                  |
| R(merge)%           | 5.8 (37.5)                 | 4.5 (26.7)                  |
| Completeness %      | 99.8 (99.7)                | 88.7 (41.2)                 |
| **Refinement**      |                            |                             |
| R-factor            | 17.5%                      | 19.4%                       |
| R$_{free}$          | 19.5%                      | 22.6%                       |
| RMSD from ideality  |                            |                             |
| for bond lengths    | 0.010 Å                    | 0.010 Å                     |
| for bond angles     | 1.6 °                      | 1.6 °                       |
Table II. Raman *trans*-enamine peak width and B-factors for the linearized inhibitor intermediates.

|                  | Raman peak width (cm⁻¹)ᵃ | B-factors (Å²) |        |        |
|------------------|---------------------------|----------------|--------|--------|
|                  |                           | N4 atom        | tailᵇ  |        |
| Tazobactam       | 18                        | 25             | 32     |        |
| Sulbactam        | 25                        | 29             | 44     |        |
| Clavulanic acid  | 32                        | 41             | 54     |        |

ᵃThe Raman band widths listed are for the main enamine feature –N4-C5H=C6H-C7=O8 stretching motion in E166A β-lactamase crystals with tazobactam, sulbactam, and clavulanic acid.

ᵇThe tail is defined as the atoms beyond the N4 atom for each of the inhibitors and the value listed is the average of these tail atoms.
Figure 2

A.

B.
High resolution crystal structures of the trans-enamine intermediates formed by sulbactam and clavulanic acid and E166A SHV-1 β-lactamase

Pius S. Padayatti, Marion S. Helfand, Monica A. Totir, Marianne P. Carey, Paul R. Carey, Robert A. Bonomo and Focco van den Akker

*J. Biol. Chem.* published online July 29, 2005

Access the most updated version of this article at doi: [10.1074/jbc.M505333200](http://dx.doi.org/10.1074/jbc.M505333200)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/) to choose from all of JBC's e-mail alerts