Evaluation of inhibition of the carbenicillin-hydrolyzing β-lactamase PSE-4 by the clinically used mechanism-based inhibitors

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Abstract Characterization of the biochemical steps in the inactivation chemistry of clavulanic acid, sulbactam and tazobactam with the carbenicillin-hydrolyzing β-lactamase PSE-4 from *Pseudomonas aeruginosa* is described. Although tazobactam showed the highest affinity to the enzyme, all three inactivators were excellent inhibitors for this enzyme. Transient inhibition was observed for the three inactivators before the onset of irreversible inactivation of the enzyme. Partition ratios (k_{cat}/k_{inact}) of 11, 41 and 131 were obtained with clavulanic acid, tazobactam and sulbactam, respectively. Furthermore, these values were found to be 14-fold, 3-fold and 80-fold lower, respectively, than the values obtained for the clinically important TEM-1 β-lactamase. The kinetic findings were put in perspective by determining the computational models for the pre-acylation complexes and the immediate acyl-enzyme intermediates for all three inactivators. A discussion of the pertinent structural factors is presented, with PSE-4 showing subtle differences in interactions with the three inhibitors compared to the TEM-1 enzyme.

Key words: β-Lactamase; PSE-4; Inhibition; *Pseudomonas aeruginosa*

1. Introduction

Infections caused by *Pseudomonas aeruginosa* are increasingly becoming a serious problem due to multiple-drug resistant strains of these bacteria [1]. In the mid 1980s, infections caused by most of the *P. aeruginosa* strains were manageable by antibiotics. However, by the early 1990s, 25% of these strains developed various resistant mechanisms [2]. The treatment strategies for the infections of *P. aeruginosa* are a cause of concern, especially in immunocompromised and cystic fibrosis patients, and in hospitals [3,2]. As in other bacteria, *P. aeruginosa* expresses β-lactamases as part of its resistance arsenal [4]. Production of β-lactamase by bacteria is the major mechanism of resistance to β-lactam antibiotics. These enzymes hydrolyze the lactam ring of the β-lactam drugs, neutralizing their antibiotic activity [5].

P. aeruginosa produces various β-lactamases including so-called *Pseudomonas*-specific β-lactamases, or PSE (for '*Pseudomonas*-specific enzyme'), in addition to other plasmid and chromosome-mediated enzymes [3,6]. PSE-4 is a class A plasmid-mediated β-lactamase and belongs to the family of 'carbenicillinases' which hydrolyze carbenicillin as efficiently or better than aminopenicillins [7–9]. PSE-type β-lactamases are also known to be part of transposable elements and transferred to other bacterial species altering their resistance profiles [10–13]. The PSE-4 enzyme is the most frequent β-lactamase found in carbenicillin resistant clinical strains of *P. aeruginosa* [7].

β-Lactam antibiotics are the most commonly used group of antibiotics in treatment of infections. This extensive use of β-lactam antibiotics creates a high selective pressure for the bacterial ecosystems, where new variants of common β-lactamases have emerged. These variants can hydrolyze β-lactam molecules which traditionally resisted the hydrolytic action of β-lactamases, or resist inactivation by the clinically used inhibitors, such as clavulanic acid (**1**), sulbactam (**2**) and tazobactam (**3**) [14–20]. A total of 67 variants of the TEM-1 β-lactamase, which is the most common enzyme among the class A β-lactamases, have been identified to date from clinical strains ([8,21,22], http://www.lahey.hitchcock.org/pages/lhc/studies/webt.htm). The prevalence of these variants and the continuous discovery of newer β-lactamases represent a serious threat to the treatment of bacterial infections. In most cases, the novel catalytic behavior of these mutants is due to mutations that alter the enzyme such that the substrate specificity is often broadened [23,24].

The understanding of the inactivation chemistry of β-lactamase inhibitors is the first step for the rational design of future generations of potent inhibitors [25,26]. The TEM-1 and PSE-4 β-lactamases are both from Gram-negative bacteria and the enzymes share some similarity in their sequences [7]. However, in light of the fact that the responses of *Escherichia coli* and *P. aeruginosa* to antibiotic treatment are quite distinct from one another, it is critical that the inhibition behavior of the β-lactamases from these organisms with the clinically used inhibitors are compared and contrasted to each other. We have undertaken such an effort and a structure-based study of the inactivation process of PSE-4 by inhibitors clavulanic acid, sulbactam and tazobactam. A discussion is presented on the similarities and subtle differences between the inhibition processes for PSE-4 and TEM-1.

2. Materials and methods

Ticarcillin and clavulanic acid were kindly provided by Smith Kline Beecham Pharmaceuticals. Sulbactam and tazobactam were provided

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by Pfizer and SynPhar Laboratories, respectively. All other antibiotics were purchased from Sigma.

2.1. Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by the broth microdilution method. An inoculum of 105 colony-forming units of *E. coli* DH5α (pMON711) expressing PSE-4 or the plasmidless strain was prepared by dilutions and inoculated into microtiter wells containing 100 μl of a binary dilution of an antibiotic in the Mueller–Hinton broth. The range of concentrations tested varied from 10 to 20 000 μg/ml for carbenicillin, ampicillin and piperacillin. The concentrations of the inhibitors clavulanic acid, sulbactam and tazobactam were maintained at 8 μg/ml and plates were incubated for 24 h at 37°C. The lowest concentration which inhibited bacterial growth (monitored by visual inspection) was recorded as the MIC.

2.2. Purification of β-lactamases

β-Lactamases were prepared from 6 l of Terrific Broth (Difco Laboratories, Detroit, MI, USA) supplemented with 50 μg/ml of kanamycin and 50 μg/ml of ampicillin and inoculated with *E. coli* JM101 expressing PSE-4. The β-lactamases were isolated with a standard osmotic shock procedure and purified to homogeneity (99.9%) according to a previously described procedure [27]. Protein concentrations were determined with a Bradford protein assay using Bio-Rad concentrated reagent for protein assays and calculated with a linear regression analysis.

2.3. Kinetic analysis

Kinetic measurements were determined with a Hewlett-Packard 8453 diode-array instrument. The data were analyzed with the Hewlet-Packard 845X UV-VIS and Microsoft Excel software. All enzymatic reactions were performed in 50 mM sodium phosphate buffer at pH 7.0. In standard assays, hydrolysis of benzylpenicillin was monitored at 240 nm ($\varepsilon = 540\ M^{-1}\ cm^{-1}$) at room temperature. All determinations were done in duplicate.

The partition ratio was determined using the titration method [4,28]. Various molar ratios of inhibitor to enzyme mixtures (*I*/*E*) ranging from 0.5 to 100, 50 to 150 and 0.05 to 10 for clavulanic acid, sulbactam and tazobactam, respectively, were prepared and incubated at 4°C for approximately 20 h. The residual enzyme activity was measured by adding 20 μl of each mixture to 980 μl of a buffered solution of 2 mM benzylpenicillin at pH 7.0. The rates of hydrolysis of benzylpenicillin were measured after complete recovery from transient inhibition since a gradual increase in the rate of hydrolysis was time-dependent, particularly with high ratios of *I*/*E*. An inhibitor-free mixture was also included in the assays as a negative control to estimate non-specific inactivation.

The first-order rate constants for inactivation (k_{inact}) were determined by the protocol described by Imtiaz et al. [28]. Increasing concentrations of inhibitors, 20–1000 nM, 200–4000 nM and 20–400 nM for clavulanic acid, sulbactam and tazobactam, respectively, were added to 200 nM of enzyme in 50 mM sodium phosphate buffer at pH 7.0 and incubated on ice. At regular time intervals of 90 s, for a total of five measurements, a 20 μl aliquot of enzyme–inhibitor mixture was removed and added to 980 μl of 2 mM of benzylpenicillin in sodium phosphate buffer (pH 7.0) to measure the remaining activity. The value for k_{inact} was determined using a double-reciprocal plot of the observed rates of inactivation (k_{obs}) versus inhibitor concentrations [28].

The dissociation constants (K_i) for the three inhibitors were determined using a Dixon plot. Two concentrations of benzylpenicillin (300 and 500 nM) were used with various concentrations of inhibitors, 0.5–20 nM, 0.5–15 nM and 0.1–0.75 nM for clavulanic acid, sulbactam and tazobactam, respectively. The enzyme was added to the substrate–inhibitor mixture giving a final concentration of 5 nM in a total reaction volume of 1 ml. The activity was measured immediately and calculated from the highest linear rate of substrate hydrolysis.

2.4. Molecular modeling of the pre-acylation complexes and the acyl-enzyme intermediates

The amino acid sequence of *P. aeruginosa* β-lactamase (PSE-4) was obtained from the Swiss-Prot data bank (accession number P16897). Similarity search was performed to extract closely related sequences in the Brookhaven protein data bank, using the BLASTP program [29]. The TEM-1 β-lactamase was the closest enzyme based on this criterion, and was used as the template to build the three-dimensional model for the PSE-4 β-lactamase. The COMPOSER program (Tripos Associates, St. Louis, MO, USA) was used to align the sequences and construct the three-dimensional model of PSE-4 β-lactamase. This program can successfully be used to predict the three-dimensional structure of a protein sequence using the three-dimensional structure of another if the two sequences show at least 30% homology [30]. PSE-4 β-lactamase showed 40% identity with the TEM-1 β-lactamase. The structure of PSE-4 β-lactamase from the residue Phe-22 through Tyr-283 (numbering according to the Swiss-Prot sequence; corresponds to Phe-27 through Tyr-289 according to Ambler nomenclature [31]) was predicted using the structurally conserved regions based on the alignment of these sequences. The numbering of the residues used henceforth is in correspondence with that of TEM-1, according to [31]. The significant difference between the active site of PSE-4 and TEM-1 β-lactamase is that Lys-234 in TEM-1 corresponds with Arg-234 in PSE-4 β-lactamase. All other residues, viz. Ser-70, Lys-73, Tyr-105, Ser-130, Asn-132, Glu-166, Asn-170, Ser-235, Arg-244 and Asn-276 [37], in PSE-4 β-lactamase correspond to similar spatial locations as those seen in TEM-1 β-lactamase, adding confidence to the reliability of the predicted three-dimensional structure of PSE-4.

The predicted model of PSE-4 β-lactamase was carefully analyzed for any bad orientations of the side chains and the structure was relaxed using the Sibyl molecular modeling program for 200 iterations. Subsequently, the crystallographic water molecules from the X-ray structure of TEM-1 β-lactamase (1btl) were added to the PSE-4 model (total of 196 waters were retained out of 199) and the model was energy-minimized using the AMBER program for 20 000 iterations using all-atom force field. The Cα carbons of the energy-minimized structure of PSE-4 β-lactamase showed an rms deviation of 1.2 Å when compared with those of the X-ray crystal structure of TEM-1 β-lactamase (1btl). Inhibitor molecules (clavulanic acid, sulbactam and tazobactam) were either obtained from the Cambridge Structural Database or constructed in SYBYL and the ESP charges were calculated using the MNDO method in the MOPAC program. The enzyme–substrate pre-acylation complexes and the acyl-enzyme complexes (total of six) were constructed and energy-minimized to obtain the final models.

2.5. Molecular dynamics

To get insight on the movements of the putative linear species of inhibitor in the active site and to verify the proximity of active site nucleophile to iminium carbon of inhibitors, we used molecular dynamics simulations. The minimized complexes were heated to 310 K over 16 ps and equilibrated for 40 ps at 310 K. The simulation was carried for 56 ps using a time step of 1 ft. A total of eight conformations were sampled every 5 ps for each linearized inhibitor complex. The 24 conformations were then minimized to relieve the energy constraint until the energy gradient reached a value smaller or equal to 0.001 kcal/mol/Å.

All the computational work was performed with Sibyl package version 6.3 on a Silicon Graphics Octane workstation. Distances between relevant active site residues, water molecules and functional groups of inactivators were determined with the functions of the viewer module of Insight II package version 95.0 (MSI, San Diego, CA, USA) on a Silicon Graphics Elan R4000 workstation. Generation of the putative inactivating species and dynamic simulations of the linearized inhibitors were performed with the MOE package version 1998 (Chemical Computing Group, Montreal, Que., Canada) on a Silicon Graphics workstation. The linear species for each inhibitor were generated by opening the bond between atom C5 and the oxygen/sulfur using MOE and based upon previous structures [28,32,33]. Prior to molecular dynamics, each linear inhibitor was energy-minimized.

3. Results and discussion

3.1. Susceptibility testing

Expression of the β-lactamase PSE-4 in *E. coli* DH5α conferred high level resistance towards ampicillin, piperacillin and ticarcillin, as shown by the MIC values depicted in Table 1. The use of clavulanic acid in combination with ampicillin or ticarcillin decreased the MICs by 400-fold and 80-fold, respec-

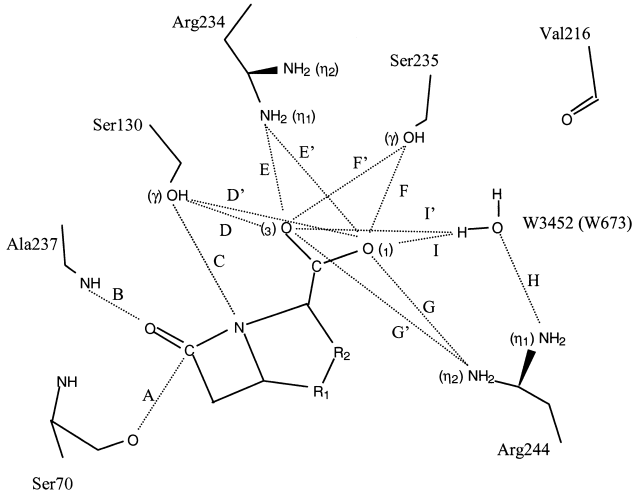
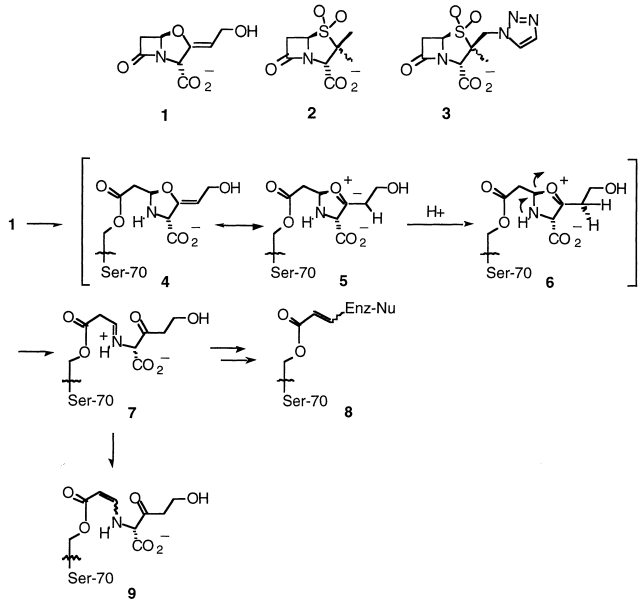


Fig. 1. A schematic drawing of the active site of the PSE-4 enzyme illustrating the distances among important amino acids and functional groups of the inactivator(s) for the pre-acylation complex and the immediate acyl-enzyme species.



Scheme 1.

tively, thus restoring susceptibility to ampicillin (MIC of 12.5 μg/ml); while resistance to ticarcillin was still maintained (MIC of 250 μg/ml). This difference is essentially due to the higher level of catalytic activity of PSE-4 towards ticarcillin. The combination of ampicillin with sulbactam or tazobactam failed to restore susceptibility to ampicillin for *E. coli* expressing PSE-4. Overall, the most potent inactivators used in combination with ampicillin were in order of increasing efficacy sulbactam, tazobactam and clavulanic acid. These results are consistent with the kinetics of inactivation with the purified β-lactamase (described below), where the kinetics followed a similar pattern. Finally, the protective effect of tazobactam for piperacillin was obvious as the MIC of piperacillin decreased by 256-fold, restoring complete susceptibility to piperacillin.

3.2. Molecular modeling

The pre-acylation complexes and the immediate acyl-enzyme intermediates for clavulanic acid, sulbactam and tazobactam in the active site of PSE-4 β-lactamase (a total of six complexes) were generated according to the procedure published earlier [34], and the complexes were energy-minimized. Schematic diagrams of the pre-acylation complexes and the acyl-enzyme intermediates are depicted in Fig. 1A,B. Table 2 gives the distances among the relevant functional groups of inactivators and the enzyme. Fig. 2 depicts these complexes in stereo projections.

3.3. The pre-acylation complex

In all the three computed pre-acylation complexes (Fig. 2A–C), the carbonyl moiety of the lactam ring of all three inhibitors formed hydrogen bonds with the backbone nitrogen of Ser-70 and Ala-237 in the oxyanion hole (Table 2). A hydrogen bond network is present between the C3 carboxylate of the inhibitors and the residues Arg-234, Ser-235, Arg-244 and Ser-139 (located in the β-3 and β-4 strands, and loop between helices α-H4 and α-H5, respectively). It is noteworthy that PSE-4 has an Arg-234 (like other enzymes of group 2c of Bush's classification, [10]) instead of a Lys residue which is present in most class A β-lactamases [8]. The guanidinium group of Arg-234 is expected to participate actively in substrate recognition in the catalytic activity of PSE-4 [7]. The correct positioning of the β-lactam molecule is crucial to permit the nucleophilic attack of the susceptible carbonyl atom of the lactam ring by Ser-70. On the basis of the calculated distances, the C3 carboxylate of the inhibitors and Arg-244 interact via a hydrogen bond in the pre-acylation complex of all three inhibitors in the PSE-4 active site. Furthermore, a weak hydrogen bond between Ser-130 and the nitrogen of the lac-

Table 1
MICs (μg/ml) of *E. coli* DH5α expressing the wild-type PSE-4

β-Lactams in combination with β-lactamase inactivators	MIC (μg/ml)	
	DH5α alone	DH5α with wild-type PSE-4
ampicillin	< 2.4	5 000
piperacillin	1.0	1 000
ticarcillin	< 10	20 000
ampicillin+clavulanic acid 8a	3.1	12.5
ampicillin+sulbactam 8b	1.6	400
ampicillin+tazobactam 8c	0.78	100
ticarcillin+clavulanic acid 8d	2.0	250
piperacillin+tazobactam 8e	1.0	4.0

a, b, c, ampicillin+8 μg/ml of clavulanic acid or sulbactam or tazobactam; d, ticarcillin+8 μg/ml of clavulanic acid; e, piperacillin+8 μg/ml of tazobactam.

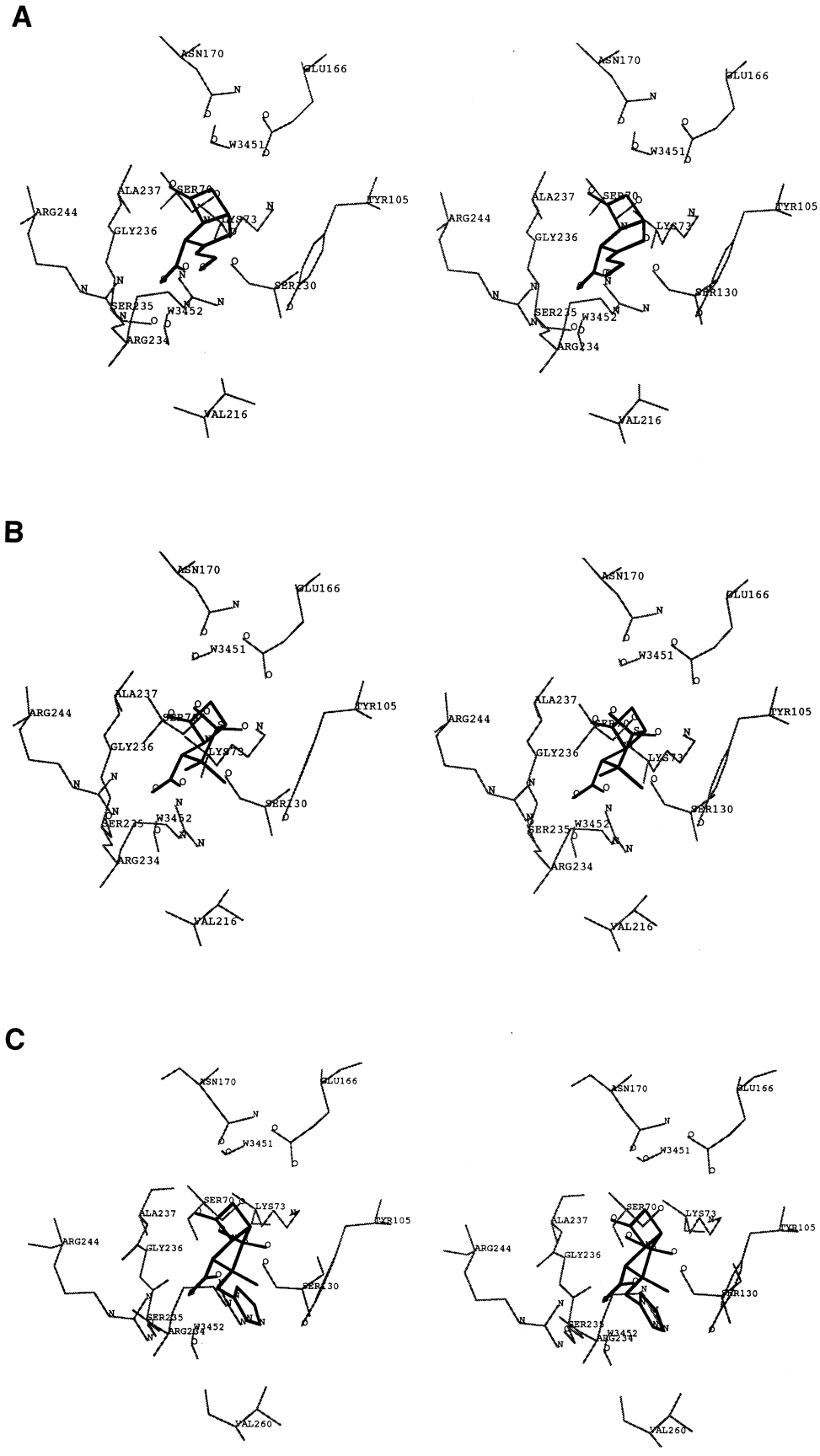


Fig. 2. Computational models of the pre-acylation complex and the immediate acyl-enzyme intermediates of the PSE-4 β-lactamase and the inhibitors, clavulanic acid (A and D, respectively), sulbactam (B and E, respectively) and tazobactam (C and F, respectively). The active site is shown for the regions within the vicinity of the inhibitor complex of PSE-4 β-lactamase. The inhibitor is shown in bold and the protein residues are in gray.

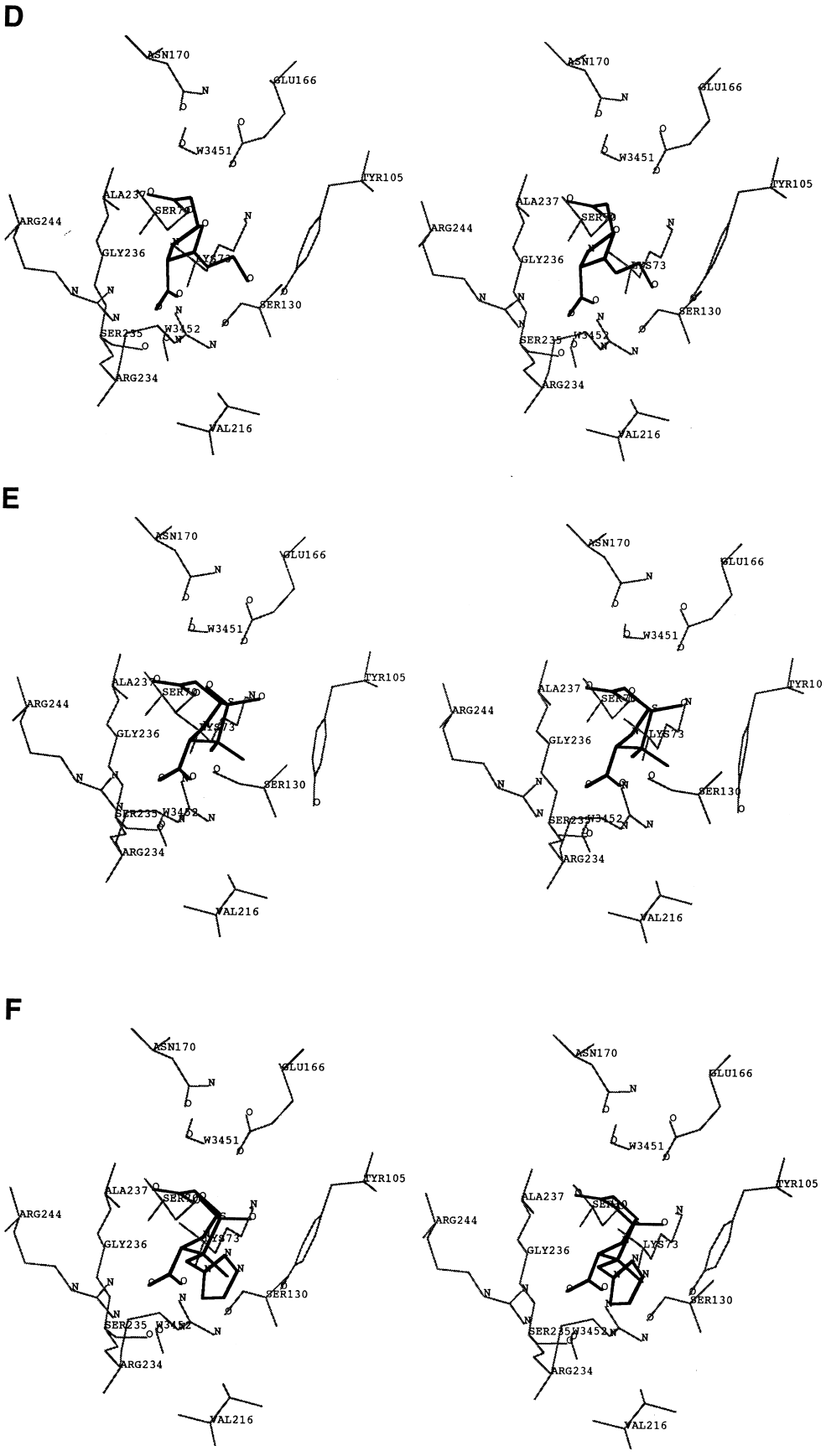


Fig. 2 (*continued*).

Table 2
Calculated distances between non-hydrogen atoms in the minimized complexes

Distances in Fig. 1	Groups involved	Pre-acylation complex (Å)			Acyl-enzyme complex (Å)		
		clv	slb	taz	clv	slb	taz
A	Ser-70 N to lactam CO	**3.0**[a]	**3.2**	**3.0**	**2.7**	**2.7**	**2.7**
B	Ala-237 N to lactam CO	**2.8**	**2.8**	**2.7**	**2.9**	**2.77**	**2.77**
C	Ser-130 Oγ to lactam N	**3.3**	**3.1**	**3.3**	4.6	3.5	4.0
D′	Ser-130 Oγ to O01	4.6	4.8	4.8	**2.6**	4.8	**2.6**
D	Ser-130 Oγ to O03	**2.6**	**2.7**	**2.7**	4.0	**2.7**	4.4
E′	Arg-234 N1 to O01	4.6	4.6	4.6	**2.7**	6.3	**2.8**
E	Arg-234 N1 to O03	**2.7**	**2.8**	**2.9**	4.6	4.6	**2.6**
F	Ser-235 Oγ to O01	**2.6**	**2.7**	**2.7**	**3.0**	**2.8**	**3.1**
F′	Ser-235 Oγ to O03	**3.1**	4.3	4.3	**2.6**	**2.8**	**2.6**
G	Arg-244 O2 to O01	**2.7**	**2.8**	**2.8**	4.5	**2.6**	4.8
G′	Arg-244 N2 to O03	4.7	4.9	4.9	**2.7**	4.8	**2.6**
H	Arg-244 N1 to W3452	**2.7**	**2.7**	**2.7**	**2.7**	**2.7**	**2.7**
I	W3452 to O01	**2.7**	**2.7**	**2.7**	4.6	**2.7**	4.3
I′	W3452 to O03	4.4	4.4	4.4	**2.6**	4.1	**2.9**

clv, clavulanate; slb, sulbactam; taz, tazobactam.
[a]Values in bold indicate potential hydrogen bonding between the corresponding groups. Average distance of a hydrogen bond: 2.7–3.1 Å.

tam ring was observed in the pre-acylation intermediates of sulbactam and tazobactam.

3.4. The acyl-enzyme intermediates of the three inhibitors

The immediate acyl-enzyme species of clavulanic acid, sulbactam and tazobactam in the active site of PSE-4 β-lactamase revealed interesting features (Fig. 2D–F). This species for clavulanate is depicted by structure **4** in Scheme 1. The ester carbonyl moiety was retained in the oxyanion hole through hydrogen bonds to the backbone nitrogens of Ser-70 and Ala-237. Whereas the ring opening from the immediate acyl-enzyme species to the inactivating linear species for sulbactam and tazobactam is a relatively simple event, this step is more complicated for clavulanic acid. Clavulanic acid would benefit from a protonation process in the ring opening process (such as **5, 6, 7**). This process is made possible by the arrangement of functionalities in the active sites of class A β-lactamases. The hydrogen bonds between the C3 carboxylate of the inhibitors with Ser-130, Arg-234, Ser-235 and Arg-244 were seen in all three acyl-enzyme species. Interestingly, Wat-3452 (corresponding spatially to Wat-673 in Imtiaz et al. [28]) forms a bridge between the carboxylate of all three inhibitors used in this study and the side chain of Arg-244. This water molecule was implicated to play a function as a source of a critical proton in the inactivation chemistry of clavulanate with the TEM-1 enzyme (Scheme 1; **5, 6** [24]). Formation of the linear inactivation species **7** (**6, 7**) would be triggered by protonation of the C8 carbon of the carbanion species **5** and the likely source of this proton was attributed to be Wat-673 [24]. It was proposed that the clavulanate carboxylate might function as a general base, facilitating the transfer of a proton from Wat-673 to the C8 of the carbanion species [24]. Due to the similar interactions observed in the models of TEM-1 and PSE-4 β-lactamases, the inactivation chemistries are probably similar in both cases.

Species **5** would trap an active site nucleophilic residue to give species **8**, which accounts for the irreversible inhibition of the enzyme. This nucleophilic amino acid was shown to be Ser-130 for the case of inactivation of the TEM-1 enzyme [32,35]. The linear species for each inhibitor were generated by opening the bond between atom C5 and the oxygen/sulfur using MOE and based upon previous structures [28,32,33]. The three linear species were generated, energies were minimized for each individually. Simulations were then performed followed by selection of structures in the time course of simulation and minimization of energies for each individually.

Molecular dynamic simulations were carried out to determine which of the active site nucleophiles of PSE-4 β-lactamase were implicated in the capture of the iminium carbon of the three inhibitors. The mean distances of active site nucleophiles to C5 of inhibitors are depicted in Table 3. Based upon the inter-atomic distances measured from the eight minimized conformations sampled during the dynamic simulation, we noted that Lys-73 or Arg-244 could be probable candidates responsible for the capture of the iminium species of clavulanic acid; however, Arg-244 cannot function as a nucleophile. Also, the participation Ser-130 in that reaction could be ruled out with an average distance of 6.1 Å. Interestingly, two different amino acids were identified to participate in the irreversible inactivation of PSE-4 by the penicillin sulfones. In the case of sulbactam, Lys-73 at an average distance of 4.8 Å is the most probable candidate, while Ser-130 at 4.5 Å would perform the same function with tazobactam. The interactions of the triazole moiety of the latter clearly affect the inactivation chemistry of tazobactam. Hence, the conformation of the PSE-4 active site contains subtle differences with

Table 3
Average distances in Å between potential active site nucleophiles of PSE-4 and C5 of the linearized inhibitor complex

	Lys-73 Nε	Ser-130 Oγ	Arg-244 N1	Arg-244 N2
Clavulanic acid	**5.7**[a]	6.1	7.5	6.2
Sulbactam	**4.8**	5.4	10.6	8.9
Tazobactam	7.2	**4.5**	6.7	5.5

[a]Values in bold indicate potential interactions between the concerned nucleophile and C5 of the inhibitor.

Table 4
Kinetic parameters for interactions of clavulanate, sulbactam and tazobactam with the PSE-4 β-lactamase

Kinetic parameter	Clavulanate	Sulbactam	Tazobactam
K_i (μM)	4 ± 1	12 ± 1	0.15 ± 0.01
k_{cat} (s^{-1})	3.8 ± 0.4	5.2 ± 1.3	7.4 ± 1.1
k_{inact} (s^{-1})	0.35 ± 0.02	0.04 ± 0.01	0.18 ± 0.02
k_{cat}/k_{inact} PSE-4	11 ± 1	131 ± 2	41 ± 4
k_{cat}/k_{inact} TEM	125 ± 36	1×10^4	475 ± 42

TEM-1 which can only be confirmed by crystallography studies.

3.5. Kinetic analyses

Kinetic evaluation of the three clinically used inhibitors, clavulanic acid, sulbactam and tazobactam, was performed with PSE-4 (Table 4). The binding affinity of the inhibitors in the active site of PSE-4 was investigated by determining the dissociation constant K_i. Clavulanic acid and sulbactam showed similar K_i (4 and 12 μM, respectively). It is noteworthy that tazobactam showed a considerably higher affinity for this enzyme (K_i of 150 nM); 80-fold higher affinity than for sulbactam. The triazole moiety of tazobactam is the only structural difference. Examination of the pre-acylation model of tazobactam in the PSE-4 active site indicated the existence of hydrogen bonds between the N3 and N4 nitrogen atoms of the triazole ring with Tyr-105. The interactions of the enzyme with the triazole of tazobactam are clearly important in enhancing affinity.

Transient inhibition was observed with each inhibitor, as seen previously with other β-lactamases, especially at high inhibitor to enzyme ratios because a gradual increase in the rate of hydrolysis was observed during the course of substrate turnover. Clavulanic acid, sulbactam and tazobactam were individually turned over 11, 131 and 41 times, respectively, before each inactivation event of the enzyme (Table 4). Consistent with previous kinetic studies on other class A enzymes, clavulanic acid appeared to be the most potent inhibitor against PSE-4 both in terms of exhibiting the best (lowest) partition ratio (i.e. k_{cat}/k_{inact}) as well as the most rapid first-order rate constant for inactivation (i.e. k_{inact}). The most striking result is the partition ratio of sulbactam measured with PSE-4 ($k_{cat}/k_{inact} = 131$) which is 76-fold lower compared to the value measured with TEM-1 ($k_{cat}/k_{inact} = 10\,000$); [28,33]. Another interesting observation is that the three inactivators are turned over with comparable rate constants (i.e. k_{cat}). The partition ratios of clavulanic acid and tazobactam were 14-fold and 3-fold lower than the values calculated with TEM-1 and TEM-2 enzymes, respectively [33,36]. The PSE-4 enzyme appears to be exquisitely sensitive to the three clinical inhibitors of β-lactamases, generally more so than other common class A enzymes such as the TEM variants which have been the subject of similar analysis. This fact bodes well for intervention in the clinic, since Pseudomonal infections are generally hard to treat and would support the tazobactam–piperacillin combination recommended. An in-depth analysis of the PSE-4 active site and its interaction with inhibitors should assist in defining the mechanism of inhibition for carbenicillin-hydrolyzing β-lactamases [3]. Optimization by energy minimization of stable complexes occurring along the pathway of hydrolysis of benzylpenicillin and cephalosporin C explained the mechanism of acyl transfer by the class A β-lactamase of *Streptomyces albus* G and the differences found in the K_{cat} values with these substrates [38]. The data reported here extend modeling studies on the hydrolytic mechanism of class A β-lactamases, and those done with the three clinically useful inhibitors [20,27,28,33,39].

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