A Structure-Based Analysis of the Inhibition of Class A β-Lactamases by Sulbactam†

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ABSTRACT: From the crystal structure of the *Bacillus licheniformis* 749/C β-lactamase, energy-minimized structures for the precatalytic, the acyl–enzyme intermediate, and the acylated linear inactivating species for sulbactam—a clinically useful mechanism-based inactivator for class A β-lactamases—were generated. The effect of individual Ser-235-Ala and Arg-244-Ser point mutations on the inactivation and turnover processes was consistent with the existence of hydrogen bonds between the side chains of these residues and the sulbactam species. The departure of the sulfinate leaving group from the acyl–enzyme intermediate of sulbactam is believed to be a prerequisite for the inactivation process. In order to explore the influence of the leaving group, penicillanic acid (**2**), penicillanic acid α-*S*-oxide (**3**), and penicillanic acid β-*S*-oxide (**4**) were synthesized and studied in kinetic experiments with the TEM-1 β-lactamase. Penicillanic acid is only a substrate, but penicillanic acid *S*-oxides were both substrates and inactivators for the enzyme. An argument is presented to rationalize these observations on the basis of the leaving ability of thiolate, sulfenate, and sulfinate from the acyl–enzyme intermediates of penicillanic acid (**2**), the penicillanic acid *S*-oxides (**3** and **4**), and sulbactam, respectively. The departure of the leaving group does not appear to be rate limiting in the inactivator process, but is an indispensable component of the irreversible inactivation of the enzyme. Molecular dynamics calculations of the putative inactivating species suggest that Lys-73, Lys-234, and Ser-130 are three likely residues that may be modified in the course of the inactivation chemistry. A discussion is presented of the mechanism of formation of the transiently inhibited enzyme species, which comes about as a consequence of the tautomerization of the double bond of the inactivating iminium moiety. In addition, the mechanistic details presented for sulbactam are compared and contrasted with those of clavulanic acid, another clinically used inactivator for class A β-lactamases.

β-Lactamases (EC 3.5.2.6) are bacterial defensive enzymes that hydrolyze β-lactam antibiotics. This hydrolytic activity is the most common means by which bacteria become resistant to β-lactam drugs. Two strategies have been employed to counter the clinical problem posed by the enzyme and the spread of β-lactamase-mediated resistance: (1) New β-lactam drugs have been developed that are inherently less susceptible to β-lactamases (Pratt, 1989; Fisher, 1984). (2) Mixtures of a mechanism-based inactivator for β-lactamase and typical penicillins have been used (Moellering, 1991; Leigh *et al.*, 1981). The rationale in the latter strategies centers on the expectation that the inactivators would destroy the β-lactamase activity, whereby the penicillin would survive to kill the bacteria by inhibiting cell wall biosynthesis.

Frequent clinical use of β-lactam antibiotics leads to selection and dissemination of novel microbial β-lactamase activities. New variants of β-lactamases are being identified at an alarming rate. Many of these are mutational variants of prevalent β-lactamases which thereby gain enhanced activity against the second- and third-generation cephalosporins (Moellering, 1991). New β-lactamases have been identified which show the ability to hydrolyze carbapenems, a class of β-lactam molecules which previously were resistant to β-lactamases (Sanders, 1992). A clear example of the power of clinical selection is that at least 21 variants of the plasmid-encoded TEM-1 β-lactamase have been identified over the past decade as a result of the resistance to broad-spectrum cephalosporins they confer (Jacoby & Medeiros, 1991).

The combination drugs of β-lactamase inactivators and penicillins have been relatively free from selection of new resistance. However, we have shown recently that the Arg-244-Ser mutant derivative of the TEM-1 β-lactamase is essentially resistant, both *in vivo* and *in vitro*, to inactivation by clavulanate (Manavathu *et al.*, 1991; Imtiaz *et al.*, 1993a), a mechanism-based inactivator for class A β-lactamases. Interestingly, this mutant enzyme remains catalytically competent in hydrolysis of typical substrates (Zafaralla *et al.*, 1992). The Arg-244-Ser TEM-1 β-lactamase has recently been detected in a clinical isolate by French workers (Vedel *et al.*, 1992); this finding may herald a more widespread compromise in the clinical utility of clavulanate in the near future.

A first step toward an understanding of the β-lactam resistance problem is an appreciation of the details of interactions of substrates or inactivators with the active sites of β-lactamases. Toward that goal, we have reported recently a detailed analysis of the chemistry of turnover of and inactivation by clavulanic acid of the TEM-1 β-lactamase (Imtiaz *et al.*, 1993a). We report here such an analysis for sulbactam (**1**), another mechanism-based inactivator for class A β-lactamases that is used widely in clinical therapy, thereby discussing the similarities and points of divergence in the chemical processes of sulbactam and clavulanate.

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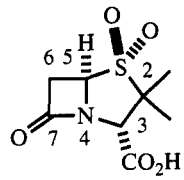

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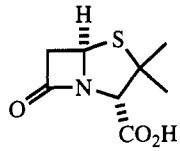

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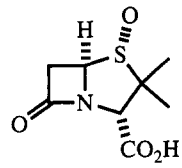

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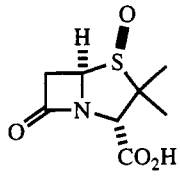

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MATERIALS AND METHODS

Benzylpenicillin was purchased from Sigma. DEAE-cellulose was obtained from Fisher, and Sephadex G-100 was a Pharmacia product. 6-Aminopenicillanic acid was purchased from the Aldrich Chemical Co. Sulbactam was obtained from the Roerig division of Pfizer, Inc. The procedure for site-specific mutagenesis at position 244 has been described (Zafaralla *et al.*, 1992); similar experiments produced the Ala-235 mutant enzyme (Imtiaz *et al.*, 1993b). A three-step purification procedure using two ion-exchange columns (DEAE-cellulose) and one sizing column (Sephadex G-100), as described by Zafaralla *et al.* (1992), was used to purify the wild-type and mutant enzymes to homogeneity. An extinction coefficient of 29 400 M^{-1} cm^{-1} at a wavelength of 281 nm was used for the determination of protein concentrations. Infrared spectra were recorded on a Nicolet DX spectrometer. The proton NMR and the mass spectra were obtained on Nicolet QE-300 and Kratos MS 80RFA spectrometers, respectively.

Modeling. The crystal structure of the TEM-1 β-lactamase was reported recently (Strynadka *et al.*, 1992; Jelsch *et al.*, 1993), but the atomic coordinates are not available in the Protein Data Bank. Therefore, the active site of the TEM-1 enzyme was constructed from the refined crystal structure of the structurally homologous *Bacillus licheniformis* 749/C β-lactamase (4BLM, Protein Data Bank; Knox & Moews, 1991) by replacing three residues near the active site: Thr-216 was changed to Val; Thr-235, to Ser; and Tyr-274, to Glu. Hydrogen atoms were added to the crystal structure for pH 7.0 using Discover 2.1.0. Whereas a direct comparison of our model and the TEM-1 β-lactamase is not possible at present, Strynadka *et al.* (1992) state that the active sites of the TEM-1, *B. licheniformis*, and *Staphylococcus aureus* enzymes are very similar and that mechanistic understanding from one enzyme should be valid for the other two. The sulbactam X-ray structure (Brenner & Knowles, 1981) was initially positioned in the active site in agreement with the earlier experimental findings and modeling studies (Imtiaz *et al.*, 1993a; Juteau *et al.*, 1992). The complex was solvated with both crystallographic and Monte Carlo water molecules. Crystallographic waters within 5 Å of the enzyme, or 15 Å of the inactivator, were included. With sulbactam sequestered, the active site was then capped with a 20-Å layer of randomly generated water molecules.

The complexes were minimized using the AMBER 4.0 (Pearlman *et al.*, 1991) force field. Figure 1 shows the general topology of the protein with the precatalytic species of the sulbactam sequestered in the active site. The use of CVFF and SYBYL parameters for the β-lactam structure resulted in an unacceptable planar nitrogen and a pyramidal carbonyl carbon for the β-lactam moiety. New parameters for the sulbactam structure were developed for the AMBER force field, which provided an RMS fit of the minimized bicyclic atoms to the sulbactam crystal structure of less than 0.07 Å. Charges for each of the intermediate species were fitted to an electrostatic surface potential generated by MOPAC 6.0 (Stewart, 1992; Bessler *et al.*, 1990). The catalytic Ser-70 atomic charges for the acylated species were also fitted using MOPAC 6.0 for the acylated species.

Table 1: Protocol for Energy Minimization and Dynamics

step	type of minimization/dynamics	steps/fs
1	only hydrogens were allowed to move	3000
2	active-site minimization, backbone restrained	10 000
3	active-site minimization	20 500
4	active-site minimization, 10-Å cutoff	4000
5	heat active site to 310 K	16 000
6	active-site simulation at 310 K	40 000
7	active-site minimization	5000

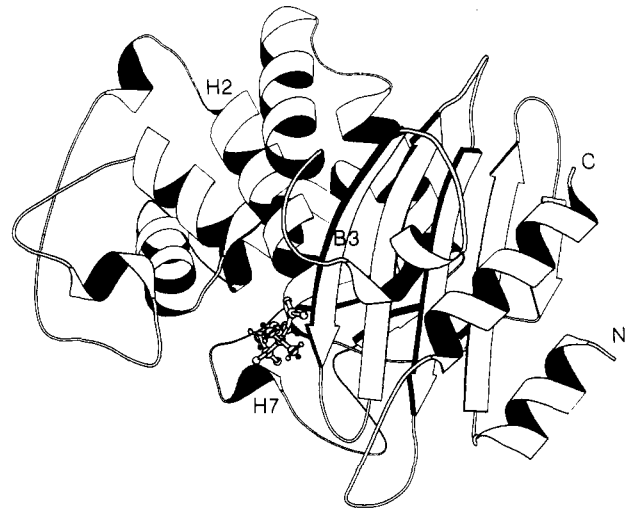


FIGURE 1: Ribbon drawing of a class A β-lactamase showing sulbactam in the active site near the α-helix H2 and the β-strand B3.

The precatalytic, acyl–enzyme intermediate, and acylated linear complexes were minimized with the AMBER force field using steps 1–4 of the protocol listed in Table 1. The more flexible linear species was further studied with molecular dynamics. The linear complex was heated over a 16-ps period (step 5), followed by a 40-ps simulation (step 6). Four conformations, randomly chosen from the simulation, were then minimized and plotted for visualization (step 7). A constant dielectric of 1 was used in all cases.

Kinetic Measurements. Kinetic measurements were carried out in Perkin-Elmer Lambda 3B or Hewlett-Packard 452 diode-array instruments. Assays were carried out by monitoring hydrolysis of benzylpenicillin at 240 nm ($\Delta\epsilon$ = 570 M^{-1} cm^{-1}).

The dissociation constants (K_i) for sulbactam were calculated for the wild-type TEM-1, Ser-244, and Ala-235 enzymes by the method of Dixon (1953), according to the protocols developed for clavulanate (Imtiaz *et al.*, 1993a). Two concentrations, 400 and 500 μM, of benzylpenicillin were used in each case at room temperature. A series of mixtures were prepared in 100 mM sodium phosphate buffer, pH 7.0, containing the substrate and varying concentrations of sulbactam (1–15 μM with the wild-type and Ala-235 enzymes and 2–40 μM with the Ser-244 enzyme). These mixtures were assayed immediately upon the addition of a 10-μL aliquot of the respective enzymes, affording a final concentration of 5 nM enzyme in the cuvette.

The partition ratios for sulbactam with the wild-type TEM-1 and mutant enzymes were determined by the titration method (Silverman, 1988). Several buffered mixtures containing various $[I]_o/[E]_o$ molar ratios, ranging from 1 to 8000 for the wild-type, Ala-235, and Ser-244 enzymes, were incubated at 4 °C in 100 mM sodium phosphate buffer, pH 7.0. After a period of approximately 20 h, the remaining enzyme activity was assayed by monitoring the rate of hydrolysis of 2 mM benzylpenicillin at 240 nm. The extent of nonspecific inactivation of the enzymes was taken into account by means of a control experiment in which the enzyme activity was measured under identical conditions, but in the absence of the inactivator.

Hydrolysis of sulbactam was monitored directly at 236 nm ($\Delta\epsilon$ = 1780 M^{-1} cm^{-1}), according to the method of Brenner *et al.* (1981). Rates of hydrolysis were measured for various sulbactam concentrations between 0.2 and 1.0 mM at room temperature; the enzyme concentration was approximately 200 nM in each measurement. Typically, a progressive decrease in the rate of hydrolysis was observed in the course of the reaction, indicating the simultaneous events of enzyme inactivation and sulbactam hydrolysis. Rates were calculated from the initial linear portion of the reaction, which allowed for the evaluation of V_{max}, from which k_{cat} was calculated. This analysis gave reliable rates, since the partition ratio for sulbactam was high for each enzyme.

The values for the kinetic parameters (k_{cat} and K_m) for penicillanic acid and the α- and β-*S*-oxides of penicillanic acid were determined from Lineweaver–Burk plots. The spectrophotometric assays were carried out in 100 mM sodium phosphate buffer, pH 7.0, at room temperature. Initial rates were determined in duplicate from the first 5–10% of the reactions with six or seven substrate concentrations. The change in extinction coefficients for the substrates at pH 7.0 were as follows: penicillanic acid, $\Delta\epsilon_{240}$ = 94 M^{-1} cm^{-1}; penicillanic acid α-*S*-oxide, $\Delta\epsilon_{240}$ = 87 M^{-1} cm^{-1}; penicillanic acid β-*S*-oxide, $\Delta\epsilon_{220}$ = 370 M^{-1} cm^{-1}.

The inactivation experiments to determine the partition ratio for penicillanic acid and the α- and β-*S*-oxides of penicillanic acid were carried out by incubating 10–100 mM concentrations of the three compounds with 2.3 μM TEM-1 enzyme at room temperature. For inactivator concentrations of 20 mM or less, incubations were made in 100 mM phosphate buffer, pH 7.0; in cases in which the concentration of the inactivator exceeded 20 mM, incubations were made in 600 mM sodium phosphate buffer, pH 7.0. The buffered mixtures were incubated for a period of approximately 20 h at 4 °C, after which the remaining enzyme activity was assayed. The assays were carried out by monitoring hydrolysis of 1 mM benzylpenicillin at 240 nm in 100 mM sodium phosphate buffer, pH 7.0, at room temperature. Total loss of activity was never seen with these compounds; therefore, the partition ratios were determined by extrapolation of the data to the point of no activity.

6,6-Dibromopenicillanic Acid (**6**). 6,6-Dibromopenicillanic acid was prepared according to the procedure of Volkmann *et al.* (1982). A solution of bromine (4.28 mL, 83.4 mmol), 2.5 N H_2SO_4 (22.3 mL), and sodium nitrite (3.84 g, 55.6 mmol) in 60 mL of methylene chloride was stirred in an ice bath. 6-Aminopenicillanic acid (6 g, 27.8 mmol) was added portionwise over a 1/2-h period, and the mixture was stirred for an additional 1/2 h at 0–4 °C. A 1.0 M solution of sodium bisulfite (45.5 mL) was added dropwise to the red solution until the color changed to pale yellow. The two layers were separated, and the aqueous layer was washed with methylene chloride (2×). The combined organic solution was washed with saturated NaCl solution (2×). The resulting organic layer was concentrated *in vacuo* to dryness to give the title compound (7.9 g): yield, 80%; IR (KBr) 1780, 1767 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.57 (3 H, s, methyl), 1.66 (3 H, s, methyl), 4.58 (1 H, s, C_3 methine), 5.78 (1 H, s, C_5 methine); MS EI 359 (M, 36%).

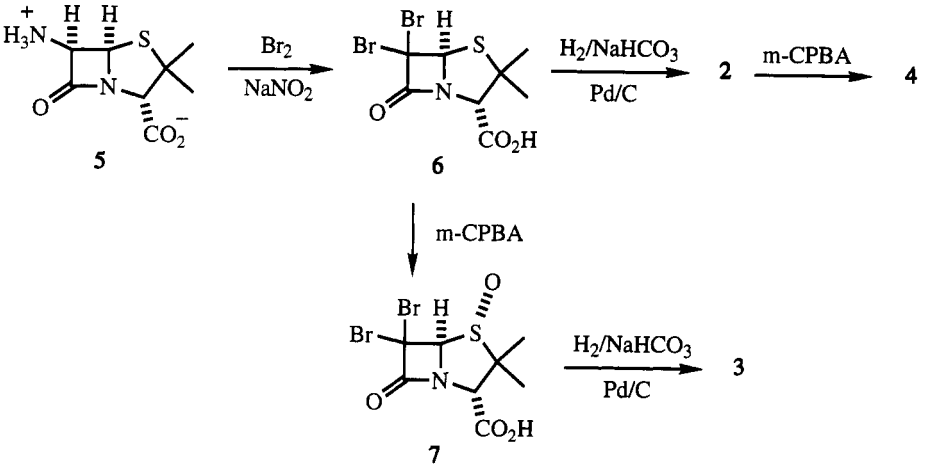
Penicillanic Acid (**2**). 6,6-Dibromopenicillanic acid (2 g, 9.95 mmol) was dissolved in saturated $NaHCO_3$ solution (300 mL) and was then mixed with 1.0 g of 10% Pd/C catalyst. Dehydrohalogenation of the mixture was carried out under 20 psi hydrogen for a period of 30 min. The reaction was subsequently stopped, and the mixture was filtered through celite. The filtrate was cooled to approximately 5 °C, acidified to pH 2.0 with 6 N HCl, and extracted with ethyl ether (2×). The organic layer was dried over magnesium sulfate and evaporated to dryness *in vacuo* to give 0.8 g of the desired product: yield, 70%; IR (KBr) 1780, 1753 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.52 (3 H, s, methyl), 1.66 (3 H, s, methyl), 4.36 (1 H, s, C_3 methine), 5.29 (1 H, dd, J = 1.5, 4.0 Hz, C_5 methine), 2.99 (1 H, dd, J = 1.5, 16 Hz, $C_{6\beta}$ hydrogen), 3.60 (1 H, dd, J = 4.0, 16 Hz, $C_{6\alpha}$ hydrogen); MS CI^+ 202 (M + H, 0.5%).

6,6-Dibromopenicillanic Acid α-S-Oxide (**7**). A solution of 6,6-dibromopenicillanic acid (7.4 g, 20.7 mmol) in 200 mL of chloroform was cooled in an ice bath prior to the addition of a solution of *m*-chloroperoxybenzoic acid (*m*-CPBA, 3.6 g, 20.7 mmol) in 30 mL of chloroform over a 5-min period. The reaction mixture was stirred at room temperature for 1 h. The product precipitated from the mixture, which was filtered and dried *in vacuo* to give 5.8 g of the product: yield, 75%; IR (KBr) 1816, 1745 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.28 (3 H, s, methyl), 1.51 (3 H, s, methyl), 4.90 (1 H, s, C_3 methine), 5.45 (1 H, s, C_5 methine); MS CI^+ 316 {M (^{81}Br, ^{81}Br) – COOH – O, 1.4%}, 314 {M (^{81}Br, ^{79}Br) – COOH – O, 2.7%}; 312 {M (^{79}Br, ^{79}Br) – COOH – O, 1.3%}; MS EI 234 (M – COOH – Br – O, 2.2%), (M – C_2Br_2O, 8.1%, *retro*-[2 + 2]-lactam ring opening).

Penicillanic Acid α-S-Oxide (**3**). 6,6-Dibromopenicillanic acid α-*S*-oxide (1.0 g, 2.7 mmol) was dissolved in a saturated solution of $NaHCO_3$ (200 mL). A portion of 10% Pd/C (0.1 g) was added to the solution, and subsequently hydrogenation was carried out under 20 psi hydrogen for a period of 15 min. The reaction mixture was filtered through a layer of celite, and the aqueous filtrate was washed with ethyl acetate (2×). The aqueous layer was cooled in an ice bath, and its pH was adjusted to 1.2 with 6 N HCl. The aqueous layer was saturated with brine, and extraction was carried out with ethyl acetate (2×). The organic layer was dried over magnesium sulfate and concentrated to afford 0.18 g of the product: yield, 30%; IR (KBr) 1786, 1732 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.21 (3 H, s, methyl), 1.45 (3 H, s, methyl), 4.40 (1 H, s, C_3 methine), 4.58 (1 H, dd, J = 1.8, 4.2 Hz, C_5 methine), 3.44 (1 H, dd, J = 1.8, 16.5 Hz, $C_{6\beta}$ hydrogen), 3.56 (1 H, dd, J = 4.2, 16.5 Hz, $C_{6\alpha}$ hydrogen); MS CI^+ 218 (M + H, 3%).

Penicillanic Acid β-S-Oxide (**4**). Penicillanic acid (0.3 g, 1.5 mmol) was dissolved in 100 mL of a 20:1 mixture of chloroform and methanol. The solution was cooled in an ice bath prior to the addition of a solution of *m*-chloroperoxybenzoic acid (*m*-CPBA, 0.26 g, 1.5 mmol) dissolved in 5 mL of chloroform. The reaction mixture was stirred at room temperature for 90 min, and then the solvent was removed *in vacuo*. A portion of benzene (40 mL) was added to the flask and stirred for 2–3 min in order to dissolve the reaction byproduct, *m*-chlorobenzoic acid. The title compound was collected by filtration (0.26 g) and dried *in vacuo*: yield, 80%; IR (KBr) 1780, 1737 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.17 (3

Scheme 1



H, s, methyl), 1.52 (3 H, s, methyl), 4.12 (1 H, s, C_3 methine), 5.24 (1 H, dd, J = 1.5, 4.5 Hz, C_5 methine), 2.96 (1 H, dd, J = 1.5, 15.9 Hz, $C_{6\beta}$ hydrogen), 3.38 (1 H, dd, J = 4.5, 15.9 Hz, $C_{6\alpha}$ hydrogen); MS CI$^+$ 218 (M + H, 1.3%).

RESULTS

Syntheses. Penicillanic acid (**2**), penicillanic acid α-*S*-oxide (**3**), and penicillanic acid β-*S*-oxide (**4**) were synthesized as described. 6,6-Dibromopenicillanic acid (**6**) was prepared from 6-aminopenicillanic acid (**5**) according to the procedure of Volkmann *et al.* (1982). Dehydrohalogenation of 6,6-dibromopenicillanic acid by a modification of the procedure of Clayton (1969) gave penicillanic acid (**2**). It is known that oxidation of penicillanic acid derivatives leads to α- or β-*S*-oxide isomers depending on the nature of the 6β-substituent (Harrison & Hodge, 1976). In the absence of a 6β-substituent, or if the 6β substituent is an acetamido (or substituted acetamido) group, the β-sulfoxide is the main product of peroxyacid oxidation. In contrast, the steric effects due to the presence of a 6β-substituent, such as 6β-bromo, lead to the formation of the α-*S*-oxide upon peroxyacid oxidation. Therefore, for the formation of penicillanic acid β-*S*-oxide (**4**), oxidation of penicillanic acid was carried out with 1 equiv of *m*-CPBA. To obtain the α-*S*-oxide, 6,6-dibromopenicillanic acid was oxidized with 1 equiv of *m*-chloroperoxybenzoic acid (*m*-CPBA) to give 6,6-dibromopenicillanic acid α-*S*-oxide (**7**), which on subsequent dehydrohalogenation afforded the desired penicillanic acid α-*S*-oxide (**3**). We add that a somewhat different route for compounds **3** and **4** has been reported earlier (Kemp *et al.*, 1979) (Scheme 1).

Preacylation Complex. A set of important interactions in the energy-minimized complex of sulbactam with the enzyme is that between the β-lactam carbonyl group and the oxyanion hole. Two hydrogen bonds to the backbone amide groups of residues Ser-70 and Ala-237 (2.82 and 2.75 Å, respectively; Table 2) may polarize the lactam carbonyl bond for attack by the reactive Ser-70 (Figure 2A). The geometry of this interaction contrasts somewhat with the binding of clavulanate (Imtiaz *et al.*, 1993a), where binding in the oxyanion hole is perhaps weaker because of the involvement of a water molecule (Wat-712) in place of the Ser-70 amide group. Strong electrostatic attraction is indicated for the C_3 carboxylate with Lys-234, Ser-235, and Arg-244. As in the preacylation complex of clavulanate, the model does not indicate an interaction of Ser-130 with the nitrogen of the β-lactam ring, which is at a distance of 3.69 Å.

The sulfone group of sulbactam is exposed at the opening of the active site and appears to be free of any interactions

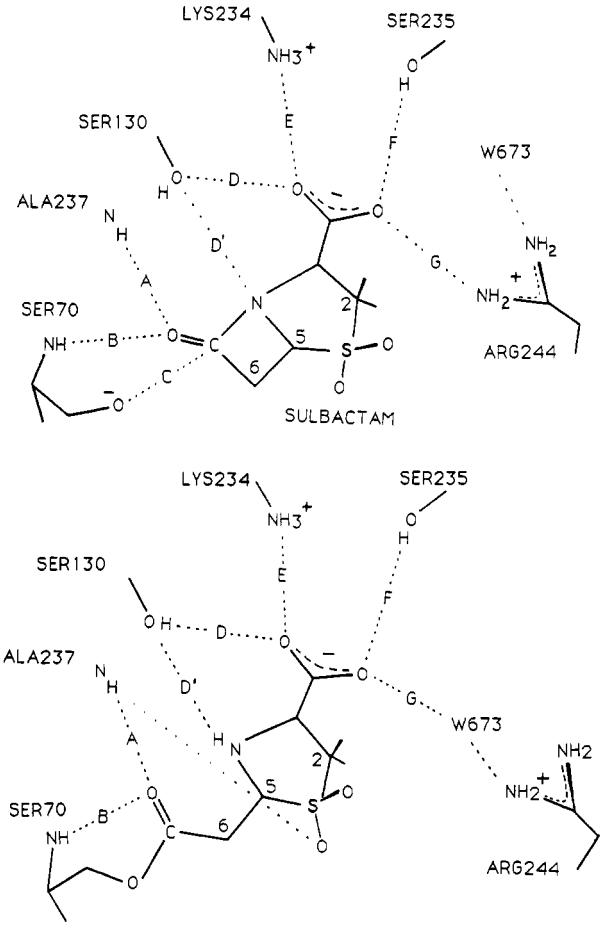


FIGURE 2: Diagram of the distances in Table 2 for (A) the preacylation complex and (B) the acyl–enzyme intermediate of sulbactam with the β-lactamase.

with the active-site residues in this complex (Figure 3A). It is interesting to note, however, that were an amino acid with a side chain capable of hydrogen bonding present at the 237 position instead of alanine, binding to the sulfone β-oxygen atom could take place. The *S. aureus* β-lactamase, for example, has a glutamine at position 237 and would be expected to have a slightly different binding interaction at this site.

*Acyl–Enzyme Intermediate (**8**).* In the energy-minimized acyl–enzyme model, the ester carbonyl group that originated in the β-lactam carbonyl remains positioned in the oxyanion hole after acylation, but the binding to the amide groups of Ser-70 and Ala-237 is now much weaker (2.98 and 3.16 Å, respectively; Figures 2B and 3B). Hydrogen bonding of the

Table 2: Calculated Distances between Non-Hydrogen Atoms in the Minimized Complexes[a]

distance in Figure 2, panels A and B	groups involved	preacylation complex (Å)	acylated complex (Å)
A	Ala-237 to lactam CO	2.75	3.16
B	Ser-70 to lactam CO	2.82	2.98
C	Ser-70 to lactam CO	2.89	
D	Ser-130 to COO^-	3.34	2.69
D′	Ser-130 to lactam N	3.69	2.92
E	Lys-234 to COO^-	2.74	2.70
F	Ser-235 to COO^-	2.64	2.73
G	Arg-244 or W673 to COO^-	2.70	2.72

[a] The estimated uncertainty in distances is 0.1 Å.

carboxylate group to Lys-234 and Ser-235 remains strong. Arg-244 is no longer directly bonded to the carboxyl group, but rather is bonded through a bridging crystallographic water molecule (Wat-673).

Hydrogen bonding of the Ser-130 side chain to the C_3 carboxylate is preserved, and actually strengthened, upon acylation; however, rotation of the five-membered ring about the C_5–C_6 bond brings the NH function of the ring sufficiently close to donate a hydrogen bond to the side-chain hydroxyl of Ser-130. This alignment contrasts with that seen in the acyl–enzyme intermediate generated for clavulanate, where the Ser-130 side-chain hydroxyl donates a hydrogen bond to the newly formed ring amine (Imtiaz *et al.*, 1993a). The above mentioned rotation of the five-membered ring positions the sulfone β-oxygen atom such that the formation of a hydrogen bond with the main-chain amide nitrogen of Ala-237 (2.84 Å) is now possible, although the N–H···O hydrogen bond angle (121°) is not optimal for strong binding. An overlay of the orientations of the preacylated sulbactam complex and the acyl–enzyme intermediate is shown in Figure 4.

The Inactivating Species (9). Expulsion of the sulfinate from C_5 generates the linear acyl–enzyme intermediate (**9**), the putative inactivating species. The energy minimization indicated that the carboxylate of this species maintains its interactions with the side chains of Ser-130, Ser-235, and Lys-234 and Wat-673, which in turn is coordinated to the side chain $N^{\eta 1}$ of Arg-244. The $N^{\eta 2}$ of Arg-244 is close enough (2.7 Å) to form a hydrogen bond to one of the sulfinate oxygens; however, the angle for such bonding (148°) is rather unfavorable. The sulfinate remains solvated by the medium at the opening of the active site.

We expanded the analysis of the intermediate **9** to include the use of molecular dynamics simulation. In this computation, the extended and flexible linear species is allowed to sample much more of the conformational space within the active site than is possible with simple energy minimization of a single initial conformation. A simulation period of 40 ps was deemed reasonable for examination of this rather small conformational domain. With dynamics, the particular starting position of sulbactam, as well as the crystallographic conformations of

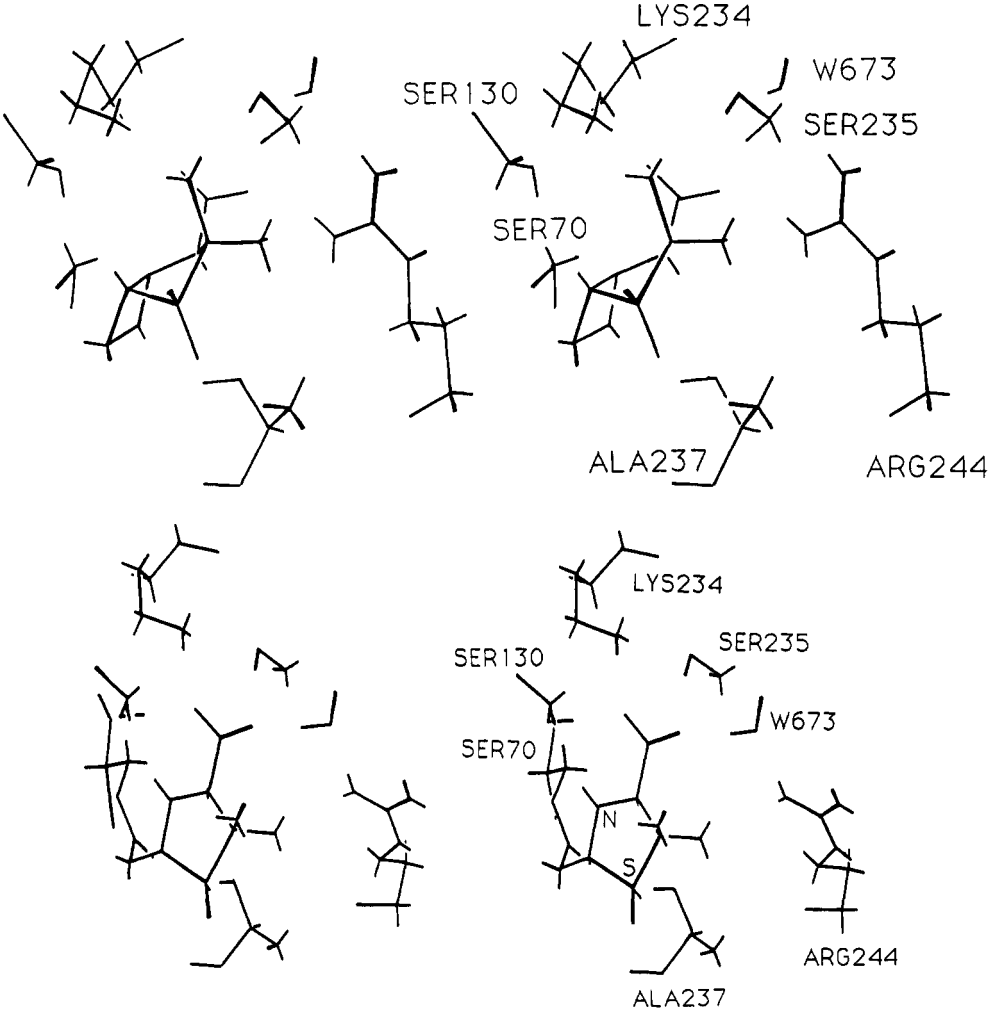


FIGURE 3: Stereoviews of the two energy-minimized structures for (A) the preacylation complex and (B) the acyl–enzyme intermediate of sulbactam with the β-lactamase. A crystallographic water molecule, Wat-673, is included. The α-face of the molecule is presented to the viewer.

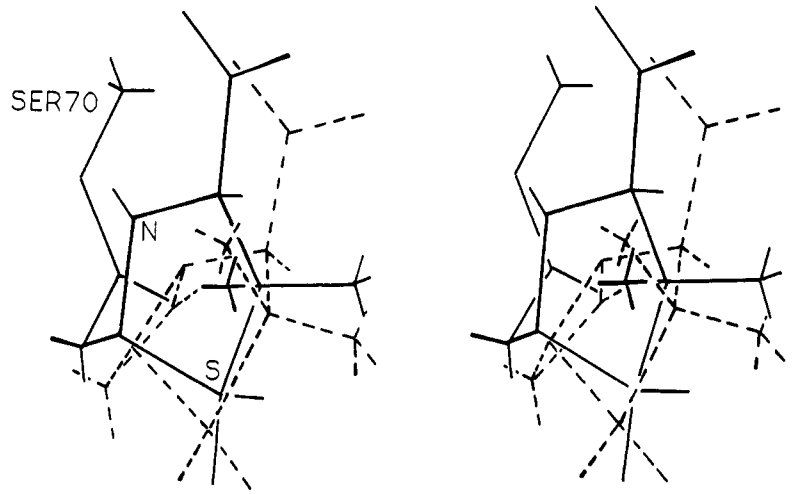


FIGURE 4: Stereoview of the overlay of the relative orientations of the preacylation (dashed lines) and the acyl–enzyme intermediate species (**8**, solid lines) for sulbactam, each in its energy-minimized position near Arg-244. The viewer's perspective is generally the same as that shown in Figure 3.

Table 3: Calculated Distances in the Complex of the Acyclic Species **9** during the Dynamics Simulation and a Comparison with the Corresponding Distances in the Clavulanate Complex

	sulbactam (Å)		clavulanate (Å)[a]	
	range	average	range	average
Lys-73 N^{ϵ} to C_5	4.7–7.4	6.3	4.8–5.8	5.3
Ser-130 O^{β} to C_5	5.5–6.7	6.1	3.6–5.4	4.6
Lys-234 N^{ϵ} to C_5	5.9–7.5	6.4	4.2–5.8	4.9
Arg-244 $N^{\eta 1}$ to C_5	5.6–7.5	6.4	6.0–8.2	7.2

[a] Reported previously (Imtiaz *et al.*, 1993a).

active-site residues, is less likely to bias the outcome of the interactions. In effect, not one, but many trajectories are sampled. Dynamics is, therefore, an ideal way to estimate which of the several possible nucleophiles might be more likely to undergo reaction with the electrophilic inactivating species. During the dynamics simulation, the energy-minimized interatom distances between the reactive species and important active-site nucleophiles are noted (Table 3). The dynamics simulation indicated that Lys-73 is a likely candidate for nucleophilic attack at the electrophilic carbon of **9**. Though the average distance of the ϵ-amine of Lys-73 (Table 3) is not the shortest among the potential nucleophilic amino acid side chains, its approach to the C_5 of **9** comes closer than the other potential nucleophiles (Figure 5). This finding contrasts with the results of a similar simulation for the corresponding linear species of clavulanate (Imtiaz *et al.*, 1993a), in which the Ser-130 approached the site of nucleophile capture the closest. We believe that the additional negative charge from the sulfinate group in the linear species from sulbactam makes the interactions with the enzyme active site significantly different from those calculated for the clavulanate species. Another trend that is noted from the comparison of the dynamics results for the two inactivators is that in general the average distances between the side chains of residues 73, 130, and 234 and the C_5 of **9** are longer in the sulbactam complex than the corresponding distances for the clavulanate species (Table 3). This finding may be a factor in the fact that sulbactam experiences many more turnovers before each inactivation event compared to clavulanate.

Fink *et al.* (1989) suggested that the nature of the substituent at the C_6 of penam sulfones influences the mechanism of inactivation. We note that the presence of a large substituent at the β-position at C_6 might hinder the transition of the imine to the enamine species. From the dynamics calculations we conclude that a $C_{6\beta}$ substituent other that hydrogen—which is found in sulbactam—would be oriented downward in the active site (Figure 3B). For the tautomerization to the enamine species (**9** → **12**) to take place, a necessary clockwise rotation of the $C_{6\beta}$ substituent would be encumbered by the enzyme active site (Asn-132, groups on the H7 helix, such as Glu-166). If the tautomerization were to occur, however, electrostatic anchoring of the carboxylate and the sulfinate functions in the active site may produce the conformational distortion of the enzyme structure observed by circular-dichroic experiments of the Fink group for the sulfones of nafcillin and cloxacillin.

Kinetic Analyses. The circular-dichroic spectra of the mutant TEM-1 enzymes Arg-244-Ser (Zafaralla *et al.*, 1992) and Ser-235-Ala (Imtiaz *et al.*, 1993b) were identical to that of the wild-type TEM-1 β-lactamase in the pH range 4.0–9.0, indicating that the overall collective secondary structural elements of the proteins did not undergo perturbations due to mutagenesis at positions 244 and 235.

Modeling had indicated that there are hydrogen bonds between the C_3 carboxylate of sulbactam and the side chains of Arg-244 and Ser-235 in both the precatalytic and the acyl–enzyme intermediate. In the latter model, Arg-244 interacts with the inactivator carboxylate via an intervening structurally conserved water molecule (Wat-673). Consistent with this view, the mutant enzymes Ser-235-Ala and Arg-244-Ser showed a decrease in affinity for sulbactam as a consequence of the loss of a hydrogen bond in each case; this effect is reflected in their respective K_i values of 5 and 44 μM, compared to 1.6 μM for the wild-type TEM-1 enzyme (Table 4). There were also decreases of approximately 9- and 22-fold in k_{cat} for sulbactam hydrolysis by the Ala-235 and Ser-244 mutant enzymes, respectively.

Sulbactam is turned over by the TEM-1 β-lactamase, but it also causes both transient inhibition and irreversible inactivation of the enzyme (Knowles, 1985, and references cited therein). The partition ratio (k_{cat}/k_{inact}) for sulbactam with the TEM-1 β-lactamase has been reported as 415 (Delaire *et al.*, 1992), 525 (Labia *et al.*, 1980), and approximately 7000 (Fisher *et al.*, 1981); we report a value of 10 000 in this manuscript. We believe that the discrepancy in the reported values is due to the relative uncertainty in the value of k_{inact}. Whereas the k_{cat} of sulbactam can be determined directly, the same does not hold true for the determination of k_{inact}. The complicating factor is the existence of the transiently inhibited species (**12**). This species was reported by Knowles as one that does not result in irreversible inactivation of the enzyme;

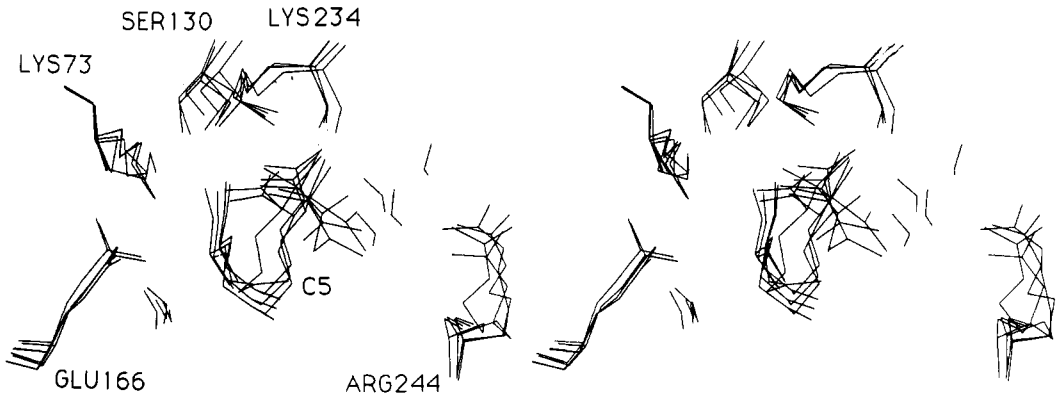


FIGURE 5: Stereoview of four overlays of the minimized complexes of the acyclic species **9**, chosen randomly from the 40-ps dynamics simulation. Distances are given in Table 3. Hydrogen atoms are not shown.

Table 4: Kinetic Parameters for Sulbactam with the TEM-1 β-Lactamase and the Arg-244-Ser and Ser-235-Ala Mutant Enzymes (100 mM Sodium Phosphate, pH 7.0)

kinetic parameters	wild type (TEM-1)	Ser-235-Ala	Arg-244-Ser
K_i (μM)	1.6	5	44
k_{cat} (s^{-1})	1.8–2.1	0.22–0.24	0.07–0.11
k_{inact} (s^{-1})	2.0×10^{-4}	2.4×10^{-5}	1.1×10^{-5}
k_{cat}/k_{inact}	10 000	9700	7800

Table 5: Kinetic Parameters for Penicillanic Acid and the α- and β-*S*-Oxides of Penicillanic Acid with the TEM-1 β-Lactamase (100 mM Sodium Phosphate, pH 7.0)

	penicillanic acid (**2**)	penicillanic acid α-*S*-oxide (**3**)	penicillanic acid β-*S*-oxide (**4**)
k_{cat} (s^{-1})	110	30	115
K_m (μM)	1670	1340	775
k_{cat}/K_m (M^{-1} s^{-1})	6.6×10^4	2.3×10^4	1.5×10^5
k_{cat}/k_{inact}		55 000	64 000
k_{inact} (s^{-1})		5.5×10^{-4}	1.8×10^{-3}

yet, it forms with a rate comparable to that of the irreversibly inactivated species in the TEM-1 β-lactamase. This observation makes it necessary to allow for the complete recovery of the enzyme from transient inhibition in order to measure accurately the inactivation rate constant, k_{inact}. We have found that in our hands such rate determinations carry relatively large error due to a lack of clarity in ascertaining the exact time point where a full recovery from the transiently inhibited species has taken place. Therefore, we decided to avoid the determination of k_{inact} by the commonly used approach involving a double-reciprocal plot of the observed rates of inactivation (k_{obs}) versus the inhibitor concentration; instead, the k_{inact} values were calculated from the partition ratios (*i.e.*, k_{cat}/k_{inact}). The partition ratios for sulbactam for the wild-type, Ser-244, and Ala-235 TEM β-lactamases (Table 4) were determined directly by the titration method and are not calculated values. The partition ratios for the wild-type and Ala-235 enzymes were virtually identical, 10 000 and 9700, respectively. Interestingly, the value for the partition ratio decreased to 7800 for the Ser-244 enzyme. This decrease in the partition ratio was mostly the consequence of a larger reduction of k_{cat} from the mutation at position 244, rather than enhancement of the k_{inact} value. Indeed, the k_{inact} values were found to be attenuated by 8- and 17-fold for the Ala-235 and Ser-244 enzymes, respectively.

Compounds **2**, **3**, and **4** were tested as substrates and potential inactivators for the wild-type TEM-1 β-lactamase. All three compounds were found to be substrates for this enzyme, with k_{cat}/K_m values in the 10^4–10^5 M^{-1} s^{-1} range (Table 5). However, none of the compounds showed any significant inactivation of the wild-type enzyme at concentrations of up to 20 mM (*i.e.*, $[I]_o/[E]_o = 8500$). Whereas the *S*-oxides of penicillanic acids inactivated the enzyme at concentrations of 50 mM and above, the penicillanic acid itself failed to inactivate significantly (<10%) the wild-type β-lactamase, even at concentrations as high as 100 mM. The values for partition ratios for the α- and β-*S*-oxides of penicillanic acid were estimated at 55 000 and 64 000, respectively; that is a minimum of 5–7-fold higher than that for sulbactam. However, in light of the fact that very high concentrations (>20 mM) of the α- and β-sulfoxides are needed to show any inactivation of the wild-type β-lactamase, and since such high concentrations of a compound are not attained *in vivo*, the sulfoxides are substantially poorer inactivators than sulbactam.

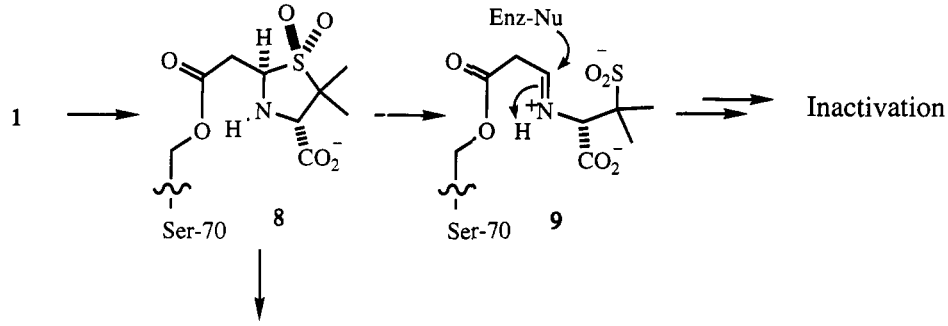
DISCUSSION

In light of the rapid clinical selection of β-lactam-resistant bacteria harboring new variants of β-lactamases in recent years (Moellering, 1991), it has become increasingly necessary that we understand the details of the interactions at the molecular level of β-lactamases with β-lactams, including those which are primarily substrates and others that function as mechanism-based inactivators. This knowledge not only would be instrumental in discerning the mechanisms of acquired resistance but also would help in rational design/redesign of β-lactam molecules as antibacterials and/or β-lactamase inactivators. The recent availability of high-resolution crystal structures for class A β-lactamases (Dideberg *et al.*, 1987; Herzberg, 1991; Knox & Moews, 1991; Strynadka *et al.*, 1992; Jelsch *et al.*, 1993) and class C β-lactamases (Oefner *et al.*, 1990; Lobkovsky *et al.*, 1993) should prove helpful, and indeed critical, for future developments in this area.

In our efforts toward the goal of understanding β-lactam–β-lactamase interaction at the molecular level, we recently reported a detailed account of the processes of clavulanate (Imtiaz *et al.*, 1993a), the first clinically useful mechanism-based inactivator for class A β-lactamases. On the basis of the mechanistic detail provided, we demonstrated that a point mutation could impart effective resistance to clavulanate inactivation by the TEM-1 β-lactamase both *in vitro* and *in vivo* (Manavathu *et al.*, 1991; Imtiaz *et al.*, 1993a). This mutation was subsequently identified in a resistant clinical isolate (Vedel *et al.*, 1992).

Sulbactam is a synthetic mechanism-based inactivator (English *et al.*, 1978) which has also been put to clinical use. The mechanistic details of the processes of sulbactam with β-lactamases are indeed quite complicated (Knowles, 1985), and a quantitative understanding of all the individual chemical steps is not feasible at the present state of the art. Our analysis

Scheme 2



herein, however, provides a structure-based discussion of these individual steps in the catalytic processes with sulbactam. We add that Matagne *et al.* (1993) summarized the kinetic results of interactions of sulbactam with three class A β-lactamases recently.

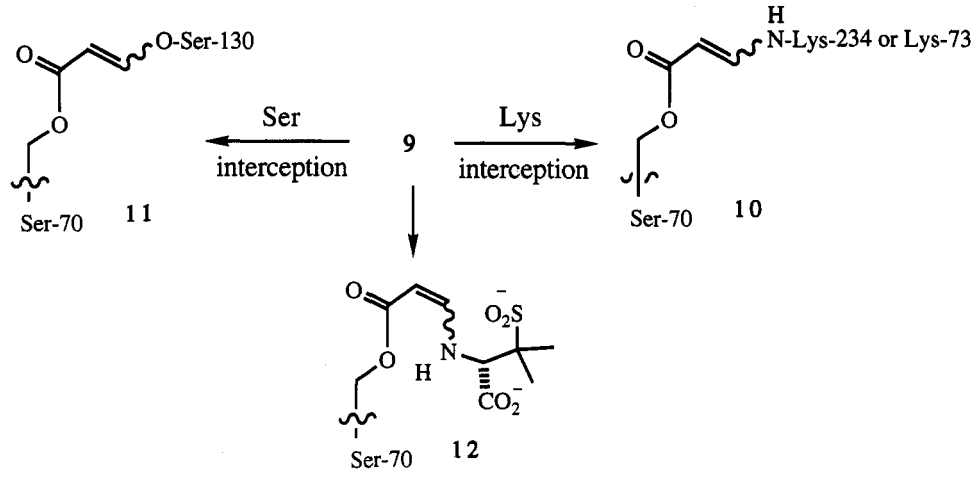
Active-Site Interactions with Sulbactam Carboxylate and Kinetic Observations. The energy-minimized models generated for the precatalytic and acylated complexes with clavulanate revealed that residues Ser-130, Ser-235, and Arg-244, along with a water molecule, Wat-673, are involved in hydrogen bonding with the clavulanate carboxylate. Similar results are essentially noted for such energy-minimized models as discussed here for sulbactam. Furthermore, the same residues were proposed to be involved in interactions with the invariant carboxylates of penicillin and cephalosporin substrates (Zafaralla *et al.*, 1992; Imtiaz *et al.*, 1993b). Fully solvated substrate carboxylates in the active sites of enzymes have been estimated to have substantial solvation energies, with stabilization energy being contributed almost equally by both atomic polarizability and hydrogen bonding to protein (Warshel & Levitt, 1979; Friedman & Kirshnan, 1973). Therefore, active-site interactions with the carboxylates of β-lactam molecules, such as those described above, are important recognition events. To provide kinetic support for the models reported here for the sulbactam–lactamase species, we undertook to evaluate the contribution of the residues involved in substrate interactions with the sulbactam carboxylate. Toward this goal, we have utilized purified mutant derivatives of the TEM-1 β-lactamase with individual replacements at positions 235 and 244. We decided deliberately to avoid mutagenesis at positions 234 and 130 for the following reasons. Ser-130, as suggested by us (this paper; Imtiaz *et al.*, 1993a) and others (Lamotte-Brasseur *et al.*, 1991, 1992; Strynadka *et al.*, 1992), plays a key role beyond hydrogen bonding to substrate carboxylate (*vide infra*) in the course of formation of the acyl–enzyme intermediate. Furthermore, side chains of both residues, Ser-130 and Lys-234, serve important functions in maintaining the proper active-site topology of the enzyme by hydrogen bonding to each other (Herzberg, 1991; Knox & Moews, 1991; Lamotte-Brasseur *et al.*, 1992; Strynadka *et al.*, 1992). Mutagenesis at the Ser-130 and Lys-234 positions, therefore, could result in the disruption of active-site topology, which would thus obscure interpretation of the kinetic results. Nonetheless, such mutagenesis experiments at positions 234 (Ellerby *et al.*, 1990; Lenfant *et al.*, 1991) and 130 (Jacob *et al.*, 1990; Juteau *et al.*, 1992) have been reported for β-lactamases by other groups. Kinetic analyses of mutant β-lactamase derivatives at positions 130 and 234 show large effects on turnover of typical substrates for β-lactamases.

The *in vitro* kinetic characterization of the mutant TEM-1 β-lactamases Arg-244-Ser and Ser-235-Ala showed a decrease in affinity for the inactivator, as evidenced by their K_i values (Table 4). The k_{cat} and k_{inact} values for sulbactam were also found to be lowered by 9- and 8-fold, respectively, for the Ser-235-Ala enzyme, and by 21- and 17-fold for the Arg-244-Ser mutant enzyme, indicating that these residues not only play a role for effective binding of sulbactam but also exhibit an effect in the inactivation chemistry. It is our view that multiple interactions with the carboxylate by the active-site functions serve a "buffering" role, in that the mutations do not have an "all-or-none" effect. That is to say, the loss of a hydrogen bond at either position 244 or 235 is compensated by strengthening the other existing hydrogen bonds in the active site. Hence, the effect of mutations is not as large as would be expected for the loss of a hydrogen bond in each case.

Inactivation Chemistry of Sulbactam and the Roles of Residues 237 and 130. Sulbactam resembles typical β-lactamase substrates, and as such acylates the active-site Ser-70 (**1** → **8**). The occurrence of **8** has not been documented experimentally with β-lactamases, although considerable indirect evidence supports its existence. Crackett and Stoodley (1984) demonstrated the formation of the thiazolidine dioxide, the product of lactam ring opening, by acid degradation of sulbactam. Also, similar intermediates have been shown for both penicillins and cephalosporins (Strynadka *et al.*, 1992; Grabowski *et al.*, 1985; Page, 1984; Page & Proctor, 1984; Faraci & Pratt, 1984). The departure of the sulfinate from C_5 of **8** is believed to generate the putative inactivating species **9**. With a pK_a of 1.5–2.0, sulfinate is not an excellent leaving group (Fujihara & Furukawa, 1990). Fersht and colleagues have showed that hydrogen bonding between enzyme and substrate is not used merely for improving binding affinity, but rather for lowering of energy barriers for chemical conversions (Fersht *et al.*, 1984; Wells & Fersht, 1985; Avis & Fersht, 1993). We looked for the possibility of hydrogen bonds from the active site of β-lactamase to the sulfone moiety of species **8**. Our energy-minimized model for **8** reveals that the main-chain NH of Ala-237 is capable of such an interaction with the β-oxygen of sulfone; this potential hydrogen bonding may conceivably facilitate the departure of the sulfone as a sulfinate (Scheme 2).

It was suggested by the Frère group that Ser-130 may serve as a leg in the proton relay from Ser-70 to the lactam amine, via Lys-73, in the course of the acyl–enzyme intermediate formation (Lamotte-Brasseur *et al.*, 1991, 1992). Subsequently, Strynadka *et al.* (1992) showed that in the crystal structure of the acyl–enzyme intermediate for the Glu-166-Asn mutant of the TEM-1 β-lactamase, which is catalytically deficient in deacylation, the Ser-130 side-chain hydroxyl is near the amine in the penicillin ring. These authors interpreted the role of Ser-130 in the same light as the Frère group. We have offered a distinct role for Ser-130 which does not require

Scheme 3



any unusual reactivity for this residue. We have suggested (Imtiaz *et al.*, 1993a) that Ser-130 facilitates the collapse of the tetrahedral intermediate—the product of Ser-70 addition to the lactam carbonyl—by the mere hydrogen bonding to the lactam amine via its side chain, according to the thesis of Fersht (*vide supra*). Our view of this mechanistic role for Ser-130 is strengthened by our observations of the energy-minimized structure of **8**. A salient feature of the side-chain hydroxyl of a serine is its ability to either accept or donate a hydrogen bond. In the case of the acyl–enzyme intermediate for clavulanate, Ser-130 donated a hydrogen bond to the ring nitrogen (Imtiaz *et al.*, 1993a). For the corresponding intermediate with sulbactam (structure **8**), Ser-130 accepts a hydrogen bond from the newly formed ring amine. A protonation is a prerequisite for the departure of the nascent amine from the tetrahedral intermediate in the course of formation of the acyl–enzyme intermediate. It would appear that the Ser-130 β-hydroxyl, in the case of sulbactam, may accept a hydrogen bond from the now-protonated amine to facilitate its departure. This may be an adaptation by β-lactamases that equips them with the catalytic machinery for turnover of substrates with diverse structures. Depending on the topology of the high-energy tetrahedral intermediate with any given substrate, it may be necessary for the Ser-130 side chain to assume one or the other hydrogen bonding role in the course of turnover.

Subsequent to the formation of the acyl–enzyme intermediate, the departure of the sulfinate from **8** would give rise to the linear acyl species **9**. We carried out molecular dynamics simulations on the structure of species **9** to survey the possible active-site nucleophilic residues that could be intercepted by the iminium carbon in **9**. As shown in Table 3, Lys-73, Lys-234, and Ser-130 are three such residues. Nucleophilic attack by the side chains of the two lysine residues would result in transimination reactions with **9**, which, subsequent to tautomerization, via deprotonation at C_6, would result in species **10**. In the case of clavulanate, the β-hydroxyl of Ser-130 was suggested by us as the most likely site of modification (Imtiaz *et al.*, 1993a). Ser-130 remains a candidate for active-site modification by **9**. This residue might experience interception by **9**, which, subsequent to deprotonation at C_6 and elimination of the amine, would give rise to species **11**. Brenner and Knowles (1981) had documented a deuterium isotope effect of 3 on the rate of inactivation with sulbactam on deprotonation at C_6. This deprotonation is necessary for formation of either **10** or **11**. Both **10** and **11** would give rise to the chromophore at 280 nm typical for α,β-unsaturated ester (Brenner & Knowles, 1981; Charnas *et al.*, 1978). The UV spectrum of the inactivated enzyme would not allow one to distinguish between **10** and **11** [for a survey of these types of chromophores, consult Ostercamp (1970)]. Deprotonation at C_6 is most likely carried out by Glu-166 via the structurally conserved water Wat-712, as proposed for clavulanate (Imtiaz *et al.*, 1993a).[1] The dynamics calculations revealed that both the 6α- and 6β-hydrogens may be abstracted by Glu-166 through Wat-712, although the 6β-hydrogen appears more favored for this reaction, as was demonstrated experimentally by Brenner and Knowles (1981).

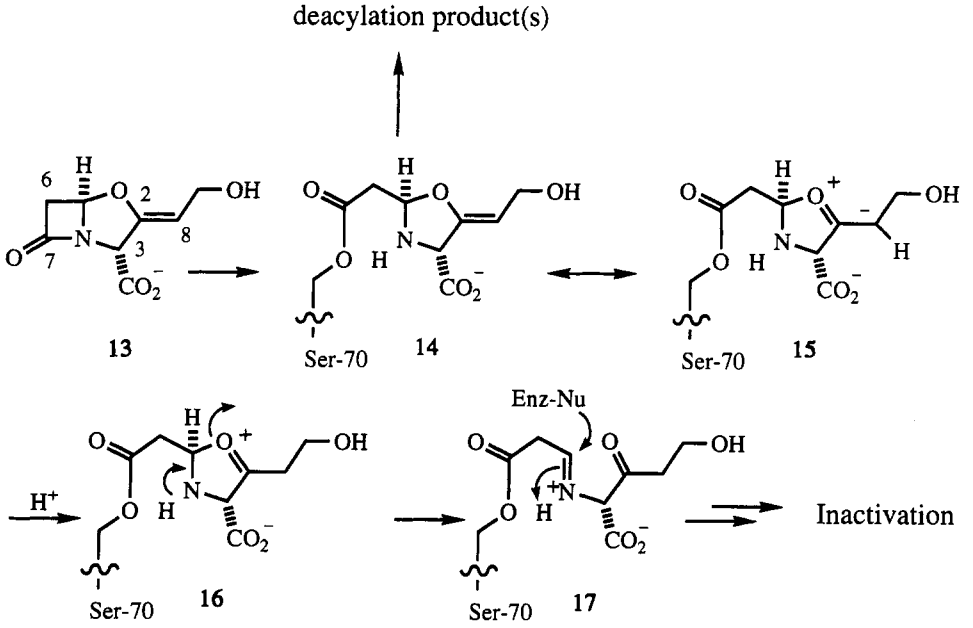
A single inactivated TEM β-lactamase has been identified after enzyme inactivation by sulbactam by isoelectric focusing (Brenner & Knowles, 1984), which appears to be identical to one of the three modified protein species identified after inactivation of β-lactamase with clavulanate (Brenner & Knowles, 1984). These observations are consistent with the results summarized in Table 3 for the minimum distances between the C_5 of species **9** and the potential active-site nucleophiles for covalent bond formation—Lys-73, Lys-234, and Ser-130 (Scheme 3).

The existence of a so-called transiently inhibited species is described by different laboratories (Brenner & Knowles, 1984; Sawai & Yamaguchi, 1989). This species has been assigned the structure **12**, which arises from tautomerization of the double bond in **9**. This tautomerization is likely to be carried out by Glu-166 through Wat-712, as was similarly described for clavulanate chemistry (Imtiaz *et al.*, 1993a). Whether deprotonation at C_6 occurs before or after the nucleophile capture determines the fate of species **9** to give the reversible or irreversible inhibition, respectively.

A Significant Difference in the Inactivation Chemistry of Sulbactam and Clavulanate. The details of the chemistry of inactivation by clavulanate and sulbactam share much similarity. However, they diverge somewhat in the step that requires the departure of the leaving group at C_5 (*e.g.*, **8** → **9** for sulbactam and **14** → **17** for clavulanate) (Scheme 4). The incipient enolate at C_8 of clavulanate has a pK_a of approximately 19–20 (Tapuhi & Jencks, 1982; Cuthrie *et al.*, 1984), which makes the enolate a substantially poorer leaving group than the sulfinate function (pK_a 1.5–2.0) from species **8**. We proposed elsewhere that a prerequisite for the five-membered ring opening is the protonation at C_8 of species **15** in the clavulanate chemistry. Subsequent to such a protonation step, the resultant oxonium ion would have a fleeting existence,

[1] Substitution of residue 166 with the other 19 amino acids has been reported [Delaire, M., Lenfant, F., Labia, R., Masson, J. M. (1991) *Protein Eng. 4*, 805–810]. Only the Glu-166-Tyr enzyme showed some marginal activity, underscoring the importance of residue 166 in the catalytic process.

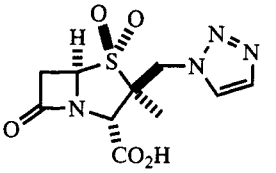
Scheme 4



and this would lead to the rapid formation of the inactivating species **17**. Indeed, we showed experimentally that a structurally conserved water molecule (Wat-673) was the likely source of this critical proton in the clavulanate chemistry (Imtiaz *et al.*, 1993a). Therefore, this protonation converts a poor leaving group (the enolate) into a very good one (the oxonium species); hence, one limitation of the chemistry of clavulanate is the rate for this protonation step. On the other hand, the sulfinate moiety is not an excellent leaving group, as discussed earlier. It would appear that the enzyme might facilitate its departure by hydrogen bonding of residue 237 at the β-oxygen of the sulfone of **8**, but the overall activation cannot be as significant as the protonation event for the clavulanate chemistry. One possible consequence of this difference in the chemistry is reflected in the partition ratios (k_{cat}/k_{inact}) of 10 000 for sulbactam and 160 for clavulanate. The sulbactam acyl–enzyme intermediate would have the opportunity to turn over many more times than the corresponding species from clavulanate before the onset of the inactivation chemistry, in part as a result of the differences in the leaving ability of the two leaving groups. This is depicted by the deacylation branching points from species **8** and **14** for sulbactam and clavulanate, respectively.

To explore further the issue of the leaving ability of the heteroatom from C_5, we studied three additional compounds. The thiolate and sulfenate leaving groups from the acyl–enzyme intermediates for compounds **2**, **3**, and **4** would have pK_a values of 9–11 (Crampton, 1974) and 7.5 (Kice *et al.*, 1989), respectively. All three compounds would be expected to be much poorer inactivators for β-lactamases, if the rate of departure of the leaving group would influence the overall rate of inactivation. Indeed, compound **2** is solely a substrate. This observation is consistent with the relatively poor leaving ability of the thiolate from the acyl–enzyme intermediate of compound **2** and the inability of the active site to facilitate the departure of this leaving group. Its k_{cat} of 110 s^{-1} indicates that it is hydrolyzed approximately 55-fold faster than sulbactam. On the other hand, the sulfenate function would serve as a much better leaving group, as evidenced by its pK_a of 7.5, compared to the thiolate. Accordingly, the sulfoxide analogues are inactivators for β-lactamase, albeit substantially poorer inactivators than sulbactam, whose sulfinate functionally is a much better leaving group. If the acyl–enzyme intermediates for the sulfoxide compounds should bind the enzyme active site comparably to the binding of the sulfone analogue (*i.e.*, the sulbactam species **8**), the α- and β-sulfoxide compounds **3** and **4** should permit evaluation of the effect—or lack thereof—of the hydrogen bond between the backbone NH of residue 237 and the β-*S*-oxide in the inactivation chemistry. Otherwise, the α- and β-sulfoxides are equivalent on electronic grounds. We feel that the values of k_{inact} for the α- and β-sulfoxides are essentially the same (3.3-fold difference in favor of the β-sulfoxide), within the limits of the experimental error in the estimation. Furthermore, the k_{inact} for sulbactam is not substantially different from the corresponding values for the two sulfoxides. These observations argue for a lack of contribution of the rate of departure of the heteroatom from C_5, be it a thiolate, sulfenate, or sulfinate, in the overall rate of inactivation (which is a macroscopic rate constant). The rate-limiting chemical step in the process of inactivation may indeed be the energetically difficult covalent modification of an active-site nucleophile, as shown by the dynamics calculations. Nonetheless, the facility of departure of the C_5 heteroatom substituent is an indispensable component of the inactivation chemistry, as evidenced by a lack of inactivation by compound **2**.

Finally, tazobactam (**18**), a variant of the structure of sulbactam, shows a considerably different kinetic behavior with β-lactamases (Bush *et al.*, 1993). The partition ratio



18

(k_{cat}/k_{inact}) for tazobactam with the TEM-2 enzyme is 125, which is in the same range as that of clavulanate (Charnas & Knowles, 1981; Imtiaz *et al.*, 1993a). The low partition ratio for tazobactam might come about largely as a consequence of a lowering of the value for k_{cat}, although neither k_{cat} nor k_{inact} have been reported for tazobactam at the present. We expect that the triazole ring of tazobactam would interact strongly with the structurally conserved Wat-673 and the side

chain of Arg-244. It is plausible that this interaction would distort the binding of both the acyl–enzyme intermediate and the linear inactivating species in the active site, such that facile hydrolysis of the ester moiety in these species is no longer possible. If so, k_{cat} would be retarded. Alternatively, the presence of the triazole function and its aforementioned interactions in the active site may influence the conformation of the inactivating species such that the side chains of the nucleophilic residues come closer to the C_5 of the inactivating species to facilitate covalent modification of the active site.

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