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Interaction of Azthreonam and Related Monobactams with β-Lactamases from Gram-Negative Bacteria

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Monobactams containing 3β-aminothiazolyl oxime side chains (SQ 81,377, SQ 81,402, azthreonam, and SQ 26,917) have poor affinities for the broad-spectrum β-lactamases TEM-2 and K1. Addition of a 4-methyl substituent significantly increased stability to hydrolysis by these enzymes. P99 cephalosporinase from *Enterobacter cloacae* was strongly inhibited by the monobactams. Interaction of azthreonam with the P99 enzyme in equimolar concentrations resulted in a single covalent complex which retained less than 3% catalytic activity. On incubation, enzymatic activity was slowly regained. Chromatographic studies of the incubation mixtures revealed the presence of a single ring-opened product. It is concluded that monobactams act as poor substrates for broad-spectrum β-lactamases and tight-binding competitive substrates for the P99 β-lactamase.

Monobactams are monocyclic β-lactam antibiotics which were originally discovered as natural products from bacteria (11, 23, 24). Azthreonam (SQ 26,776) is the first synthetic monobactam to be selected for clinical development (22). An important feature of this molecule is its outstanding stability in the presence of a variety of β-lactamases from gram-negative organisms.

β-Lactamases are highly specific catalysts which bind only β-lactams at the active site. Until a recent report described the poor binding of nocardicin A and desthiobenzylpenicillin to the *Bacillus cereus* β-lactamase (18), only bicyclic β-lactams were known to bind to these enzymes. Although 3-methoxylated monobactams do not show measurable affinity for broad-spectrum β-lactamases, nonmethoxylated monobactams bind readily to a variety of these enzymes (23, 25). Hydrolysis of monobactams has also been observed, although catalytic efficiency is considerably less than that observed for cephalosporins such as cephaloridine and cefoperazone (22).

In this paper, four monobactams containing aminothiazolyl oxime side chains (Fig. 1) are described with respect to their interactions with β-lactamases possessing both cephalosporinase and broad-spectrum activities. Hydrolytic and inhibitory properties are examined and compared with those of other β-lactam antibiotics of current interest.

MATERIALS AND METHODS

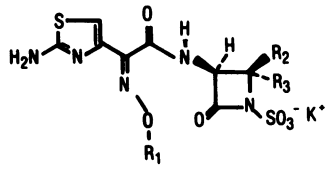
Antibiotics. Monobactams SQ 81,377, SQ 81,402, SQ 26,917, and azthreonam were synthesized at the Squibb Institute. Cephaloridine and moxalactam were gifts from Eli Lilly & Co., Indianapolis, Ind.; cefoperazone was from Pfizer Inc., New York, N.Y.; cefotaxime was from Hoechst-Roussel, Somerville, N.J.; and ceftazidime was from Glaxo Laboratories, Greenford Middlesex, England. SQ 24,902, a chromogenic cephalosporin similar to nitrocefin (4), and aminothiazolyl [^{14}C]azthreonam were prepared at the Squibb Institute.

Preparation of β-lactamases. TEM-2 β-lactamase from *E. coli* W3310 was purified as described by Sykes et al. (22). The final preparation had a catalytic activity of 1,300/mol of cephaloridine hydrolyzed per mol of enzyme per s at 25°C.

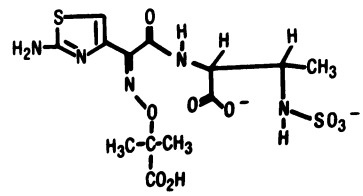
P99 β-lactamase from *Enterobacter cloacae* SC 10,435 was a homogeneous preparation purified as described by Ross and Boulton (20), with a catalytic activity of 845/s with cephaloridine at 25°C. The purified protein had a molecular weight of 39,000 as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis (15) and gel filtration chromatography on Sephadex G-75 (0.1 M phosphate buffer, pH 7.0). K1 β-lactamase from *Klebsiella pneumoniae* SC 10,436 was purified as follows. A cell suspension, 1:10 (wt/vol) in 0.1 M phosphate buffer, pH 7.0, was sonicated for 2 min and centrifuged; the resulting supernatant was passed through Sephadex G-75 (2.5 by 107 cm) with 0.1 M phosphate buffer, pH 7.0, as the eluent. A single β-lactamase activity (pI of 6.5) was identified by isoelectric focusing; specific activity was 65 nmol of cephaloridine hydrolyzed per mg of protein per min at 25°C.

β-Lactamases were characterized by isoelectric focusing by using an LKB Multiphor (LKB Instruments, Inc., Rockville, Md.; pH, 3.5 to 9.5); enzyme activity was detected on the gels by using a chromogenic cephalosporin substrate (17). Gels were also stained to detect protein, using Coomassie brilliant blue R250. pIs were determined by using an LKB Multiphor electrode, standardized from pH 4 to pH 10.

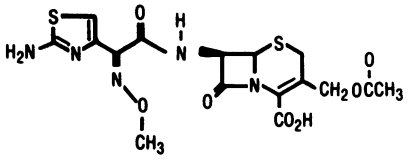
Hydrolysis studies. β-Lactamase hydrolysis studies



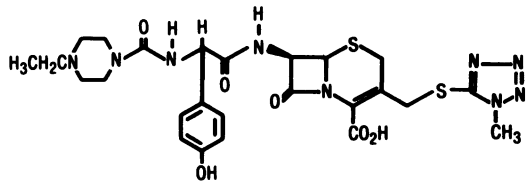
Compound	R_1	R_2	R_3
SQ 81,377	CH_3	H	H
SQ 81,402	$C(CH_3)_2CO_2^-K^+$	H	H
SQ 26,917	$C(CH_3)_2CO_2^-K^+$	CH_3	H
Azthreonam	$C(CH_3)_2CO_2^-K^+$	H	CH_3



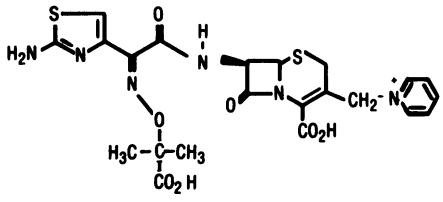
SQ 26,992



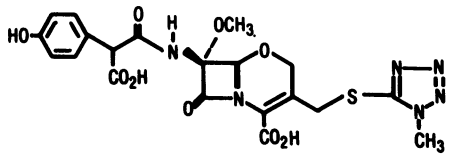
Cefotaxime



Cefoperazone



Ceftazidime



Moxalactam

FIG. 1. Structures of selected β-lactam antibiotics and related compounds.

were performed spectrophotometrically on a Gilford 250 spectrophotometer. UV spectra were recorded for each compound either in the presence and absence of a selected β-lactamase or, before and after the addition of sodium hydroxide (final pH of reaction, 11.7). From the resulting difference spectrum, an appropriate wavelength was selected for assay, and a molar extinction coefficient ($\Delta\varepsilon$, $M^{-1}cm^{-1}$) was calculated from the differential absorbance change at that wavelength (21).

Spectral parameters used in these studies were: cephaloridine, 295 nm, $\Delta\varepsilon$ = 890; SQ 81,377, 320 nm, $\Delta\varepsilon$ = 374; SQ 81,402, 320 nm, $\Delta\varepsilon$ = 493; SQ 26,917, 318 nm, $\Delta\varepsilon$ = 720; azthreonam, 318 nm, $\Delta\varepsilon$ = 660 (±7%); cefotaxime, 267 nm, $\Delta\varepsilon$ = 6,700; ceftazidime, 260 nm, $\Delta\varepsilon$ = 8,660; cefoperazone, 275 nm, $\Delta\varepsilon$ = 8,460; moxalactam, 245 nm, $\Delta\varepsilon$ = 10,900; and chromogenic cephalosporin, 495 nm, $\Delta\varepsilon$ = 16,200. All compounds were prepared in 0.1 M phosphate buffer (pH 7.0) immediately before use. Kinetic studies were performed at 25°C. A range of substrate concentrations was selected for each compound by estimating K_ms from direct linear plots of raw data (7). Each determination of kinetic parameters included at least five concentrations of substrate spanning the K_m where possible. Linear regression analysis of Lineweaver-Burk plots was used to obtain the reported kinetic values. V_{max} was then normalized with respect to cephaloridine. In cases where K_m could not be determined (no detectable hydrolysis), a maximal value for V_{max} was estimated.

Inhibition studies. Inhibition of P99 β-lactamase was studied, using a computerized Gilford 202 spectrophotometer to calculate initial reaction rates. Enzyme (100 μl) and inhibitor (10 μl) were incubated 5.0 min at room temperature (20°C); 1.0 ml of cephaloridine was added, and substrate hydrolysis was monitored for 1.0 min at 25°C. Inhibition by azthreonam was also studied in the absence of preincubation. K_is were determined by using Dixon plots (6) where 1.0 mM substrate was maintained or Lineweaver-Burk plots where substrate concentrations were varied.

Deacylation studies. P99 β-lactamase was incubated in a volume of 230 μl with an excess of inhibitor at 25°C. After 20 min of incubation, mixtures containing enzyme and either SQ 26,917 or moxalactam were dialyzed at least 16 h at 4°C against 1 liter of 0.1 M phosphate buffer, pH 7.0 (two buffer changes) to remove excess inhibitor. Incubation mixtures were then maintained at 25°C. Studies involving cefotaxime, azthreonam, and SQ 81,402 did not involve dialysis. Recovery of enzymatic activity was monitored periodically by adding 2 μl of incubation mixture to 1.0 ml of cephaloridine (1.0 mM) and following initial reaction

rates as a function of time. Chromogenic cephalosporin (0.33 mM) was also used as substrate as noted in the text.

Stoichiometry of azthreonam binding to P99 β-lactamase. P99 β-lactamase (1.3 nmol) was incubated in duplicate with 20 nmol of [^{14}C]azthreonam (8.0 μCi/mg) for 20 min at 20°C in a volume of 630 μl. Two samples of 500 μl each were dialyzed against 400 ml of 0.05 M phosphate buffer, pH 7.0, for 17.5 h at 4°C (two buffer changes). Samples were assayed for enzymatic activity before and after dialysis by diluting 2 μl of the incubation mixture into 3.0 ml of chromogenic cephalosporin (0.33 mM) and comparing cephalosporin hydrolysis rates with appropriate controls. Dialyzed enzyme samples were washed into scintillation vials and counted, using a cocktail containing 0.5% PPO (2,5-diphenyloxazole) and 10% naphthalene in dioxane. Samples were counted by using an LKB model 1215 Rackbeta liquid scintillation counter.

Modification by methanesulfonyl fluoride. Stock methanesulfonyl fluoride (Aldrich Chemical Co., Milwaukee, Wis.) was prepared in dry 2-propanol. Reaction mixtures containing 20 to 25 nM enzyme and 0 to 30 mM methanesulfonyl fluoride were incubated at 25°C in 0.1 M phosphate buffer, pH 7.0 containing 5% 2-propanol. Samples were assayed for residual enzyme activity with 1.0 mM cephaloridine as substrate for P99 β-lactamase and 1.0 mM benzylpenicillin as substrate for TEM-2 β-lactamase. Second-order rate constants were determined by the method of Kitz and Wilson (13).

Analysis of azthreonam hydrolysis products. High-pressure liquid chromatography analyses of azthreonam and related products were conducted as described by Pilkiewicz et al. (F. G. Pilkiewicz, S. M. Fisher, B. J. Remsburg, and R. B. Sykes, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 882, 1981.) Mixtures containing β-lactamase and azthreonam (49 μM) were prepared in 2.0 ml of 0.1 M phosphate buffer, pH 7.0, and allowed to incubate at 25°C. Standards included azthreonam alone, neutralized azthreonam after hydrolysis by NaOH (no enzyme), and SQ 26,992, the ring-opened monobactam corresponding to azthreonam. Before high-pressure liquid chromatography, samples were centrifuged with Amicon Centriflo membrane cones CF25 (Amicon Corp., Lexington, Mass.) to separate azthreonam from enzyme.

Incubation mixtures of [^{14}C]azthreonam and K1, TEM-2, or P99 β-lactamase were also analyzed by thin-layer chromatography. [^{14}C]azthreonam or unlabeled azthreonam at 1.0 mg/ml were incubated in the presence and absence of TEM-2, K1, or P99 β-lactamase for 20 h at 20°C. Volumes of 2 to 10 μl were spotted on Merck silica gel 60F and developed, using *n*-butanol–ethyl acetate–acetic acid–water (1:1:1:1). A standard of SQ 26,992 was also included. Chromatograms were analyzed by using densitometry at 295 nm and fluorography (3) followed by visual inspection of the X-ray film for radiolabeled samples.

RESULTS

Hydrolysis studies were performed by following changes in the UV spectrum of each compound. Small changes in spectral characteristics were observed when azthreonam was hydrolyzed; identical spectra were obtained in the region of 235 to 350 nm, regardless of whether hydrolysis was effected by sodium hydroxide (pH 11.7) or by K1 or TEM-2 β-lactamase (Fig. 2A). The difference spectrum for azthreonam which exhibits a wavelength maximum of 318 nm (Fig. 2B) is typical of that observed with all four monobactams in this study.

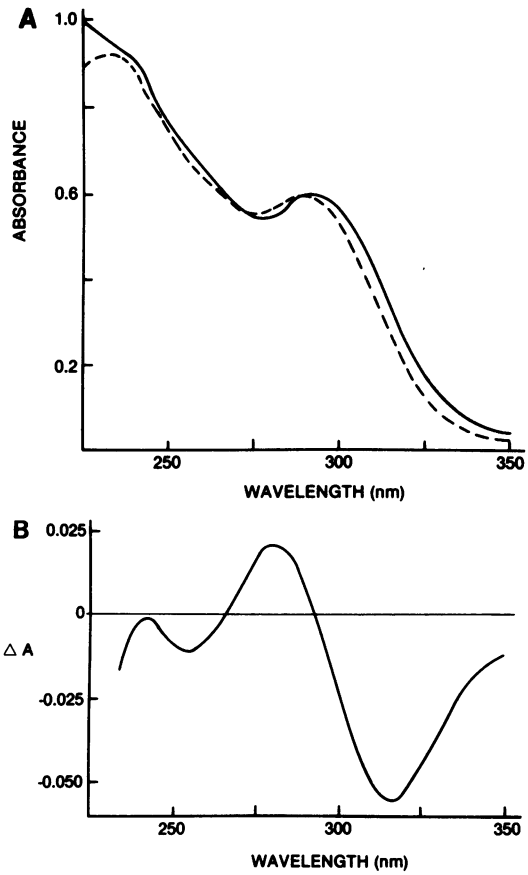


FIG. 2. (A) Ultraviolet spectrum of azthreonam (98 μM) before (——) and after (– – –) hydrolysis by 82 nM TEM-2 β-lactamase in 0.1 M phosphate buffer (pH 7.0). (B) Difference spectrum of azthreonam (98 μM) after hydrolysis by 82 nM TEM-2 β-lactamase. ΔA, Change in absorbance.

Kinetic parameters for enzymatic hydrolysis of the monobactams are presented in Table 1. TEM-2 β-lactamase which exhibits broad-spectrum activity readily hydrolyzed SQ 81,377 which has a methoxime (ether) aminothiazolyl side chain. Stability to enzymatic hydrolysis was improved considerably when an acidic oxime side chain was introduced in the monobactam (SQ 81,402); this stability was enhanced even more by methyl substitution at the 4-position on the monobactam ring (azthreonam and SQ 26,197). The 4β-methyl derivative, SQ 26,917,

TABLE 1. Kinetic constants for selected β-lactams with β-lactamases

Compound	Kinetic constant for following β-lactamase (producing organism):						
	TEM-2 (*E. coli*)		K1 (*K. pneumoniae*)		P99 (*E. cloacae*)		
	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	K_i (μM)[a]
Cephaloridine	100	660	100	150	100	580	ND[b]
SQ 81,377	46	950	780	940	0.07	350	0.40
SQ 81,402	8.9	870	370	1,500	0.0003	ND	0.0035
Azthreonam	0.4	2,900[c]	55	800	<0.0001	ND	0.0019
SQ 26,917	0.003	750	1.4	3,000	<0.0001	ND	0.0045
Cefotaxime	0.06	510	5.1	150	0.003	0.32	0.035
Ceftazidime	0.01	480	0.03	700	0.002	6.6	3.0
Cefoperazone	17	130	0.9	0.6	0.23	16	9.0
Moxalactam	<0.01	ND	<0.01	ND	<0.001	ND	0.0051

[a] Inhibition constants were determined after a 5.0-min preincubation of inhibitor and enzyme. The final enzyme concentration was 0.28 nM.

[b] ND, Not determined.

[c] Biphasic Lineweaver-Burk plots. A higher apparent K_m was obtained at low substrate concentrations.

exhibited the greatest resistance to hydrolysis. Thus, azthreonam and SQ 26,917 are poor substrates for the TEM-2 β-lactamase.

Similar behavior was observed with the K1 β-lactamase, another β-lactamase with both cephalosporinase and penicillinase activities. Although this enzyme hydrolyzed SQ 81,377 quite rapidly, susceptibility to hydrolysis was decreased substantially with the 4-methyl-substituted monobactams azthreonam and SQ 26,917.

K_ms for the monobactams with the broad-spectrum B-lactamases were higher than the K_ms observed with cephaloridine and the third-generation cephalosporins cefotaxime and cefoperazone. Therefore, binding of monobactams such as azthreonam, would be minimal at the relatively low substrate concentrations which are observed clinically. For example, an azthreonam concentration of 50 μg/ml corresponds to 100 μM substrate, a level which would be hydrolyzed much more slowly than the maximum rate of hydrolysis observed for this compound with either the TEM-2 or K1 β-lactamase.

A similar pattern of stability was observed with P99 β-lactamase, an enzyme which hydrolyzes cephalosporins almost exclusively. However, only SQ 81,377 was hydrolyzed at a rate detectable by routine spectrophotometric methods. Stability to P99 β-lactamase was greatest for azthreonam and SQ 26,917 in comparison with other cephalosporins and monobactams.

Because significant hydrolysis of these monobactams did not occur in the presence of P99 β-lactamase, inhibitory properties were studied. SQ 81,402, azthreonam, and SQ 26,917 were observed to be potent inhibitors of the enzyme, with K_is equal to or better than that of moxalactam. Extensive studies with azthreonam showed that the initial phase of inhibition was competitive, as observed from Lineweaver-Burk plots of initial reaction rates obtained when enzyme was added last to reaction mixtures containing azthreonam and cephaloridine as substrate. However, if enzyme and azthreonam were preincubated before substrate was added, a kinetic diagnostic plot for noncompetitive inhibition was observed. These results are consistent with the behavior of a tight-binding competitive inhibitor which binds virtually stoichiometrically to the enzyme or an irreversible inhibitor which binds by forming an initial reversible complex. A poor substrate would also exhibit the same kinetic pattern.

P99 β-lactamase was fully inhibited by equimolar concentrations of azthreonam. However, enzymatic activity was slowly regained from mixtures of enzyme and inhibitor incubated at 25°C (Fig. 3). On addition of excess azthreonam (100- to 200-fold) to P99 β-lactamase, overnight dialysis of the inactive enzyme mixtures resulted in a recovery of enzyme activity similar to that observed in Fig. 3. Enzymatic recovery rate (0.10/h) was the same, within experimental error, for inhibitor to enzyme ratios of 0.6 to 200. In all cases, full enzymatic activity was restored, even when P99 β-lactamase was incubated with a 200-fold excess of azthreonam for 174 h before dialysis.

Nonlinear progress curves were observed if an excess of azthreonam was present during the assay for β-lactamase activity, using a chromogenic cephalosporin as the substrate (Fig. 4). Enzyme was observed to regain activity slowly during assay. However, after dialysis, or in the presence of less than stoichiometric concentrations of inhibitor, linear kinetics were obtained. These results indicate a shift of overall equilibria in the presence of excess azthreonam, resulting in the availability of enzyme to hydrolyze substrate. However, this slow recovery of activity amounted to no more than 5% of control activity.

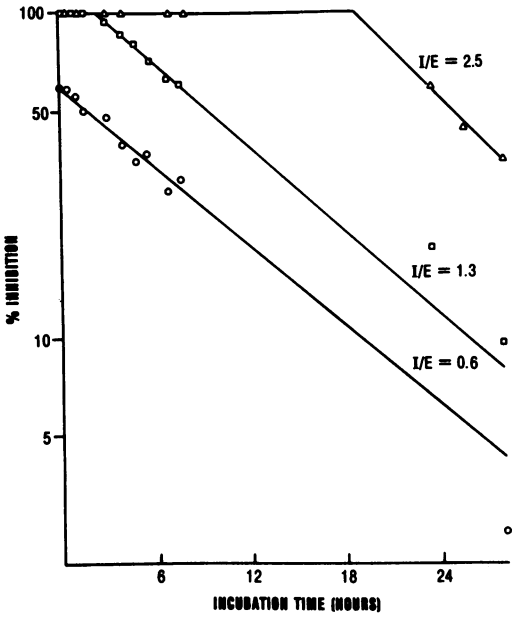


FIG. 3. Recovery of enzymatic activity after incubation of P99 β-lactamase with azthreonam. Enzyme (0.35 nmol) was incubated at 25°C with 0, 0.22 (○), 0.44 (□), or 0.88 (△) nmol of azthreonam in a volume of 60 μl. Samples were assayed periodically by diluting 2 μl of the incubation mixture into 1.0 ml of cephaloridine (1.0 mM). I/E, ratio of inhibitor to enzyme.

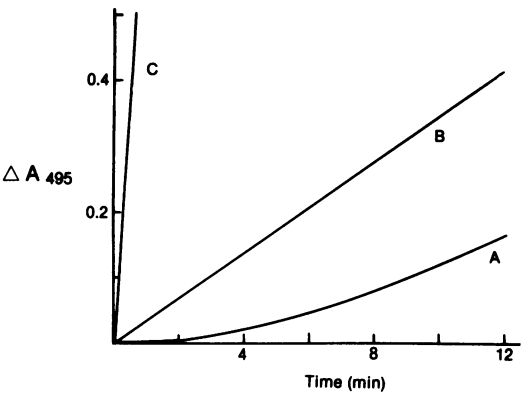


FIG. 4. Hydrolysis of chromogenic cephalosporin by P99 β-lactamase after inhibition by azthreonam. Assays were performed by diluting 2 μl of an enzyme solution (2.2 μM) into 3.0 ml of chromogenic cephalosporin (0.33 mM). (A) Enzyme with 33 μM azthreonam after 0.15 h of incubation; (B) enzyme incubated with 33 μM azthreonam and dialyzed for 16 h; and (C) control enzyme containing no azthreonam. ΔA_{495}, change in absorbance at 495 nm.

The existence of a transiently stable enzyme-azthreonam complex was demonstrated by isoelectric focusing (Fig. 5). After short time intervals, enzyme inhibited by azthreonam at molar ratios of 1 and 100 exhibited a single band more acidic than native enzyme. Enzyme activity in the azthreonam-inhibited samples was less than 3% of the control. After 16 h at 20°C, enzyme incubated with equimolar azthreonam had regained 60% of its activity. Isoelectric focusing of this sample showed two bands, one corresponding to native enzyme and one band identical to that observed in the initial inactive enzyme mixture. After 16 h, the sample containing a 100-fold excess of azthreonam still exhibited a single band identical to that of the original mixture. Inhibition remained greater than 99%. Therefore, a single enzyme-inhibitor complex forms between azthreonam and P99 β-lactamase; the isoelectric focusing characteristics of this complex were altered only on recovery of enzyme activity as native enzyme was regenerated.

Further evidence for a single covalent enzyme-inhibitor complex was provided by experiments which resulted in the isolation of a [^{14}C]azthreonam-P99 β-lactamase. Enzyme incubated with a 15-fold excess of [^{14}C]azthreonam was dialyzed to remove excess inhibitor. The resulting enzyme, which had regained 5% of its activity, contained 0.72 mol of azthreonam per mol of enzyme, confirming the formation of a specific enzyme-azthreonam adduct.

Acyl intermediates have been identified for TEM-2 β-lactamase with clavulanic acid (5) and cefoxitin (8) and for *B. cereus* β-lactamase I with 6β-bromopenicillanic acid (14, 16). It seemed reasonable, therefore, to suspect that monobactams may also react with β-lactamases via acyl enzymes. To demonstrate the presence of a reactive hydroxyl group at the active site, TEM-2 and P99 β-lactamase were treated with methanesulfonyl fluoride, an amino acid-modifying reagent which reacts with active center serine residues (10). TEM-2 β-lactamase, which can form acyl intermediates via serine-70 (9), was inactivated by methanesulfonyl fluoride with a second-order rate constant of 3.1 liters/mol per min. P99 β-lactamase was inactivated with a second-order rate constant of 0.2 liters/mol per min. Because both enzymes were modified by this reagent, they exhibited the ability to form irreversible complexes with an active site serine.

Deacylation half-times were determined for several β-lactams which effectively inhibited P99 β-lactamase: SQ 81,402, 4 min; azthreonam, 6.8 h; SQ 26,917, 18.5 h; moxalactam, 3.5 h; and cefotaxime, <1 min (samples of SQ 26,917 and moxalactam were dialyzed before analysis). SQ 26,917 and azthreonam exhibited the slowest rates for deacylation, followed by moxalactam. Although moxalactam has been described as a "suicide inhibitor" of P99 β-lactamase (19), our

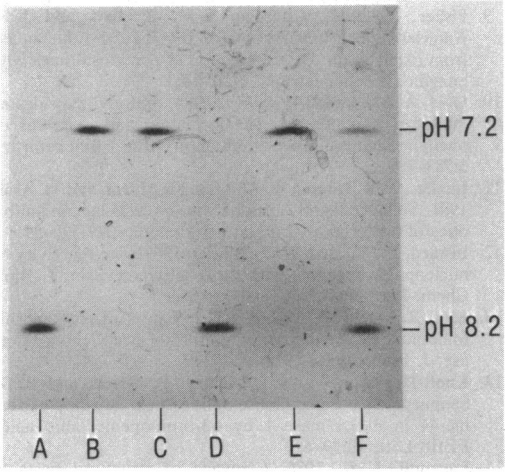


FIG. 5. Isoelectric focusing pattern of P99 β-lactamase with azthreonam. Incubation of 2.8 μM P99 β-lactamase was performed at 20°C in 0.1 M phosphate buffer containing azthreonam as shown. (A) Control, no azthreonam, 0.1 h; (B) 280 μM azthreonam, 0.1 h; (C) 2.8 μM azthreonam, 0.1 h; (D) control, no azthreonam, 16 h; (E) 280 μM azthreonam, 16 h; and (F) 2.8 μM azthreonam, 16 h. Gel was stained for protein with Coomassie brilliant blue R250.

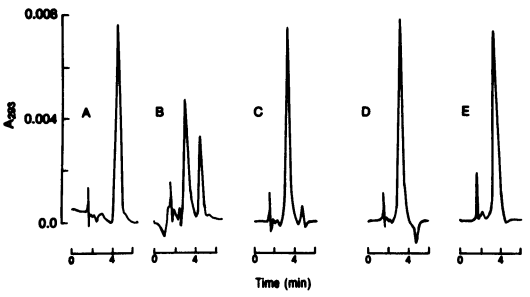


FIG. 6. Analysis by high-pressure liquid chromatography of azthreonam after incubation with base or β-lactamases. (A) 49 μM azthreonam in 0.1 M phosphate buffer, pH 7.0; (B) azthreonam and base hydrolysis product, initial azthreonam concentration of 41 μM; (C) 49 μM azthreonam incubated for 21 h with 41 nM TEM-2 β-lactamase; (D) 49 μM azthreonam incubated for 21 h with <40 nM K1 β-lactamase; and (E) 49 μM azthreonam incubated for 72 h with 9 μM P99 β-lactamase. High-pressure liquid chromatography was performed by using a μBondapak C_{18} column (3.9 mm by 30 cm; Waters Associates, Inc., Milford, Mass.) with a mobile phase of 80% 0.005 M tetrabutylammonium hydrogen sulfate (pH 3.0)–0.005 M ammonium sulfate and 20% acetonitrile (vol/vol). Samples of 25 μl were injected. Retention times were 3.3 min for base hydrolysis product and 4.6 min for azthreonam.

studies showed that full enzymatic activity was eventually recovered after the removal of excess inhibitor by dialysis. Full activity was also recovered after inhibition by cefotaxime and the monobactams. Therefore, none of these compounds can be classified as suicide inactivators, which, by definition (1), must irreversibly form a covalent adduct with the target enzyme, rendering it incapable of regaining catalytic activity.

Deacylation rates with azthreonam were not increased significantly in the presence of 100 mM hydroxylamine, pH 7.0. Although deacylation of serine proteases such as α-chymotrypsin can be accelerated (up to 1,000-fold) by hydroxylamine (12), the catalytic activity of TEM-2 β-lactamase with either benzylpenicillin or cefoxitin (8) is not affected by hydroxylamine. Substrate hydrolysis rates were also not affected by hydroxylamine in the case of PC1 penicillinase from *S. aureus* (2). Thus, the active center of P99 β-lactamase closely resembles that of other β-lactamases in that the acyl group appears to be shielded from external nucleophiles.

Chromatographic procedures were performed to identify the product(s) formed from the action of TEM-2, K1, and P99 β-lactamases with azthreonam. High-pressure liquid chromatography studies indicated the formation of a single product with a retention time equal to that of the base hydrolysis product (Fig. 6). The same product was identified in all studies, regardless of the β-lactamase used, and shown to possess identical spectral characteristics with those of SQ 26,992, the ring-opened analog of azthreonam. Thin-layer chromatography of [^{14}C]azthreonam also confirmed formation of a single product after reaction with TEM-2, K1, and P99 β-lactamase; this product again corresponded to SQ 26,992.

DISCUSSION

Monobactams have previously been shown to exhibit poor affinity for broad-spectrum β-lactamases (23, 24). Monobactams bearing aminothiazolyl oxime side chains exhibited K_m values higher than most cephalosporins and penicillins for TEM-2 and K1 β-lactamases. However, the efficiency of hydrolysis was quite good for SQ 81,377 in the presence of K1 β-lactamase. Addition of an acidic moiety on the oxime side chain with a methyl substituent in the 4-position stabilized the monobactams so that azthreonam and SQ 26,917 in particular exhibited impressive β-lactamase stability.

P99 β-lactamase was potently inhibited by the monobactams studied. Although covalent adducts with azthreonam were isolated, irreversible inactivation of the enzyme did not occur. After removal of excess inhibitor, full enzymatic activity could be recovered and hydrolyzed azthreonam identified in reaction mixtures. The following reaction sequence is the simplest scheme which would account for the observed inhibition by all the monobactams studied: $E + M \rightleftharpoons E \cdot M \rightarrow E - A \rightarrow E + P$ where M is

monobactam, $E \cdot M$ represents the reversible formation of a Michaelis complex, $E - A$ is the acyl intermediate, and P is released hydrolyzed monobactam.

Because of the observed nonlinearity of progress curves in the presence of excess inhibitor, it is possible that a second reversible complex is present before acylation occurs: $E + M \rightleftharpoons E \cdot M \rightleftharpoons (E \cdot M)^* \rightarrow E - A \rightarrow E + P$ where $(E \cdot M)^*$ represents a second reversible complex. A similar mechanism has been proposed by Anderson and Pratt (2) with respect to the early phases of substrate hydrolysis for the *S. aureus* PC1 β-lactamase.

In both these mechanisms, the monobactam behaves simply as a poor substrate for the enzyme. Therefore, we propose that these monobactams be classified as competitive substrates for the P99 β-lactamase.

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