ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1993, p. 851–858
0066-4804/93/040851-08$02.00/0

Vol. 37, No. 4

Kinetic Interactions of Tazobactam with β-Lactamases from All Major Structural Classes

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Received 5 October 1992/Accepted 5 February 1993

Tazobactam was shown to be a potent inhibitor of group 1, 2a, 2b, and 2b′ β-lactamases. Extended kinetic studies with class A and C serine β-lactamases showed that the PC1, TEM-2, and P99 enzymes all were reversibly inhibited prior to inactivation of the enzymes. The CcrA metallo-β-lactamase was less well inhibited, with a 50% inhibitory concentration at least 3 orders of magnitude less favorable than those for most serine β-lactamases. The numbers of hydrolytic turnovers of tazobactam before inactivation were 2 for PC1, 125 for TEM-2, 50 for P99, and 4,000 for the CcrA enzyme. In spectral studies, transient intermediates were formed after reaction of tazobactam with the PC1, TEM-2, and CcrA β-lactamases, corresponding to enzyme-associated intermediates responsible for hydrolysis of tazobactam. Chromophores absorbing at 270 nm (CcrA) and 288 nm (TEM-2 and PC1) were observed for these reaction intermediates. The P99 cephalosporinase formed a stable complex with a UV maximum at 295 nm. Incubation of tazobactam with all of the enzymes resulted in accumulation of a tazobactam reaction product with a short-wavelength absorbance. This product has characteristics similar to those of the major eucaryotic metabolite of tazobactam. Possible reaction mechanisms are presented to explain the findings. In conclusion, both serine-based and metallo-β-lactamases were irreversibly inactivated by tazobactam following an initial transient inhibition phase.

Tazobactam, a triazolyl-substituted penicillanic acid sulfone (3), is a β-lactamase inhibitor that has been successfully combined with piperacillin to protect this broad-spectrum penicillin from β-lactamase-mediated hydrolysis (17, 31). Although extensive microbiological studies have been described for the piperacillin-tazobactam combination (24, 29), little has been reported concerning the mechanism of inhibition of standard β-lactamases. In contrast to the elaborate biochemical studies describing clavulanic acid and sulbactam inactivation of the TEM-2 (7, 15, 18, 21) and PC1 β-lactamases (13), no comparative enzymological studies have been performed with tazobactam.

In this set of studies, a metallo-β-lactamase and three serine β-lactamases from different functional groups (8, 9) were examined in detail to determine the kinetics of inhibition by tazobactam. These enzymes include the P99 cephalosporinase not inhibited by clavulanic acid (Bush group 1); the PC1 penicillinase (group 2a) and the broad-spectrum TEM-2 β-lactamase (group 2b), both well inhibited by clavulanic acid; and the CcrA metallo-β-lactamase from *Bacteroides fragilis* (group 3), an enzyme not inhibited by the classical β-lactamase inhibitors. The PC1 and TEM-2 β-lactamases are members of structural class A, with serine as the active-site acylating residue (2). The CcrA enzyme is a zinc-dependent enzyme of structural class B (2, 32). P99 is a class C enzyme with a higher molecular weight, also with an active-site serine (20). Evidence is presented to show that each enzyme undergoes reversible reactions with tazobactam before inactivation eventually occurs.

MATERIALS AND METHODS

Microorganisms. Strains *Escherichia coli* W3310, *Enterobacter cloacae* P99, *Serratia marcescens* SC 9782, and *B. fragilis* TAL3636 were the original sources for TEM-2, P99, S2, and the CcrA metallo-β-lactamases, respectively. PC1 β-lactamase from *Staphylococcus aureus* was a commercial product from Porton Products, Ltd., Berkshire, England. *E. coli* J53 R1010, R6K, CF102, 2936E, and KC2X; *Klebsiella pneumoniae* KPS19; *S. marcescens* S6; and *Xanthomonas maltophilia* 1712 were sources for the SHV-1, TEM-1, TEM-3, TEM-9, TEM-10, TEM-26, Sme-1, and L1 enzymes, respectively.

Antibiotics. Nitrocefin was obtained from Becton Dickinson Microbiology Systems, Hunt Valley, Md. Piperacillin, tazobactam, and the major eucaryotic metabolite of tazobactam (26) were prepared at Lederle Laboratories, Pearl River, N.Y. Clavulanic acid and sulbactam were provided by Smith Kline Beecham Laboratories, Bristol, Tenn., and Pfizer Inc., Groton, Conn., respectively.

β-Lactamase extraction and purification. Purified β-lactamases from gram-negative organisms were prepared from extracts obtained after four freeze-thaw cycles to release the periplasmic β-lactamase (11). The TEM-1, TEM-2, TEM-9, TEM-10, and TEM-26 β-lactamases were further purified by boronic acid affinity chromatography (10, 14). The S2 cephalosporinase and the SHV-1 and TEM-3 β-lactamases were partially purified by Sephadex G75 chromatography. P99 β-lactamase was purified as previously described (12). Purification of the CcrA metalloenzyme from *E. coli* was described previously by Yang et al. (32). The Sme-1 β-lactamase, a pI 9.7 metalloenzyme, was purified from QAE-Sephadex in 10 mM phosphate buffer, pH 9.0 (33). The L1 β-lactamase was purified from CM-Sephadex C50 in pH 7.5 *N*-2-hydroxyethylpiperazin-*N*′-2-ethanesulfonic acid (HEPES) containing 1 μM $ZnCl_2$. The CcrA β-lactamase was diluted in 10 mM HEPES–10% glycerol buffer, pH 7.4, and all metalloenzyme assays were performed with 10 mM HEPES, pH 7.4. The L1 enzyme was maintained in the presence of 1.0 μM $ZnCl_2$ at all times. All nonmetalloenzymes were diluted and assayed in 0.05 M phosphate buffer, pH 7.0.

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Isoelectric focusing (IEF). IEF was performed with an LKB Multiphor apparatus with prepared PAGplates (Pharmacia LKB), pH 3.5 to 9.5. The identity of each purified enzyme was confirmed by activity staining with nitrocefin following IEF (28). IEF was also used to determine the presence of a covalently modified β-lactamase following overnight incubation of the enzyme with tazobactam. Reaction mixtures of 30 μl contained either the enzyme and tazobactam or the enzyme and buffer. Samples were assayed with nitrocefin to determine residual activity before IEF was performed. Gels were first stained with Coomassie brilliant blue and then with silver stain in accordance with the instructions from the manufacturer.

Determination of IC_{50}s. The inhibitor concentration that caused a 50% reduction in enzyme activity (IC_{50}) was determined graphically from activity data generated after 10 min of preincubation of 10 to 50 μl of the enzyme and inhibitor at 25°C. The substrate used to initiate the enzymatic reaction in these studies was nitrocefin at an assay concentration of 100 μg/ml in a volume of 1,000 μl.

Progressive inhibition determinations. Enzyme (2.2 μM PC1, 2.5 μM TEM-2, 0.21 μM P99, or 5.0 μM TAL3636) and various concentrations of inhibitors were incubated together at 25°C in a volume of 50 to 100 μl. At least two controls were prepared containing buffer in place of the inhibitor. Samples (1 to 2 μl) were withdrawn at regular intervals and immediately diluted into 600 or 1,000 μl of 200 μM nitrocefin (P99 enzyme) or 100 μM piperacillin (PC1, TEM-2, and CcrA β-lactamases). Reaction rates were monitored for 2 to 5 min with a Gilford 250 spectrophotometer. A percent control activity value was obtained by dividing an experimental hydrolysis rate by the mean value of duplicate control activities obtained at identical time points. The turnover number, equivalent to the number of inhibitor molecules required to inactivate one enzyme molecule, was based upon the inhibitor-enzyme molar ratio that resulted in >90% enzyme inactivation after 18 h of incubation.

For the class A and C β-lactamases, pseudo-first-order rate constants were obtained from semilogarithmic plots of percent control activity against time by using only the initial rates of inactivation (≤5 min, depending upon the enzyme). Second-order rate constants were estimated from the slopes of linear plots of the pseudo-first-order rate constants against the inhibitor concentration. It was also possible to calculate from the plots an apparent K_d, the dissociation constant for the initial complex formation between tazobactam and enzyme (22).

Spectrophotometric studies. PC1, TEM-2, P99, and CcrA β-lactamases (final concentrations, 3.6 to 6.9 μM) were mixed with buffer, and UV spectra were recorded periodically until the spectrum exhibited no change in absorbance. Spectra were analyzed from 200 to 300 nm at 25°C with a Beckman 7400 spectrophotometer. At a fixed time, tazobactam was added to give the desired inhibitor-to-enzyme ratio. Spectra were recorded every 0.2 to 0.5 min for the first 5 minutes of a reaction and then every 5 min until a plateau absorbance was reached. Additional tazobactam was then added, and the spectra were recorded a second time. Difference spectra were calculated electronically by subtracting the initial enzyme spectrum from the enzyme-tazobactam spectrum.

Labeling with [^{14}C]tazobactam. [^{14}C]tazobactam labeled in both carbon atoms of the triazole ring (32.6 μCi/mg) was incubated with 0.4 to 10 μM PC1, TEM-2, P99, or CcrA β-lactamase for fixed periods of time, at tazobactam concentrations sufficient to give >90% inactivation of the enzymes.

TABLE 1. Inhibition of selected β-lactamases by β-lactamase inhibitors[a]

Enzyme	Bush group[b]	IC_{50} (nM)		
		Clavulanate	Sulbactam	Tazobactam
Class A				
PC1	2a	30	80	27
TEM-1[c]	2b	90	900	97
TEM-2	2b	22	2,400	17
TEM-3	2b′	11	21	5.0
TEM-9[c]	2b′	9.0	270	77
TEM-10[c]	2b′	4.4	940	87
TEM-26[c]	2b′	8.4	350	77
SHV-1	2b	12	12,000	150
Class C				
P99	1	>100,000	5,600	8.5
S2	1	51,000	5,200	6,000
Class B				
CcrA	3	>500,000	>500,000	400,000
Sme-1	3	14,000	3,300	3,000
L1	3	>400,000	>400,000	>400,000

[a] The enzyme and inhibitor were preincubated for 10 min before substrate addition.
[b] References 8 and 9.
[c] Data are from reference 30.

The reaction mixtures were submitted to polyacrylamide gel electrophoresis under nondenaturing conditions by the procedure of Laemmli in the absence of sodium dodecyl sulfate (25); the gels were then analyzed by fluorography (5). Inactivated enzyme-tazobactam mixtures were also separated on Sephadex G50 (0.7 by 21 cm) at room temperature. Consecutive fractions of approximately 500 μl were counted for radioactivity.

RESULTS

IC_{50}s of inhibitors. IC_{50}s of clavulanic acid, sulbactam, and tazobactam against various β-lactamases are listed in Table 1. For class A β-lactamases, tazobactam showed activity comparable to that of clavulanate against the PC1, TEM-1, TEM-2, and TEM-3 enzymes. Clavulanate had stronger inhibitory activity than tazobactam against the extended-spectrum ceftazidime-hydrolyzing β-lactamases and the SHV-1 broad-spectrum enzyme, giving 9- to 20-fold decreases in the IC_{50}s. Sulbactam was the least active inhibitor for class A enzymes, with IC_{50}s of sulbactam approximately 3 times higher than those of clavulanate and tazobactam for the PC1 enzyme, 2- to 200-fold higher than those of the TEM enzymes, and 80- to 1,000-fold higher than that of the SHV-1 β-lactamase. Tazobactam showed excellent activity against class C enzyme P99 but poorer activity against S2 cephalosporinase. The sulfone inhibitors sulbactam and tazobactam were more potent than clavulanate for both enzymes. The Sme-1 metallo-β-lactamase, an enzyme functionally different from other metallo-β-lactamases (9, 32, 33), was weakly inhibited by all three inhibitors. The CcrA and L1 metalloenzymes were not effectively inhibited by any of the inhibitors, although some inhibition of the CcrA enzyme was observed with tazobactam at higher concentrations. The major metabolite of tazobactam was a poor inhibitor for the PC1, TEM-2, P99, and CcrA enzymes, with IC_{50}s of >400 μM.

Progressive inhibition of β-lactamase. The time depen-

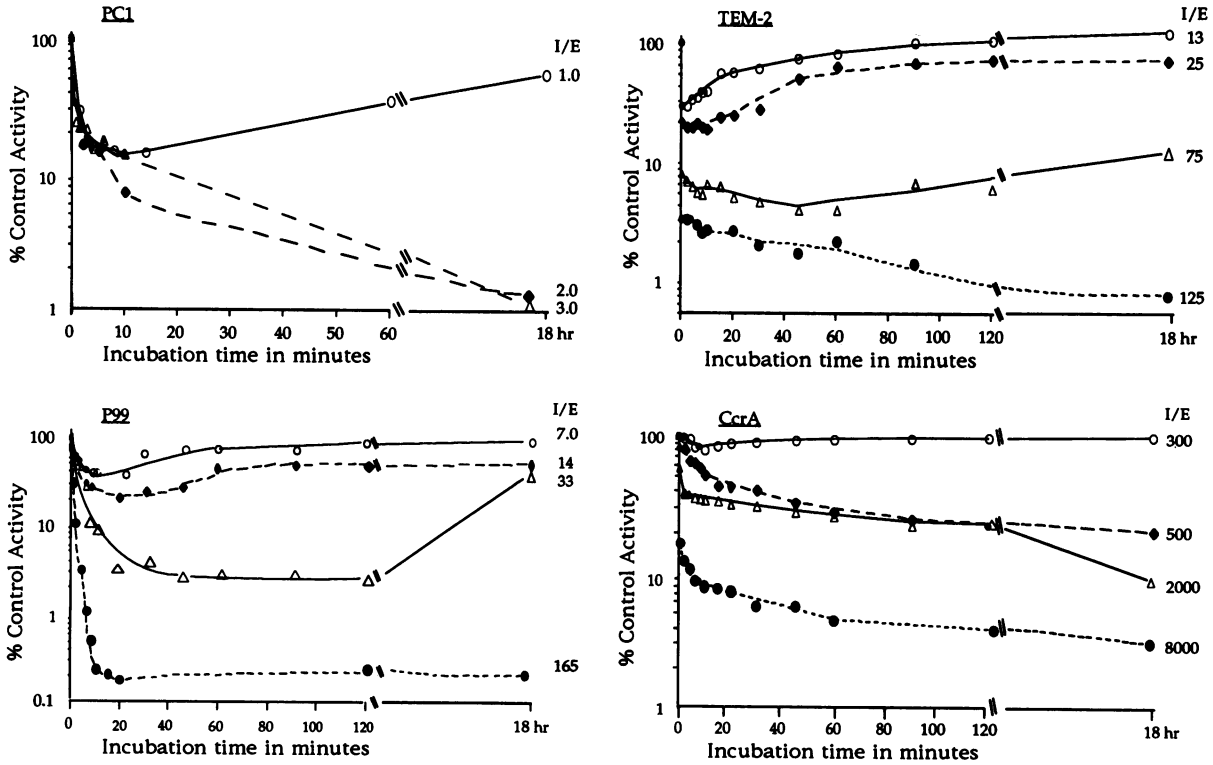

FIG. 1. Inactivation of β-lactamases by tazobactam. Inactivation mixtures contained 2.2 μM PC1 β-lactamase with 2.2 (○), 4.4 (◆), and 6.6 (△) μM tazobactam; 2.5 μM TEM-2 β-lactamase with 33 (○), 63 (◆), 190 (△), and 310 (●) μM tazobactam; 0.21 μM P99 β-lactamase with 1.5 (○), 3.0 (◆), 6.9 (△), and 35 (●) μM tazobactam; and 5.0 μM CcrA β-lactamase with 1.5 (○), 2.5 (◆), 10 (△), and 40 (●) mM tazobactam. Assays were conducted in piperacillin or nitrocefin following a 500-fold dilution for the TEM-2, P99, and CcrA mixtures and a 600-fold dilution for the PC1 mixture. I/E, inhibitor-enzyme ratio.

dence of tazobactam inhibition for the major enzymes is demonstrated in Fig. 1. The mode of β-lactamase inactivation by tazobactam differed from one enzyme to another. For the PC1 serine β-lactamase, a biphasic profile was observed: inhibition was rapid initially, followed by a slower rate after approximately 2 min of incubation. At stoichiometric inhibitor-to-enzyme concentrations, there was >50% reversal of inhibition after 18 h. At higher tazobactam concentrations, the PC1 enzyme was completely inactivated.

The TEM-2 enzyme was inhibited immediately following addition of tazobactam. Although the enzyme slowly regained activity when incubated with limiting concentrations of tazobactam, recovery of TEM-2 enzyme activity was incomplete, indicating some formation of permanently inactivated enzyme at relatively low concentrations of tazobactam. At tazobactam-to-enzyme ratios greater than 130, complete inactivation was effected.

Inhibition of the P99 β-lactamase by tazobactam was progressive, with a first-order rate of inhibition observed initially. The rate of inhibition increased gradually as more of the inhibitor was added. A certain degree of reversibility was observed in the reaction. Although 60% of P99 enzyme activity was inhibited after 10 min of incubation at a molar ratio of 7:1 (inhibitor to enzyme), enzyme activity was slowly recovered as the incubation time was extended. Complete inactivation of the P99 enzyme required tazobactam ratios of at least 75:1.

Inactivation of the CcrA metalloenzyme also exhibited a biphasic pattern: the immediate rapid inhibition became slower after approximately 2 min (Fig. 1). However, the subsequent rate of inhibition was steady and progressive at tazobactam ratios greater than 500. Only a small amount of reversibility was associated with long-term incubation of the enzyme and tazobactam. After reaction of the β-lactamase with a 4,000-fold excess of tazobactam, >90% inactivation was observed.

Inhibition kinetics of tazobactam inactivation. The kinetic parameters for tazobactam inhibition of the β-lactamases are summarized in Table 2. Because the proposed mechanism of inactivation for the serine β-lactamases is similar to those described in previous studies (6, 23), whereas the inactivation sequence for the CcrA metalloenzyme may involve additional product release and reassociation with the enzyme (see below), the assumptions used to derive the rate

TABLE 2. Kinetic characteristics of tazobactam inactivation of β-lactamases

β-Lactamase	Apparent dissociation constant (nM)	Second-order rate constant for inactivation (M^{-1} s^{-1}, 10^6)	No. of turnovers before inactivation	
			Clavulanate	Tazobactam
PC1	217	6,020	1	2
TEM-2	130	2,800	150[a]	125
P99	125	790	>500,000	50
CcrA	NC[b]	NC	>500,000	4,000

[a] Reference 19.
[b] Not calculated.

constants (22) are valid only for class A and C enzymes. Examination of the apparent dissociation constants indicated that tazobactam showed similar affinity for all of the serine enzymes. Tazobactam appeared to inactivate the PC1 enzyme more rapidly than the TEM-2 enzyme, but it should be noted that the initial rates of inactivation for the TEM-2 enzyme were so rapid that the rate constant of 2,800 $M^{-1} s^{-1}$ represents an estimated lower limit. Although the IC_{50} for the P99 class C enzyme was less than the IC_{50}s observed for PC1 and TEM-2, tazobactam inactivated P99 three- to sevenfold slower than the class A enzymes.

Turnover numbers are also given in Table 2. For the PC1 enzyme, a turnover number of 2 indicates that only two tazobactam molecules were needed to inactivate one molecule of the PC1 enzyme. For the TEM-2 β-lactamase, higher ratios of tazobactam were needed for inactivation than with the PC1 enzyme. Comparable turnover numbers were observed for clavulanate and tazobactam for the PC1 and TEM-2 enzymes. For the P99 β-lactamase, tazobactam was 10,000 times more efficient than clavulanic acid. In contrast to the serine β-lactamases that were inactivated by molar ratios of 125 or less, 4,000 tazobactam molecules were required to inactivate 1 molecule of the CcrA metalloenzyme. Clavulanate and sulbactam, however, did not inactivate the metalloenzyme, even with a 500,000-fold molar excess (32).

Formation of reaction intermediates. When the β-lactamases were incubated with tazobactam, characteristic reaction intermediates could be recognized in accordance with the calculated difference spectra. By using the UV spectrum for the native enzyme as the reference, spectra for each reaction mixture were analyzed for appearance and disappearance of specific absorbance peaks. Figure 2 shows spectra of tazobactam (see structure 1 in Fig. 5) and the major metabolite of tazobactam (see structure 4 in Fig. 5), together with the spectra observed with the CcrA and PC1 β-lactamases after addition of tazobactam (Fig. 2A and B). The calculated difference spectra for the CcrA and PC1 enzymes are shown in Fig. 2C, with λ_{max} values of 270 and 288 nm, respectively. The extinction coefficients at 218 nm (the maximum wavelength) were 3,700 $M^{-1} cm^{-1}$ for tazobactam and 6,350 $M^{-1} cm^{-1}$ for the metabolite. It is obvious that the 270 nm chromophore from the CcrA enzyme or the 288 nm chromophore from the PC1 enzyme-tazobactam complex is associated with a molecular structure different from that of the intact sulfone or the metabolic degradation product.

After addition of tazobactam, the PC1 enzyme formed a reactive intermediate that had a peak absorbance at 288 nm (Fig. 3). Formation was maximal 2 min after mixing; the complex then disappeared with time. The concentration of the 288 nm-absorbing intermediate was greatest when equimolar amounts of the enzyme and tazobactam were combined initially. A second addition of either equimolar tazobactam or a fivefold excess of tazobactam (data not shown) resulted in a second cycle of intermediate formation, followed by continued degradation of the absorbing material. Maximal absorbance of the second complex was less than that following the initial addition of tazobactam. Concomitant with the tazobactam addition was formation of a low-UV-absorbing material. This material was stable and remained present even after disappearance of the 290 nm-absorbing complex.

Two similar complexes with the TEM-2 β-lactamase were observed (Fig. 3). A complex with a λ_{max} at 288 nm was formed with tazobactam at molar ratios ranging from 1 to 10.

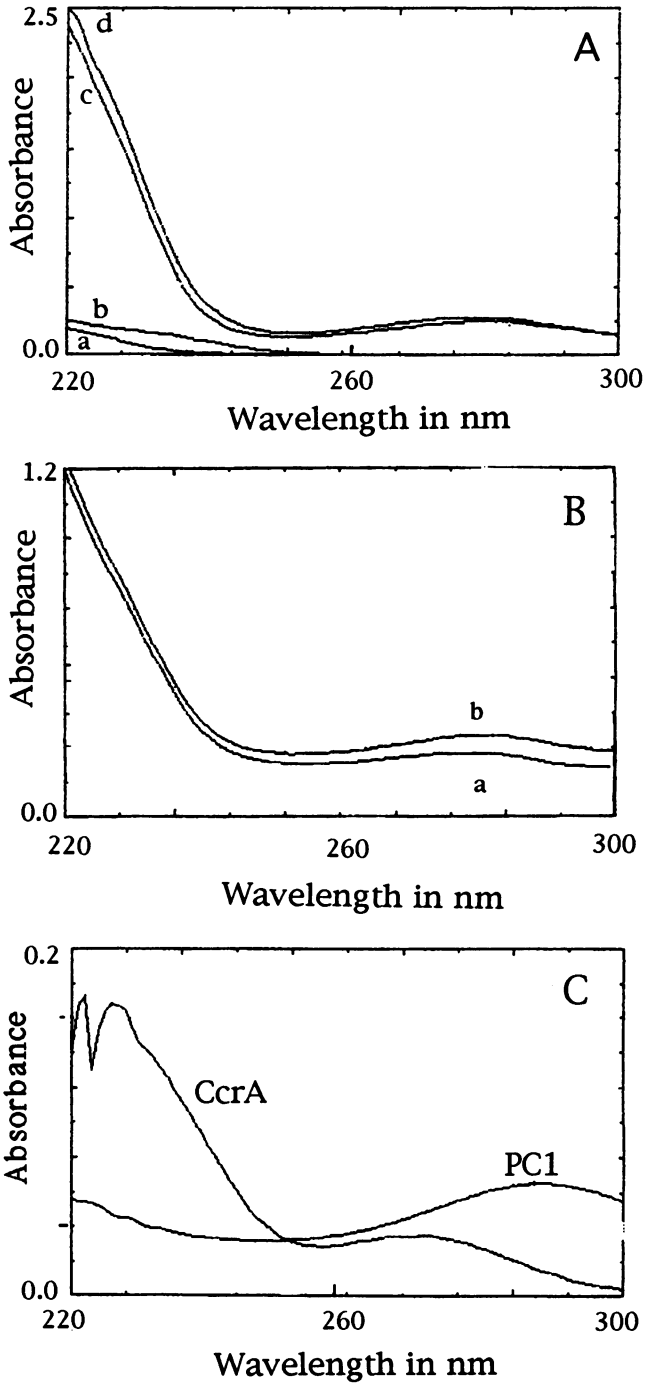

FIG. 2. UV spectra of tazobactam-related products. (A) Curves: a, 42 μM tazobactam; b, 42 μM tazobactam metabolite 4; c, 4.2 μM CcrA β-lactamase; d, CcrA β-lactamase with 42 μM tazobactam 0.5 min after mixing. (B) Curves: a, 6.2 μM PC1 β-lactamase; b, 6.2 μM PC1 β-lactamase with 6.2 μM tazobactam 1.1 min after mixing. (C) Difference spectra of CcrA and PC1 β-lactamase mixtures as shown in panels A and B.

The maximal absorbance at 288 nm was not dependent upon the amount of tazobactam added, indicating that the complex formed was enzyme associated. The extinction coefficient for this intermediate of 12,600 $M^{-1} cm^{-1}$ was based

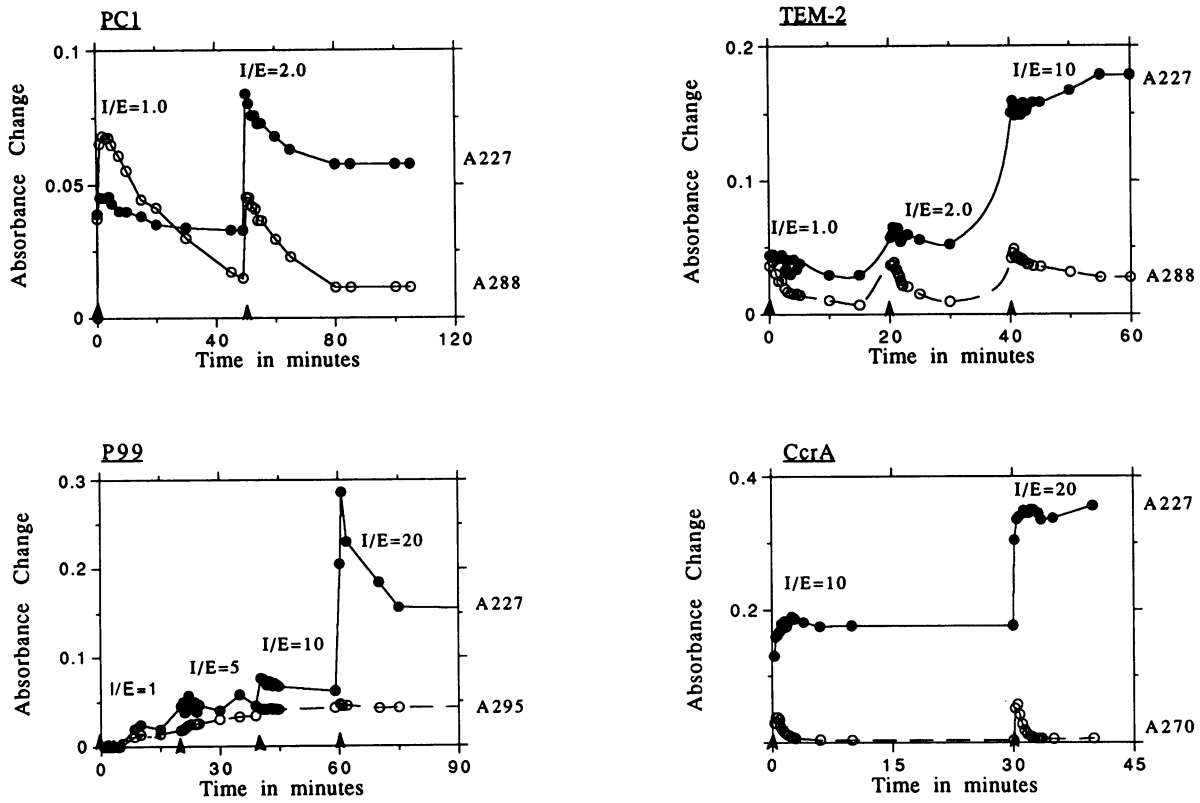

FIG. 3. Time course for appearance of UV-absorbing chromophores. Absorbance changes, high UV chromophores, (○) and low UV chromophore (●), were based upon the difference spectra obtained with the native enzyme as the reference. Arrows on the abscissa mark the addition of tazobactam to the native enzyme. Molar ratios represent the cumulative total of tazobactam added per mol of the enzyme. Enzyme concentrations were 6.9 μM PC1, 3.6 μM TEM-2, 3.8 μM P99, and 4.2 μM CcrA β-lactamase. I/E, inhibitor-enzyme ratio.

upon equimolar formation of an enzyme-inhibitor complex. Molar extinction coefficients of 23,000 (λ_{max}, 289 nm) and 16,000 (λ_{max}, 290 nm) M^{-1} cm^{-1} had previously been reported for the TEM-2 enzyme inactivated by the quinacillin sulfone (19) and sulbactam (6), respectively. As with the PC1 enzyme, formation of the complex was rapid, with a time-dependent decline in the complex concentration. The rate of decrease was fastest after the initial tazobactam addition. A second peak of absorbance was observed, with a λ_{max} of 227 nm. At high tazobactam concentrations, this component increased in absorbance as the 288 nm-absorbing material declined.

A somewhat different pattern was observed with the P99 cephalosporinase (Fig. 3). Addition of tazobactam resulted in a stable complex with a λ_{max} of 295 nm. This complex did not degrade, as observed with the class A enzymes. However, material with UV absorbance in the range of 225 to 227 nm also accumulated in the reaction.

The CcrA metalloenzyme exhibited a different UV profile (Fig. 3). Tazobactam addition caused this enzyme to exhibit a rapid increase and subsequent rapid decrease in UV-absorbing material with a peak absorbance at 270 nm. The maximum absorbance associated with this chromophore was related to both the amount of the enzyme present (data not shown) and the concentration of tazobactam added, suggesting an enzyme-substrate type of reaction. As seen with the other enzymes, however, a low UV-absorbing material with a λ_{max} in the range of 225 to 229 nm was also present in the reaction mixture. The molar extinction coefficient of 4,200 M^{-1} cm^{-1} was comparable to that observed with the PC1 and TEM-2 enzymes for the short wavelength chromophore.

Spectral parameters and molar extinction coefficients for each of the enzyme complexes are summarized in Table 3. From these data, one can propose formation of a similar chromophore as an end product of all of the inactivation reactions.

Labeling of enzymes by [^{14}C]tazobactam. None of the inactivated enzymes bound significant amounts of [^{14}C](triazole)-tazobactam after long-term incubation. The CcrA metalloenzyme showed a small amount of labeling only at high enzyme concentrations as determined by fluorography. However, when the inactivated CcrA enzyme-[^{14}C]tazobactam complex was separated from unbound tazobactam on Sephadex G50, the amount of label on the enzyme was only 5% of the theoretical amount expected for stoichiometric

TABLE 3. Spectrophotometric characteristics of reactions of β-lactamases with tazobactam

Enzyme	Enzyme-associated chromophore		Reaction product	
	λ_{max}	Extinction coefficient[a] (M^{-1} cm^{-1})	λ_{max}	Extinction coefficient[b] (M^{-1} cm^{-1})
PC1	288	10,300	227	5,000
TEM-2	288	12,600	227	4,960
P99	295	21,300	225–227	4,500
CcrA	270	13,000	225–229	4,200

[a] Based upon enzyme concentration.
[b] Based upon total tazobactam concentration.

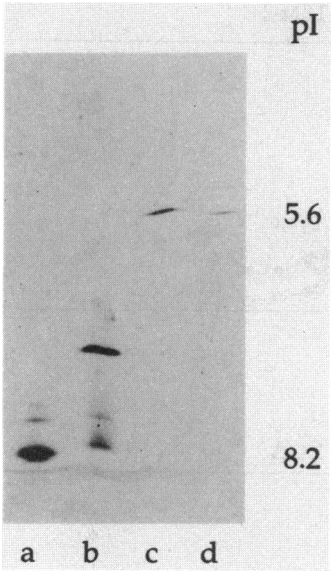

FIG. 4. IEF pattern of β-lactamases inactivated by tazobactam. The P99 and TEM-2 β-lactamases were incubated for 4 h with tazobactam. Lanes: a, 1.3 μM P99 β-lactamase; b, 1.3 μM P99 with 52 μM tazobactam; c, 2.7 μM TEM-2 β-lactamase; d, 2.7 μM TEM-2 with 125 μM tazobactam.

addition of inhibitor. These results indicate that a stable covalent intermediate containing the triazole ring was not formed with the CcrA enzyme.

When the P99-[^{14}C]tazobactam complex (20-fold molar excess of tazobactam) was incubated for only 3 h and chromatographed on Sephadex G50, the inactivated enzyme peak contained 0.4 mol of [^{14}C]tazobactam per mol of P99 β-lactamase. Samples analyzed following overnight incubation of the enzyme and inhibitor contained <0.05 mol of [^{14}C]tazobactam per mol of enzyme.

IEF of inactivated enzymes. The four major enzymes were reacted with excess tazobactam to give modified enzymes with less than 4% residual activity. As seen in Fig. 4, the P99 enzyme, when modified with tazobactam, migrated with a lower pI than the native enzyme. Although both the TEM-2 and CcrA β-lactamases had major protein bands with unaltered isoelectric points after inactivation, the intensity of the band was less for the enzymes reacted with tazobactam. The PC1 enzyme, unfortunately, did not focus well in the electrophoresis system used for the other enzymes, so no conclusions can be drawn concerning the physical state of this protein upon inactivation.

DISCUSSION

Tazobactam was an effective mechanism-based inactivator of class A β-lactamases, as evidenced by low IC_{50}s, rapid inactivation rates, and low dissociation constants. For all of the class A enzymes studied, the IC_{50}s for tazobactam were lower than for sulbactam, indicating a more favorable interaction with the triazole-substituted penicillanic acid sulfone than with the naked sulfone. Although the IC_{50}s of clavulanic acid and tazobactam were comparable for the PC1, TEM-1, TEM-2, and TEM-3 enzymes, clavulanate was a more effective inhibitor of extended-spectrum TEM-derived ceftazidime-hydrolyzing β-lactamases. Thus, the amino acid changes responsible for expanding the substrate profiles of these enzymes also increased the sensitivity to clavulanic acid more dramatically than with the sulfone inhibitors. Additional studies are required to determine whether turnover numbers or reversibility of the sulfone reactions with the extended-spectrum enzymes have also been affected by their amino acid substitutions.

The class C P99 cephalosporinase was also effectively inactivated by tazobactam, although the rate constant for complex formation indicated slower formation of the inactive enzyme than the PC1 and TEM-2 enzymes. In contrast to the class A enzymes, the turnovers observed before inactivation were lowered 3 orders of magnitude compared with clavulanic acid. Therefore, for the class C enzymes there is a major difference in the recognition of the two inhibitors. The difference in tazobactam activity between the P99 and S2 cephalosporinases must be related to the active-site changes between the two enzymes.

Correlation of the inactivation time course and formation of UV chromophores for the class A enzymes resulted in the following conclusions. Both the PC1 and TEM-2 enzymes formed a transient complex, with a λ_{max} occurring at 288 to 290 nm. Formation and subsequent degradation of this complex was faster for the TEM-2 enzyme, as might be expected from the inactivation time course. It is most probable that this transient complex represented the formation of a tazobactam-enzyme complex that subsequently was hydrolyzed. The finding of biphasic inactivation kinetics leads to the conclusion that the reaction pathway includes a branch leading to an inactivated enzyme. Because the PC1 enzyme was inactivated at much lower inhibitor-to-enzyme ratios than the TEM-2 enzyme, one would expect degradation of the PC1 complex to proceed more slowly, as a greater percentage of enzyme was partitioned into the inactivation pathway at lower tazobactam concentrations.

A possible mechanism for inactivation of the class A β-lactamases is shown in Fig. 5, where a branched pathway is proposed on the basis of similar pathways described in the literature as a result of both kinetic and crystallographic data (16, 19, 21). In this mechanism, tazobactam (structure 1) forms a noncovalent complex that then results in formation of an acyl enzyme (structure 2). The acyl enzyme can then undergo the following reactions. The first would be deacylation to yield an unstable ring-opened tazobactam (compound 3) that subsequently fragments to give the malonsemialdehyde and metabolite (structure 4), the stable product of tazobactam identified in biological studies. A second possible fate of the acyl enzyme (structure 2) would be elimination to give the imine shown in structure 5, based upon structures previously proposed by Knowles for the reaction of clavulanic acid and penam sulfones with the TEM-2 enzyme (23). Crystallographic data for the PC1-clavulanate complex support the formation of this intermediate, which can exist in either a *cis* or a *trans* conformation (16). Intermediate 2 could also undergo elimination across the C-5 and C-6 bond, resulting in an acyl enamine intermediate (compound 6). Intermediate 6 could also be formed by tautomerization of compound 5 and would be the species responsible for the 288- to 290-nm chromophores. This enzyme-bound form could deacylate to give active enzyme and metabolite 4, or it could lose fragment 4 but retain an acylated serine, as seen with the class C β-lactamase. It might be possible to undergo subsequent reaction with a lysine on the enzyme, resulting in a cross-linked, inactive β-lactamase. In all cases, an end product resembling or resulting from compound 4 would be formed, yielding material with a low UV-absorbing chromophore. It should be

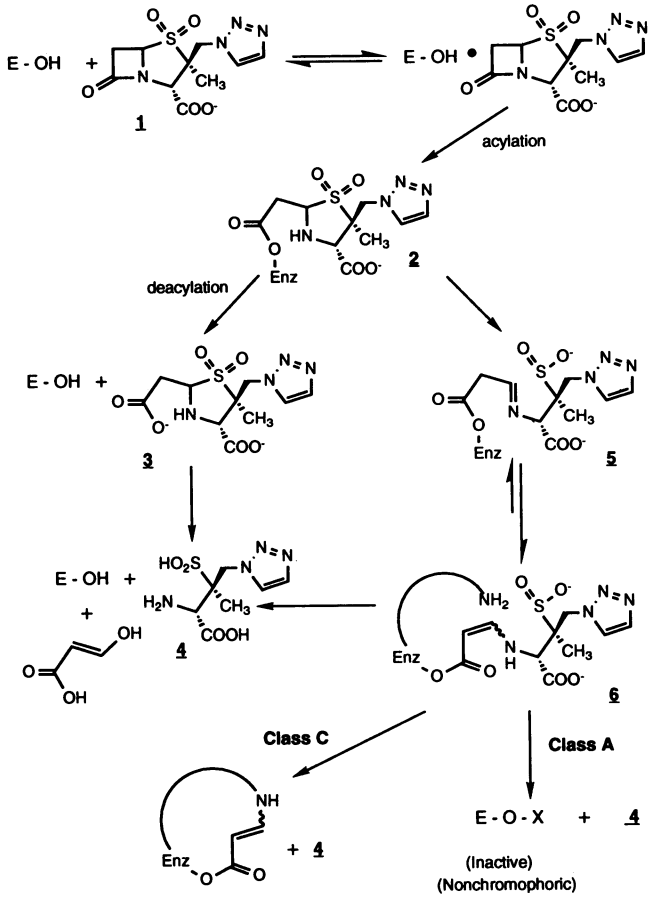

FIG. 5. Possible reaction pathways for interaction of tazobactam and serine β-lactamases. Enz, enzyme.

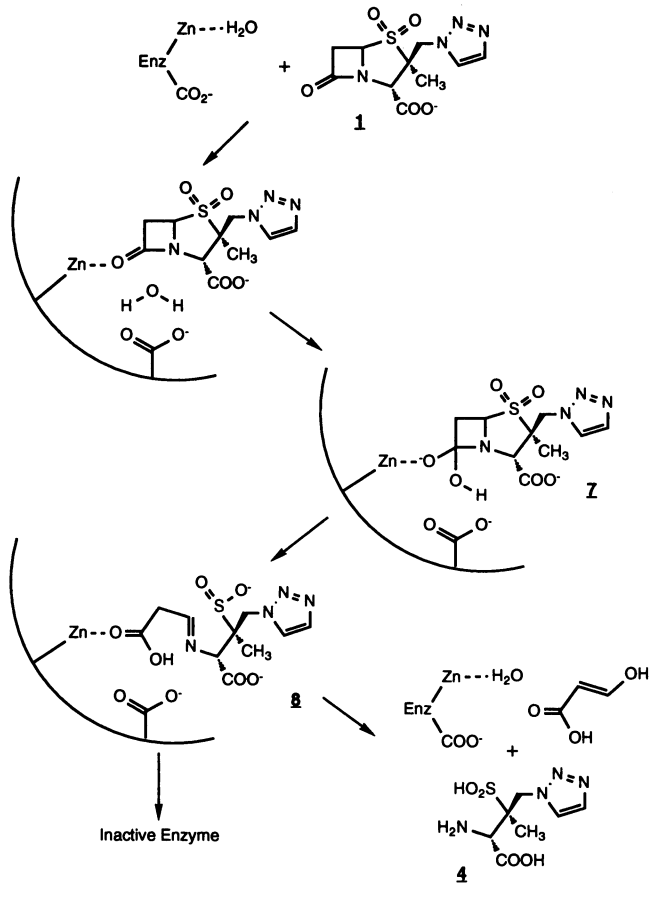

FIG. 6. Possible inactivation mechanism for tazobactam inhibition of the CcrA metallo-β-lactamase. Enz, enzyme.

noted that further degradation of compound 4 to several unidentified species has previously been recorded (27).

Inhibition behavior of the P99 cephalosporinase was consistent with earlier studies from Livermore's laboratory that described partial recovery of activity of an *E. cloacae* cephalosporinase treated with either tazobactam or clavulanic acid (1). The data obtained in this study with the P99 enzyme suggest that the inactivation pathway involves multiple branches similar to those followed by the other serine β-lactamases. As shown in the postulated mechanism in Fig. 5, formation of an acyl enzyme with partitioning through a hydrolytic reaction or inactivation via intermediates 5 and 6 would be possible. In contrast to the class A β-lactamases, however, the inactive P99 enzyme was shown to retain a stable chromophore absorbing at 295 nm. Structure 6 might be expected to exhibit UV characteristics similar to those of the proposed class C inactivated enzyme in Fig. 5, possibly accounting for the 295 nm absorbance. Because [^{14}C]triazole was bound to the enzyme after a relatively short periods of incubation, it is reasonable to propose modified enamine structure 6 as the radiolabeled intermediate that could eventually lose triazole-related metabolite 4. The long-term-inactivated, cross-linked enzyme could then form after reaction of an enzyme-bound tazobactam fragment with a reactive lysine on the enzyme. Again, an end product similar to compound 4 would be expected to correspond to the 227 nm chromophore that accumulated in the P99-tazobactam reaction mixture.

Inactivation of the metallo-β-lactamase is more difficult to explain, as less is known about the catalytic mechanism for this group of enzymes. However, a possible mechanism for the interaction of the CcrA enzyme with tazobactam is shown in Fig. 6. If the basic mechanism proposed by Bicknell and Waley is assumed (4), an active-site zinc with water as a ligand could complex with the intact tazobactam. Formation of a tetrahedral intermediate (structure 7) could then be followed by ring opening, resulting in an imine such as structure 8. This is the entity that would be most consistent with the 270 nm chromophore observed immediately after mixing of the CcrA enzyme with tazobactam. Subsequent formation of the common 227-nm chromophore would then occur. The molecular nature of the inactive enzyme is uncertain from the data presented. Because only a minute quantity of [^{14}C]tazobactam was associated with inactivated enzyme, the triazole ring could not be present in this modified protein; the small amount of the ^{14}C label observed may be due to nonspecific binding of a tazobactam end product. It is also possible that tazobactam metabolite 4 reassociates with functional enzyme to undergo further fragmentation, leading to inactive enzyme. The high IC_{50} observed for the tazobactam metabolite, however, suggests that interaction of compound 4 with free enzyme should not account for enzyme inactivation. Alternatively, further degradation of compound 4, resulting in loss of triazole, may provide a transient reactive intermediate that

can modify free enzyme to provide the final inactivated product. Studies are in progress to identify the tazobactam products and their effects upon the CcrA metalloenzyme.

In conclusion, all of the β-lactamases exhibited reversible reactions, leading to a functional enzyme after reaction with tazobactam. The extent of reversibility was dependent upon inhibitor-to-enzyme concentration ratios and varied in accordance with enzyme type. Although the PC1 penicillinase was most effectively inactivated on the basis of inhibitor ratios, the time course for inactivation was slower than that observed for the TEM-2 broad-spectrum enzyme, also a class A β-lactamase. The CcrA metallo-β-lactamase was least inhibitable, but inactivation did occur at high inhibitor ratios. Therefore, successful inhibition of all major classes of β-lactamases depends upon maintenance of a minimal level of tazobactam compared with the amount of enzyme present. Either a low concentration of tazobactam relative to that of the enzyme or an increased amount of β-lactamase could lead to clinical failures among β-lactamase-producing organisms. However, in most instances, the kinetics involved in time-dependent inactivation will eventually allow accumulation of inactive enzyme, especially for class A β-lactamases.

ACKNOWLEDGMENTS

We thank N. Bhachech for assistance in the radiolabeling experiments and B. Terman for help with purification of the CcrA enzyme. We appreciate helpful discussions with W. Curran, G. Feigelson, and C. Ziegler concerning the reaction mechanisms presented.

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