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Inactivation of the RTEM β-Lactamase from *Escherichia coli*. Interaction of Penam Sulfones with Enzyme†

Jed Fisher,‡ Robert L. Charnas,§ Scott M. Bradley, and Jeremy R. Knowles*

ABSTRACT: The characteristics of the reaction of a number of mechanism-based inactivators of the RTEM β-lactamase have suggested that a common mechanistic pathway may be followed by many of these compounds. These ideas have been tested by the synthesis and evaluation of some penam sulfones as β-lactamase inactivators. The sulfones of poor β-lactamase substrates are, as predicted, potent inactivators of the enzyme. A unique serine residue (Ser-70) is labeled by quinacillin sulfone, and it is likely that this serine acts nucleophilically in the normal hydrolytic reaction of the β-lactamase to form an acyl-enzyme intermediate.

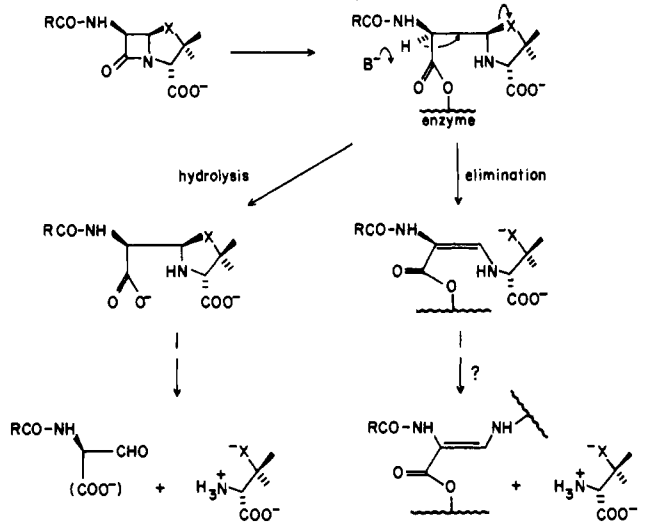
β-Lactam antibiotics exert their lethal effect on growing cells by interfering with the enzymes responsible for the synthesis and integrity of the bacterial cell wall. Resistance to the bacteriocidal action of β-lactams may develop, however, and this resistance is most frequently due to the production by the organism of a β-lactamase which catalyzes the hydrolytic cleavage of the β-lactam ring in penicillins and cephalosporins. The gene that encodes the β-lactamase is often on a plasmid, and the promiscuous transfer of plasmids among bacterial populations has sharply increased the incidence of β-lactam-resistant infections. This problem is of obvious clinical importance and was first attacked by developing β-lactam antibiotics [for example, semisynthetic penams (e.g., Doyle & Nayler, 1964), the cephamycins (Nagarajan et al., 1971), thienamycin (Kahan et al., 1979), and moxalactam (Komatsu & Nishikawa, 1980)] that were less sensitive to the hydrolytic action of the β-lactamases. More recently, however, a second approach to the β-lactamase problem has been taken; it utilizes the synergistic action of reagents that inhibit the β-lactamase when applied simultaneously with a good (though β-lactamase-sensitive) antibiotic. The first report of a β-lactamase inactivator, clavulanic acid (Brown et al., 1976), has been followed by the isolation and synthesis of a number of natural and semisynthetic β-lactam derivatives, including carbapenems (Brown et al., 1977; Maeda et al., 1977; Okamura et al., 1980), penicillanic acid sulfone (English et al., 1978; Labia et al., 1980), 6β-bromopenicillanic acid (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b), 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1979), and the sulfenimines of various cephems and penams (Gordon et al., 1980). The mechanism of action of many of these materials has been subjected to some scrutiny (Fisher et al., 1980a), and it seems likely that, in common with at least one substrate (Fisher et al., 1980b), the interaction with the enzyme involves the formation of an acyl-enzyme. The acyl-enzyme may then collapse to a more stable species, with concurrent inactivation of the enzyme. We suggested, therefore, that most, if not all, of the above β-lactam inactivators are "suicide" or "mechanism-based" inactivators of the β-lactamase (Fisher et al., 1980a).

The simplest mechanistic postulate that accommodated the kinetic and chemical behavior of clavulanic acid and the penicillanic acid derivatives [carbapenems such as the olivanates are considered elsewhere (Charnas & Knowles, 1981)] was that the first-formed acyl-enzyme suffered a β elimination across C-5 and C-6 (Scheme I). Abstraction of the 6α proton and cleavage of the bond between C-5 and the heteroatom would lead to a relatively stable β-aminoacrylate chromophore and concurrent inactivation of the enzyme. It seemed that the acyl-enzyme could partition between this β elimination and the normal hydrolysis of the ester linkage (Scheme I), and we suggested (Fisher et al., 1980a) that this class of inactivators

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received September 30, 1980.* This work was supported by the National Institutes of Health and the National Science Foundation.
‡ National Institutes of Health Postdoctoral Fellow.
§ National Science Foundation Predoctoral Fellow.

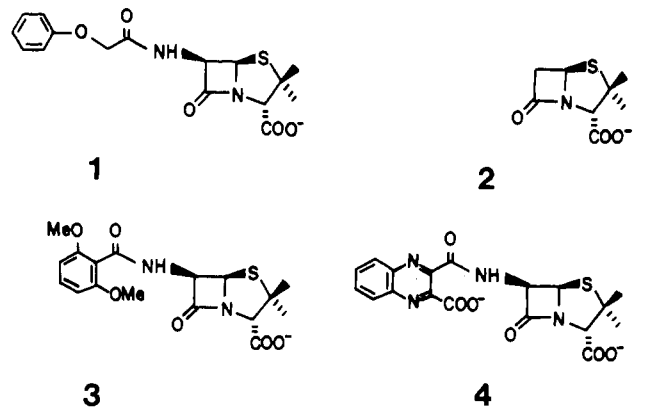
0006-2960/81/0420-2726$01.25/0

Scheme I: Mechanistic Outline for One Class of Mechanism-Based Inactivators of β-Lactamase



exhibited three characteristics: (a) a β-lactam structure that could lead to the formation of a long-lived acyl-enzyme, (b) a 6α proton of adequate acidity for the postulated β elimination, and (c) a good leaving group at C-5.

To test these ideas, we have investigated the behavior of the sulfones of the penams **1–4**, with the TEM-2[1] β-lactamase



from *Escherichia coli*. Penam sulfones are known (Guddal et al., 1962) to be substrates for the β-lactamase, and oxidation of the thiazolidine sulfur to the sulfone should satisfy both points b and c above, simultaneously acidifying the 6α proton (Pant & Stoodley, 1978) and providing a better leaving group from C-5 for the putative β elimination. [The pK_a value for a sulfinic acid is some 5 units below that of the parent thiol.] Proper choice of the β-*N*-acylamino substituent at C-6 should satisfy criterion a, and we argued that substituents that result in low substrate activity might form acyl-enzymes that hydrolyze only slowly and could partition favorably toward the inactivating elimination reaction (Fisher & Knowles, 1980). We report here the nature of the interaction of such sulfones with the plasmid-encoded RTEM[1] β-lactamase.

Experimental Procedures

Materials

β-Lactamase was isolated from *Escherichia coli* W3310 carrying the RP4 plasmid (Matthew & Hedges, 1976). The enzyme is a TEM-2 β-lactamase (Sykes & Matthews, 1976) and had a specific activity of 3800 units/A_{280nm}. A unit of enzyme activity is that amount of enzyme that will catalyze the hydrolysis of 1 μmol of benzylpenicillin per min at pH 7.0 (30 °C). The enzyme was purified as described earlier (Fisher et al., 1978) and was homogeneous as judged by isoelectric focusing in polyacrylamide gels.

Benzylpenicillin (penicillin G) and methicillin (sodium salts) and phenoxymethylpenicillin (penicillin V) (potassium salt) were from Sigma Chemical Co. Quinacillin (disodium salt) was a gift from Boots, Ltd., Nottingham, England. Penicillanic acid sulfone (sodium salt) was a gift from Pfizer Inc., Groton, CT. All β-lactams were stored desiccated at 4 °C.

Penam sulfones were prepared by the aqueous permanganate oxidation of the parent penam by the method of Johnson et al. (1963). The sulfones were purified by crystallization of the free acid from ethyl acetate–hexane.

(Phenoxymethyl)penicillanic acid sulfone had mp 160–161 °C dec (Guddal et al. (1962) give 135–136 °C; Chow et al. (1962) give 147 °C); IR (KBr disk) 3400, 3050, 2940, 1815, 1750, 1700, 1600, 1515, 1495, 1328, 1117 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.47 (s, 3 H), 1.63 (s, 3 H), 4.56 (s) and 4.58 (s) (total of 3 H), 4.86 (d, 1 H, J = 4.6 Hz), 6.17 (d, d, 1 H, J = 4.7, 10.6 Hz), 7.0 (m, 5 H), 8.30 (d, 1 H, J = 10.6 Hz), and 9.01 (s, 1 H); mass spectrum of methyl ester, m/z 396 (M^+) and appropriate fragment ions.

Methicillin sulfone had mp 170–172 °C dec (Johnson et al. (1963) give 174.5–174.8 °C); IR ($CDCl_3$) 3430, 2960, 2500, 1815, 1745, 1680, 1600, 1500, 1470, 1325, 1110 cm^{-1}; ^1H NMR ($CDCl_3$–Me_2SO-d_6, 10:1 v/v) δ 1.45 (s, 3 H), 1.57 (s, 3 H), 3.80 (s, 6 H), 4.42 (s, 1 H), 4.89 (d, 1 H, J = 4.5 Hz), 5.60 (br s, 2 H), 6.30 (d, d, 1 H, J = 4.6, 10.8 Hz), 6.52 (d, 2 H, J = 8.5 Hz), and 7.25 (t, 1 H, J = 8.2 Hz).

Quinacillin sulfone had mp 190–192 °C dec; IR ($CHCl_3$) 3380, 2970, 2450, 1815, 1740, 1700, 1325, 1115 cm^{-1}; ^1H NMR ($CDCl_3$–Me_2SO-d_6, 10:1 v/v) δ 1.45 (s, 3 H), 1.58 (s, 3 H), 4.45 (s, 1 H), 4.97 (d, 1 H, J = 4.6 Hz), 6.40 (d, d, 1 H, J = 4.7 and 10.8 Hz), 8.01 (m, 4 H), and 9.2 (d, 1 H, J = 10.8 Hz); mass spectrum of dimethyl ester; m/z 477 (M^+ + 1) and appropriate fragment ions.

Products from β-Lactamase-Catalyzed Hydrolysis of (Phenoxymethyl)penicillanic Acid Sulfone. (Phenoxymethyl)penicillanic acid sulfone (21 mM) was incubated with β-lactamase (0.3 μM) in 0.1 M sodium phosphate buffer, pH 7.0, for 30 min at room temperature. A chloroform extract of this mixture yielded only *N*-(phenoxyacetyl)glycinaldehyde, as shown by ^1H NMR ($CDCl_3$) δ 9.67 (s, 1 H), 7.1 (m, 6 H), 4.55 (s, 2 H), and 4.26 (d, 2 H, J = 5.2 Hz). The doublet at 4.26 ppm collapses to a singlet upon the addition of D_2O: IR ($CHCl_3$) 3410, 1730, 1680 cm^{-1}. The residual aqueous layer was freeze-dried, dissolved in D_2O, and freeze-dried again, and the ^1H NMR spectrum of the product (in D_2O) was consistent with the sulfinate of penicillamine: δ 3.99 (s, 1 H), 1.16 (s, 3 H), and 1.08 (s, 3 H).

Methods

Solutions of β-lactams were prepared by weight by using a Cahn 25 electrobalance. Ultraviolet measurements were made in 0.1 M sodium phosphate buffer, pH 7.0 (30 °C), by using a Perkin-Elmer 554 or 575 spectrophotometer. Enzyme activity was assayed by measuring the change in absorbance at 240 nm that accompanies the hydrolysis of the β-lactam ring of benzylpenicillin (Samuni, 1975). Proton release during β-lactam hydrolysis was followed with a Radiometer RTS 822 recording titration system. Amino acid analyses were performed on a Beckman 120 B analyzer. Protein concentration was determined according to Bradford (1976) by using native

[1] Abbreviations used: RTEM specifies the source of the plasmid [see Datta & Kontomichalou (1965)]; TEM-2 specifies the enzyme [see Sutcliffe (1978)].

β-lactamase as the protein standard. (For calibration, a value for $A^{0.1\%}_{280nm}$ of 1.0 was used.)

Time Dependence of Enzyme Inactivation by Penam Sulfones. Samples (1 μL) from an incubation mixture of the enzyme and a penam sulfone (in 0.1 M sodium phosphate buffer, pH 7.0, 30 °C) were mixed with buffered solutions of benzylpenicillin (2.0 mM, 3.0 mL) in a 10-mm optical cuvette. The dilution factor precluded any further significant reaction between enzyme and the sulfone. The hydrolysis of benzylpenicillin showed initial biphasic behavior [analogous to that seen with clavulanic acid (Fisher et al., 1978)], and to ensure that only the extent of irreversible inactivation was monitored, the rate of hydrolysis of benzylpenicillin was measured after the progress curves had become linear (approximately 15 min) and was expressed as a fraction of the enzyme activity from a similar incubation lacking the sulfone. Incubations with penam sulfones contained β-lactamase (1 μM) and methicillin sulfone (12 mM), β-lactamase (2 μM) and quinacillin sulfone (3.2 mM), or β-lactamase (5 μM) and penicillanic acid sulfone (36 mM) in a final volume of 0.5 mL.

Number of Hydrolytic Events before Enzyme Inactivation. Portions of buffered β-lactamase solution (10 μL, 50 μM) were mixed with portions of buffered penam sulfone solution (25–200 μL, 2–20 mM) in 0.1 M sodium phosphate buffer, pH 7.0 (final volume 0.5 mL), at 30 °C. After the reaction was complete (>150 min), the residual enzymatic activity was measured. From the (linear) plot of the initial molar ratio of penam sulfone to enzyme vs. the fraction of catalytic activity remaining, the number of molecules of sulfone required to cause complete enzyme inactivation was determined by extrapolation. For (phenoxymethyl)penicillanic acid sulfone, which does not lead to significant enzyme inactivation, much larger amounts (1.5–4.5 mL, 20 μM) of sulfone were used and longer incubation times were allowed, to provide a lower limit on the number of hydrolytic events before enzyme inactivation.

Isolation of Enzyme Inactivated by Quinacillin Sulfone. A solution of β-lactamase (30 mL, 50 μM) was mixed with a solution of quinacillin sulfone (17 mL, 43 mM) in 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C. Less than 5% of the original catalytic activity remained after 150 min. The protein was dialyzed exhaustively against 10 mM potassium phosphate buffer, pH 7.0, at 4 °C, and the solution was then freeze-dried. The protein was then dissolved in 0.5 M Tris-HCl buffer, pH 8.0 (6 mL), containing guanidinium chloride (6.0 M) and dithiothreitol (5 mM). The solution was purged with N_2 and left at 37 °C for 1 h. To this mixture was then added a buffered solution of iodoacetic acid (1.0 mL, 100 mM), and after 10 min, the reaction was quenched by the addition of 2-mercaptoethanol (600 μL). The protein was then dialyzed exhaustively against 25 mM ammonium bicarbonate, pH 7.8, at 4 °C before being freeze-dried.

Isolation of Labeled Peptide. The freeze-dried carboxymethylated protein was dissolved in 25 mM ammonium bicarbonate, pH 7.8 (4.0 mL), and trypsin (100 μL of 10 mg/mL) was added. The mixture was incubated at 37 °C, and the pH was maintained at 7.8 in a pH-stat. After 4 h, the digestion was complete, and the solution was applied to a column (1.6 × 100 cm) of Sephadex G-50 equilibrated with and eluted with 25 mM ammonium bicarbonate, pH 7.8. The absorbance of column fractions was monitored at 325, 280, and 226 nm. Fractions containing the 325 nm chromophore were pooled and freeze-dried. The major labeled peptide was then further purified on a column (0.9 × 6 cm) of DEAE-Sephacel equilibrated with 25 mM ammonium bicarbonate, pH 7.8, and eluted with a linear gradient (40 mL + 40 mL) of ammonium bicarbonate (25–500 mM). Fractions containing the 325-nm chromophore were pooled and freeze-dried. The concentration of the quinacillin-labeled peptide was estimated approximately from the A_{325nm}, assuming an extinction coefficient at 325 nm of 7200.

Table I: Kinetic Characteristics of Penams and Penam Sulfones with β-Lactamase

penam	k_{cat} of penam (s^{-1})	k_{cat} of sulfone[a] (s^{-1})	$t_{1/2}$ of inactivation by sulfone (min)	number of hydrolytic events before inactivation by sulfone
penicillin V (**1**)	1000	~1500		>250 000[b]
penicillanic acid (**2**)	40	~2[c]	44	~7 000
methicillin (**3**)	7	~80	1.5	10 000
quinacillin (**4**)	2	~10	<1	400

[a] These values are approximate. [b] Benzylpenicillin (penicillin G) sulfone behaves analogously. [c] This apparent exception from the trend of higher k_{cat} values for the hydrolysis of the sulfones derives from the absence of a 6β-acylamino substituent in **2**, which allows a further partioning of the acyl-enzyme in this case to a transiently stable complex of the kind observed with clavulanic acid (Fisher et al., 1978; Charnas et al., 1978) (C. Kemal and D. Brenner, unpublished experiments).

Thin-layer chromatography of peptides was done on normal and fluorescent silica plates (Merck F-254) eluted with 1-propanol–ammonium hydroxide (7:3 v/v). Portions of the purified peptide were hydrolyzed in vacuo with 5.7 N HCl containing phenol (1% v/v) for 24 h at 110 °C before amino acid analysis.

Identification of the Labeled Amino Acid. A portion (40 μL, 250 μM) of the purified labeled peptide in 25 mM ammonium bicarbonate was mixed with chromic acid (20 μL of a solution of 1.75 M chromium trioxide in 3 M H_2SO_4) and glacial acetic acid (40 μL). After 10 min at 100 °C, the solution was cooled, and ethanol (20 μL) was added. After 1 h at 37 °C, the solution was freeze-dried and subjected to acid hydrolysis and amino acid analysis.

Results and Discussion

Kinetics of Enzyme Inactivation by Penam Sulfones. Consideration of the kinetic and chemical characteristics of many of the known inactivators of the β-lactamase [especially the observations of English et al. (1978) and of Cartwright & Coulson (1979)] led us (Fisher et al., 1980a) to suggest that if the three criteria outlined in the introduction were satisfied, a "suicide" or mechanism-based inactivator would result.

For an effective inactivator, the β-elimination process must compete with the normal hydrolysis of the acyl-enzyme (Scheme I), so any factor that slows the hydrolysis of the acyl-enzyme relative to the rate of the β-elimination reaction should increase the efficacy of the inactivator. Since the rate of enzyme-catalyzed hydrolysis of the β-lactam ring of a penam depends on the nature of the substituent(s) at C-6, we chose to investigate the sulfones of both poor and excellent substrates of the enzyme. In Table I are listed the k_{cat} values for one excellent substrate (penicillin V) and three poor substrates (penicillanic acid, methicillin, and quinacillin). When the sulfones of these materials interact with the enzyme, we observe that only those that derive from poorly hydrolyzed penams are efficient inactivating reagents. No significant inactivation of the β-lactamase by the sulfone of **1** is observable

even with the latter in very large molar excess, but as the k_{cat} of the parent penam decreases (through **2**–**4**), the corresponding sulfones become increasingly good inactivators. These data bear out the view that enzyme inactivation derives from the partitioning of a reaction intermediate on the normal enzyme-catalyzed pathway.

Let us consider the reaction of methicillin sulfone in more detail. Incubation of the β-lactamase with a large (>10^4-fold) molar excess of methicillin sulfone results in the loss of catalytic activity with a $t_{1/2}$ of 1.5 min. More than 95% of the enzyme activity is ultimately lost. The rate of inactivation is the same at sulfone concentrations between 2 and 16 mM (at a constant molar ratio of sulfone to enzyme), indicating that the inactivation follows saturation kinetics. Methicillin itself has no inactivating effect on the enzyme, nor do the products of nonenzymatic decomposition of methicillin sulfone (after incubation at pH 7.0, 30 °C, for 48 h). (Analogous results were obtained with **1**, **2**, and **4** and the decomposition products of their sulfones.) It appears, then, that methicillin sulfone is a specific inactivator of the β-lactamase and that penam sulfones constitute a class of β-lactamase inactivators.

The saturating concentrations of sulfone used to demonstrate enzyme inactivation do not allow an estimate of the minimal amount of reagent that is required completely to inactivate the enzyme. Incubation of enzyme with lower levels of sulfone results in partially inactivated β-lactamase. Some 10000 molecules of methicillin sulfone are required to inactivate one molecule of enzyme, demonstrating that in terms of Scheme I the acyl-enzyme partitions 10000-fold toward the penicilloic acid product. Analogous results for the other sulfones studied are included in Table I. These data further indicate that the penam sulfones can be *better* substrates than their unoxidized parents. Thus, for methicillin sulfone, approximately 5000 hydrolytic turnovers occur in 90 s, corresponding to a k_{cat} value of about 80 s^{-1} (allowing for the concomitant loss of enzyme activity). This value is larger than the k_{cat} value for methicillin of 7 s^{-1}, and the difference in susceptibility to the β-lactamase was confirmed by following the hydrolytic cleavage of methicillin and its sulfone in the pH-stat. While the stoichiometry of proton release at pH 7.0 is not known precisely (this arises from uncertainties in the pK_a values of ionizing groups in the products as well as from possible decarboxylation and further hydrolytic reaction of the first-formed penicilloic acid derivatives),[2] it is clear that methicillin sulfone is hydrolyzed faster than is methicillin. On the reasonable basis that two protons are released on hydrolysis of the β-lactam, a k_{cat} value of about 80 s^{-1} for methicillin sulfone and of about 4 s^{-1} for methicillin can be derived. These numbers are in good agreement with the results quoted above and in Table I.

In summary, the kinetic results reported in Table I support the mechanistic notions outlined earlier. The sulfones of poor penam substrates of the β-lactamase are good inactivators of the enzyme, the acyl-enzyme intermediate evidently partitioning more favorably toward the aminoacrylate species from which the inactive enzyme is derived (Scheme I).

Chemical Nature of the Inactive Enzyme. If the inactivation of the β-lactamase results from the β-elimination reaction across C-6 and C-5 shown in Scheme I (the aminoacrylate may form by tautomerism of the first-formed imine to the enamine), then the ultraviolet spectrum of the inactivated enzyme should show the resulting β-aminoacrylic ester chromophore, which has a λ_{max} near 280 nm and a molar extinction coefficient of approximately 18 000. To test this, the enzyme was inactivated with a 600-fold molar excess of quinacillin sulfone and then freed from small molecules by gel filtration. The ultraviolet spectrum of the inactive enzyme is shown in Figure 1, from which it is clear that the enzyme contains a quinoxalinoyl group [λ_{max} 325 nm ($\epsilon \sim 7200$); λ_{max} 245 nm ($\epsilon \sim 30000$)] and has developed an intense new chromophore of λ_{max} 289 nm. (The A_{280nm} of the protein increases by 1.7-fold on inactivation). The attached chromophores are not removed by gel filtration under denaturing conditions in 1% sodium dodecyl sulfate. These results are entirely consistent with the formulation of Scheme I. Moreover, enzyme that has been inactivated by the sulfones of **2** (which is itself transparent at 290 nm) and of **3** shows analogous increases in the absorbance near 290 nm. This unusually strong chromophore at 290 nm is also seen with clavulanic acid (Charnas et al., 1978) and with the sulfone of 6-chloropenicillanic acid (Cartwright & Coulson, 1979). There is little doubt that this chromophore derives from a β-aminoacrylate ester group (Ostercamp, 1970; Bell et al., 1970; Clarke et al., 1949), though the question of whether the participating amino group is that of the original penam or is from the protein (via a transimination reaction) is still moot.

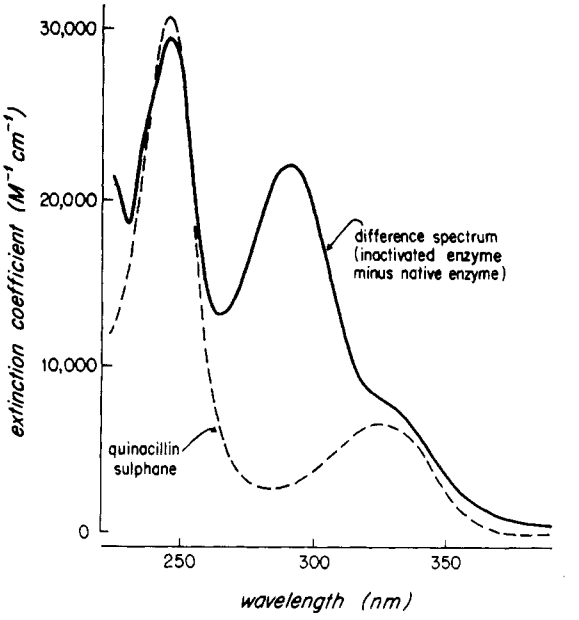


FIGURE 1: Ultraviolet spectrum of quinacillin sulfone (---) and the ultraviolet difference spectrum of enzyme inactivated by quinacillin sulfone minus native enzyme (—).

The stability of the attached quinoxalinoyl group affords the opportunity to discover the site of covalent attachment to the protein. Treatment of the β-lactamase with a 500-fold molar excess of quinacillin sulfone (150 min, 30 °C, pH 7.0) yielded protein that after dialysis had less than 5% residual catalytic activity and an absorbance at 325 nm corresponding to the attachment of 0.98 mol of quinoxalinoyl groups per mol of enzyme. After denaturation and carboxymethylation, the protein was digested with trypsin and subjected to gel filtration on Sephadex G-50. Two labeled peptides, Q1 and Q2, absorbing at 325 nm accounted for ~80% of the chromophore applied to the column. The larger peak, Q1, was further purified by ion-exchange chromatography on DEAE-cellulose, the elution profile for which is shown in Figure 2. The major

[2] The nature of these events is clear from the products resulting from the hydrolysis of penicillin V sulfone by the β-lactamase. These have been identified (see Experimental Procedures) as phenoxyacetylglycinaldehyde and the sulfinate of penicillamine (C. Kemal, unpublished experiments), and they presumably arise by decarboxylation and hydrolysis of the imine, which is formed either directly or via the enamine. Analogous products have been isolated from the chemical hydrolysis of penicillin G (Clarke et al., 1949).

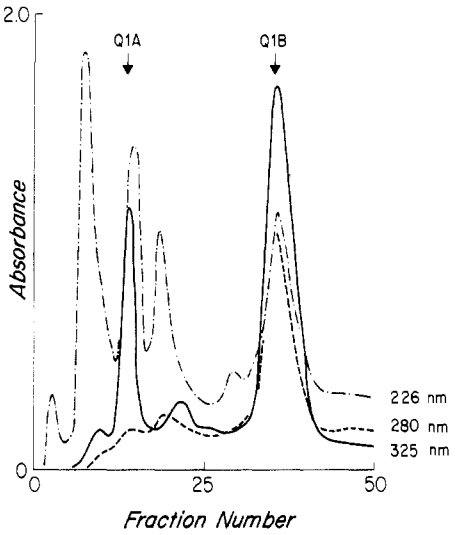


FIGURE 2: Elution profile of the major chromophoric peptide, Q1, from a column of DEAE-cellulose. For details, see Experimental Procedures. A_{226nm} (—·—); A_{280nm} (- - -); A_{325nm} (—). The trace for A_{325nm} has been scale-expanded 5-fold.

Table II: Amino Acid Composition of the Labeled Peptide from β-Lactamase Inactivated with Quinacillin Sulfone

amino acid	mol of amino acid/mol of peptide	
	observed[a,b]	expected[c]
Ser	1.2 ± 0.2	1
Thr	1.2 ± 0.2	1
Pro	0.8 ± 0.1	1
Met	1.8 ± 0.1	2
Phe	1.8 ± 0.1	2
Lys	1.1 ± 0.2	1

[a] No other amino acid was present at a level higher than 0.25, except for Asp at 0.39. [b] Average of two analyses. [c] On the basis of the sequence data of Ambler & Scott (1978) and Sutcliffe (1978). See the text.

component, Q1B (fractions 35–40), was collected and shown by thin-layer chromatography on silica gel to contain two ninhydrin-positive materials in 5:1 ratio, the predominant one of which contained the quinoxalinoyl group as shown by the quenching of fluorescent indicator. When the freeze-dried peptide Q1B was rechromatographed, most of the 325-nm chromophore now eluted as Q1A (see Figure 2), and it is evident that Q1A is a decomposition product of Q1B lacking the aminoacrylate chromophore at 290 nm. Analogously, it was shown that Q2 is a decomposition product of Q1, from which the aminoacrylate group has been lost. Inspection of Scheme I rationalizes these observations since hydrolytic loss of the sulfinate of penicillamine (presumably via the imine tautomer of the enamine) will result in the observed changes in chromatographic behavior: Q1 hydrolyzes to Q2 (≡Q1A), and Q1B hydrolyzes to Q1A. This description of events is consistent with the observed products from the hydrolysis of penam sulfones by the β-lactamase. It is clear, then, that quinacillin sulfone labels a single locus of the β-lactamase.

On amino acid analysis of Q1B (or of Q1A deriving from it), the composition shown in Table II is obtained. The peptide can be uniquely located in the known sequence of the RTEM β-lactamase (Ambler & Scott, 1978; Sutcliffe, 1978): the protein has nine Met and five Phe, and only two tryptic peptides contain both residues. The sequence corresponding to the composition of Q1B is

Phe-Pro-Met-Met-Ser-Thr-Phe-Lys

comprising residues 66–73 [numbering of Ambler (1979)].

Table III: Effect of Chromate Oxidation of Labeled Peptide Q1B on the Serine and Threonine Content

peptide	Phe[a]	Ser[a]	Thr[a]	Thr/Ser
before oxidation	14.9 (2.0)	9.9 (1.3)	9.2 (1.2)	0.93
after oxidation[b,c]	7.3 (2.0)	4.1 (1.1)	0.9 (0.2)	0.22

[a] The first number is the amount in nmol; the number in parentheses is the mol of residue/mol of peptide. [b] Purified labeled peptide (10 μM) was treated with chromic acid (0.7 M, 10 min, 100 °C) and then with ethanol (3 M, 10 min, 100 °C) to destroy the excess chromate. The peptide was then hydrolyzed in acid prior to amino acid analysis. [c] The amount of Phe provides a measure of the amount of peptide analyzed.

Confirmation of this assignment was obtained from digests of Q1B with aminopeptidase M and carboxypeptidase B, which identified N-terminal Phe and C-terminal Lys, respectively. The most likely sites for attachment of the label are Ser or Thr in this peptide. Distinction between these sites was not trivial since neither aminopeptidase M nor a mixture of carboxypeptidase A and B cleaved the Ser–Thr link (cf. Knott-Hunziker et al., 1979b). Accordingly, the peptide was oxidized with chromic acid, which will oxidize the unmodified Ser or Thr side-chain alcohols (e.g., Roček, 1960) but will not affect the esterified group. Table III shows that in the labeled peptide the serine residue is protected from chromate oxidation, thus identifying Ser-70 as the site of modification by quinacillin sulfone.

According to the chemical pathway outlined in Scheme I, the amino acid residue labeled by quinacillin sulfone will be that which is directly involved in the formation of the acyl-enzyme. This is evidently Ser-70. There has been considerable recent focus on this serine residue, and Knott-Hunziker et al. (1979a,b) first identified the homologous serine residue (Ser-70) of the *B. cereus* I β-lactamase as the site of attachment of 6β-bromopenicillanic acid. This finding has recently been confirmed by Cohen & Pratt (1980). Further, Cartwright & Coulson (1980) have found that the *Staphylococcal* enzyme is labeled by 6-chloropenicillanic acid sulfone on either the homologous serine or the adjacent threonine residue. In the case of the 6β-bromopenam, mechanistic considerations also strongly suggested that this serine residue was the enzyme nucleophile responsible for the initial formation of acyl-enzyme. It is gratifying, therefore, that there is now persuasive evidence (Fisher et al., 1980b) that the β-lactamase does proceed, in its normal catalytic reaction, via the intermediacy of an acyl-enzyme intermediate. Further, we have recently shown (R. L. Charnas, unpublished results) that the same tryptic peptide of the RTEM β-lactamase is labeled by the substrate [^{14}C]cefoxitin when the hydrolytic reaction is quenched from the steady state. The agreement of all these experiments on substrates and inhibitors of β-lactamases from different sources puts β-lactamase firmly into the class of the "serine" amidohydrolases.

Finally, the finding that the D-Ala-D-Ala-carboxypeptidases from *B. subtilis* and *B. stearothermophilus* are also labeled on a serine that is part of a sequence which is reasonably homologous with the N-terminal sequence of the β-lactamases (Yocum et al., 1979; Waxman & Strominger, 1980) suggests further mechanistic analogy and the possible ancestral relationship between the β-lactamases and the carboxypeptidases that process the peptides of the bacterial cell wall.

Acknowledgments

We are especially grateful to Boots, Ltd., and Pfizer, Inc., for gifts of compounds, to Dr. Cemal Kemal for the analysis of the products of hydrolysis of penicillin V sulfone, and to

Merck, Sharp & Dohme for support.

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