Penicillanic Acid Sulfone: Nature of Irreversible Inactivation of RTEM β-Lactamase from *Escherichia coli*†

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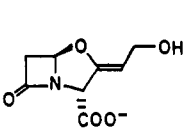
ABSTRACT: When penicillanic acid sulfone in large molar excess is incubated with the RTEM β-lactamase, the enzyme becomes inactivated irreversibly. From studies of the consequential spectroscopic changes, from the use of specifically tritiated penicillanic acid sulfone, and from comparison by isoelectric focusing of the enzyme after inactivation by the sulfone and by clavulanic acid, the inactivated enzyme appears to be cross-linked by a β-aminoacrylate fragment deriving from C-5, C-6, and C-7 of the original β-lactam. Model studies on the behavior of alcoholic solutions of penicillanic acid sulfone in the presence of amines are entirely consistent with this interpretation.

Since the discovery in 1976 of the naturally occurring β-lactamase inactivator clavulanic acid, **1** (Brown et al., 1976),



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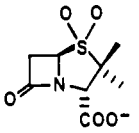

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a variety of other compounds have been found that also incapacitate this enzyme. These include the 6β-halopenams, the penam sulfones (e.g., **2**), the carbapenems, and the 6-(sulfenimino)penams [see Knowles (1983)]. On the basis of chemical and kinetic studies (Fisher et al., 1978; Charnas et al., 1979; Pratt & Loosemore, 1978; Knott-Hunziker et al., 1970; Cartwright & Coulson, 1979; Fisher & Knowles, 1980; Brenner & Knowles, 1981; Kemal & Knowles, 1981), many of these materials (clavulanic acid, the halopenams, and the penam sulfones) can be grouped economically into a single class of β-lactamase inactivator. The members of this class are characterized by a heteroatom in the 1-position that may act as a leaving group subsequent to acylation of the enzyme and cleavage of the β-lactam ring. This first-formed acyl-enzyme is a β-imino ester and has three fates available to it (Figure 1). First, it may undergo deacylation, completing the hydrolysis reaction. Second, there may be a tautomerization of the imine to an enamine, generating a β-aminoacrylate ester, which results in transient inhibition of the enzyme. Third, the acyl-enzyme may undergo other chemistry that leads to irreversible inactivation of the β-lactamase. In the case of 6β-bromopenicillanic acid, the inactivated enzyme is formed by the intramolecular attack of the liberated thiolate group upon C-6, displacing bromide ion to form a dihydrothiazine chromophore attached to the active site serine residue (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b, 1980; Loosemore et al., 1980; Cohen & Pratt, 1980; Orlek et al., 1979). Clavulanic acid, on the other hand, gives three species of inactivated protein that are distinguishable by isoelectric focusing (Charnas et al., 1978). The precise structure of each of these inactivated forms is, however, not known.

The mechanism of the interaction of clavulanic acid (**1**) with the β-lactamase has been clarified by analogous studies on the reaction of penicillanic acid sulfone (**2**) with the enzyme. Mechanistic investigations involving the sulfone have permitted the formulation of the inhibition and inactivation pathways described above, which are consistent with what is known about the behavior of clavulanic acid with the enzyme. The nature of the inactive forms of the β-lactamase formed after long incubations with penicillanic acid sulfone or with clavulanic acid remains, however, to be explored. Because the chemistry of penicillanic acid sulfone (**2**) is evidently simpler than that of clavulanic acid (**1**) and since the sulfone is more readily obtained through semisynthesis, an investigation of the inactivation process with this molecule has been made.

Three approaches have been taken to probe the chemical events that result in irreversible inactivation of the β-lactamase. First, specifically tritiated penicillanic acid sulfone has been used to evaluate which parts of the inhibitor molecule are attached to the inactivated protein. Second, the inactivated enzyme has been analyzed by isoelectric focusing, which is particularly sensitive to small changes in the ionization behavior of proteins. Third, attempts have been made to identify the enzymic residue(s) involved in the covalent modification of the protein.

Experimental Procedures

Materials

Sodium clavulanate was the generous gift of Beecham Pharmaceuticals. D_2O (99.8%) was from Bio-Rad Laboratories. ^3H_2O (5 Ci/mL) and sodium boro[^{3}H]hydride (0.55 Ci/mmol) were from Amersham. Bio-Solv was from Beckman Instruments, Liquifluor from New England Nuclear, and scintillation-grade toluene from Fisher Scientific. Constant-boiling HCl (5.7 N) was from Pierce Chemicals. Ampholine PAGplates (pH range 4–6.5) were obtained from LKB Instruments.

6α-Bromopenicillanic acid benzyl ester was prepared by the esterification of 6α-bromopenicillanic acid (Cignarella et al., 1962) (9.34 g, 33.4 mmol) with excess phenyldiazomethane in ether. The crude product (12 g, 98%) was chromatographed on silica gel by eluting with hexane–ethyl acetate (4:1 v/v) to obtain the ester as an oil: R_f (silica) 0.46 (hexane–ethyl acetate, 4:1 v/v); ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 3 H), 1.58 (s, 3 H), 4.56 (s, 1 H), 4.79 (d, 1 H, J = 1.4 Hz), 5.18 (s, 2 H), 5.40 (d, 1 H, J = 1.4 Hz), 7.36 (s, 5 H); mass spectrum, m/z 371 ($M^+ + 2$), 369 (M^+), 250, 91.

Penicillanic acid benzyl ester was prepared by the hydrogenolysis of 6α-bromopenicillanic acid benzyl ester (12 g, 32.5 mmol) in methanol (200 mL) with palladium on calcium carbonate (5% w/w, 12 g) under atmospheric pressure for 6

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 19, 1984.* This work was supported by the National Institutes of Health.

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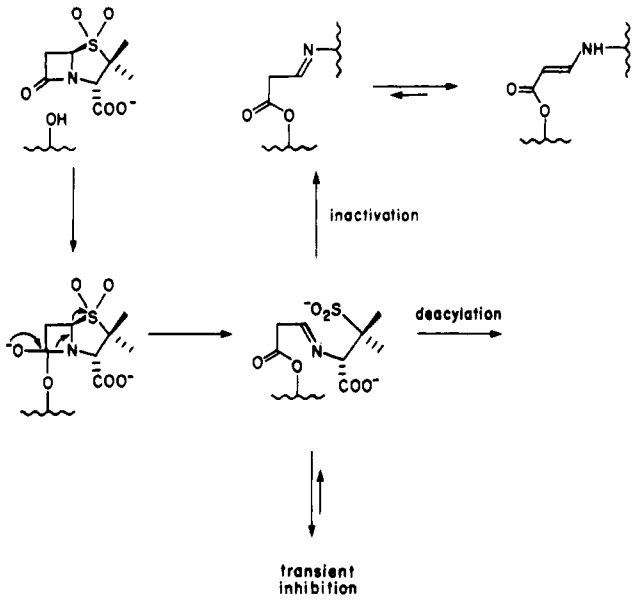


FIGURE 1: Proposed pathway for the irreversible inactivation of the β-lactamase by penicillanic acid sulfone. (Although the collapse of the first-formed tetrahedral intermediate to the β-imino ester is shown as a single event, this transformation most likely would occur in two steps.)

h at 25 °C. The mixture was filtered through Celite, and the Celite was washed well with methanol. The solvent was then removed from the combined filtrates by evaporation to give an oil (7.45 g, 80%). The crude product was then chromatographed on silica gel by eluting with hexane–ethyl acetate (4:1 v/v) to give the ester as an oil: R_f (silica) 0.31 (hexane–ethyl acetate, 4:1 v/v); IR (film) ν 1778 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.39 (s, 3 H), 1.63 (s, 3 H), 3.02 (dd, 1 H, J = 1.9 and 15.8 Hz), 3.55 (dd, 1 H, J = 4.1 and 15.8 Hz), 4.48 (s, 1 H), 5.17 (s, 2 H), 5.27 (dd, 1 H, J = 1.9 and 4.1 Hz), 7.35 (s, 5 H); mass spectrum, m/z 291 (M^+), 263, 250, 200, 91.

Penicillanic acid (S)-sulfoxide benzyl ester was synthesized from the benzyl ester of penicillanic acid (5.62 g, 19.3 mmol) by oxidation in CH_2Cl_2 (100 mL) at 25 °C with *m*-chloroperbenzoic acid (1 equiv). The reaction was monitored by thin-layer chromatography (TLC) since excess oxidant will react further with the sulfoxide to give the corresponding sulfone. When the reaction was complete, the solution was washed sequentially with 0.1 M aqueous sodium bisulfite, aqueous sodium bicarbonate (5% w/v), water, and brine. After the solution was dried over $MgSO_4$, the solvent was removed by evaporation to give the sulfoxide as a solid (5.68 g, 96%): R_f (silica) 0.26 (CH_2Cl_2–ether, 5:1 v/v); mp 115–117 °C (as white plates, from CCl_4); IR (Nujol) ν 1775, 1730, 1201, 1048, and 762 cm^{-1}; ^1H NMR (C_6D_6) δ 0.59 (s, 3 H), 1.32 (s, 3 H), 2.39 (dd, 1 H, J = 4.6 and 15.8 Hz), 2.98 (dd, 1 H, J = 2.0 and 15.8 Hz), 3.78 (dd, 1 H, J = 2.0 and 4.6 Hz), 4.64 (s, 1 H), 4.65 (d, 1 H, J = 12.1 Hz), 4.92 (d, 1 H, J = 12.1 Hz), 7.06 (s, 5 H); ^{13}C NMR ($CDCl_3$) δ 17.96 (q, J = 129.4 Hz), 19.73 (q, J = 129.3 Hz), 35.57 (t, J = 144.6 Hz), 65.16 (d, J = 146.4 Hz), 67.34 (t, J = 145.0 Hz), 70.48 (d, J = 170.2 Hz), 73.39 (s), 128.36 (d, J = 162.2 Hz), 134.47 (s), 167.78 (s), 170.47 (s); mass spectrum, m/z 307 (M^+), 291, 265, 263, 168, 91.

[10-^2H]Penicillanic acid (S)-sulfoxide benzyl ester was prepared by refluxing the unlabeled sulfoxide (0.036 g, 0.12 mmol) in benzene (10 mL) containing D_2O (99.8%, 0.5 mL) under argon for 48 h. The labeled sulfoxide was isolated quantitatively after evaporation of the solvent. The ^1H NMR of this material was identical with that described for the unlabeled material except that the signal from the downfield geminal methyl group (at 1.32 ppm in C_6D_6) was diminished in its intensity. The mass spectrum showed that the material was 43.2% d_3, 39.5% d_2, 10.9% d_1, and 6.4% d_0. This sample of the sulfoxide contained 2.2 atoms of ^2H per molecule: mass spectrum, m/z 310, 309, 308, 307, 294, 268, 91.

Penicillanic acid sulfone benzyl ester was prepared from the corresponding sulfoxide (0.10 g, 0.33 mmol) by oxidation with *m*-chloroperbenzoic acid (2 equiv) in benzene (20 mL) at 25 °C. No starting material remained after 1 h as judged by TLC. The product was isolated as described for the sulfoxide (above) to give the sulfone as a clear oil (0.10 g, 95%): R_f (silica) 0.62 (hexane–ethyl acetate, 1:1 v/v); IR (Nujol) ν 1780, 1745, 1495, 1330, and 1120 cm^{-1}; ^1H NMR (C_6D_6) δ 0.98 (s, 1 H), 1.16 (s, 1 H), 2.24 (dd, 1 H, J = 4.5 and 16.2 Hz), 2.82 (dd, 1 H, J = 1.9 and 16.2 Hz), 3.62 (dd, 1 H, J = 1.9 and 4.5 Hz), 4.34 (s, 1 H), 4.63 (d, 1 H, J = 12.0 Hz), 4.85 (d, 1 H, J = 12.0 Hz), 7.04 (s, 5 H); ^{13}C NMR ($CDCl_3$) δ 18.22 (q, J = 130.3 Hz), 19.75 (q, J = 130.4 Hz), 37.96 (t, J = 146.0 Hz), 60.82 (d, J = 174.8 Hz), 62.43 (s), 62.82 (d, J : 146.2 Hz), 67.72 (t, J = 147.5 Hz), 128.52 (d, J = 159.6 Hz), 134.27 (s), 166.55 (s), 170.69 (s); mass spectrum, m/z 323 (M^+), 259, 91.

Penicillanic acid sulfone was prepared from the corresponding benzyl ester (0.05 g, 0.15 mmol) by catalytic hydrogenolysis in methanol (10 mL) with activated palladium on carbon (10% w/w, 0.05 g) under H_2 (50 psi) for 45 min at 25 °C. After removing the catalyst by filtration through Celite, the product was extracted into cold aqueous sodium bicarbonate (2% w/v). The aqueous extract was washed twice with ethyl acetate and then acidified to pH 2 with concentrated HCl at 0 °C, before extraction of the free acid into ethyl acetate. The combined organic extracts were washed with brine and then dried over $MgSO_4$. The solvent was removed under reduced pressure to give the free acid of the sulfone as a crystalline solid (0.021 g, 60%). After recrystallization from ethyl acetate–hexane, the product showed spectroscopic and physical properties identical with those for an authentic sample of penicillanic acid sulfone (Brenner & Knowles, 1981).

[10-^3H]Penicillanic acid sulfone was synthesized from the benzyl ester of penicillanic acid (*S*)-sulfoxide (1 g, 3.3 mmol). The sulfoxide was dissolved in benzene (100 mL) containing ^3H_2O (5 Ci, 1 mL) and sealed in a heavy-walled tube. The tube was heated to 100 °C for 50 h, then cooled, and opened. The contents of the tube were transferred via canula to a round-bottom flask, leaving behind most of the water (0.8 mL). The water remaining in the benzene solution was removed by azeotropic distillation. TLC on silica (CH_2Cl_2–ether, 5:1 v/v) of the organic solution showed that the major component of the sample (R_f 0.26, 90%) comigrated with the unlabeled starting material. The benzene solution of the labeled sulfoxide was oxidized as described for the unlabeled sulfoxide, and the sulfone was isolated as an oil (0.81 g, 75%). The product of this reaction comigrated on TLC with a sample of penicillanic acid sulfone benzyl ester. The benzyl ester group of the labeled sulfone (0.7 g, 2.2 mmol) was cleaved by catalytic hydrogenolysis as described for the unlabeled compound above, to give the tritiated sulfone as a crystalline solid (0.26 g, 51%). This material was recrystallized to constant specific radioactivity (0.14 Ci/mmol).

Acetaldehyde 2,4-dinitrophenylhydrazone was prepared from penicillanic acid sulfone as follows. The sulfone (9.1 mg, 0.04 mmol) was dissolved in 0.5 N NaOH (0.5 mL) at 25 °C. After 5 min, the solution was added to a mixture of 2,4-dinitrophenylhydrazine (80%, 10.6 mg, 0.04 mmol) and ethanol

(2.0 mL) in acetic acid (2.0 mL), and the resulting solution was heated to boiling for 1 min. The solution was cooled and the product extracted into CCl_4. The combined organic extracts were washed successively with water, aqueous sodium bicarbonate (5% w/v), and brine and then dried over $MgSO_4$. The solvent was removed by evaporation to give the hydrazone as a yellow solid (9.0 mg, 94%) that was spectroscopically identical with an authentic sample of the hydrazone: R_f (silica) 0.69 (hexane–ethyl acetate, 1:1 v/v); mp 164–165 °C [needles, from ethanol; Roberts & Green (1946) give mp 164–164.5 °C]; ^{1}H NMR ($CDCl_3$) δ 2.14 (d, 3 H, J = 5.4 Hz), 7.56 (q, 1 H, J = 5.4 Hz), 7.92 (d, 1 H, J = 9.4 Hz), 8.31 (dd, 1 H, J = 2.5 and 9.4 Hz), 9.11 (d, 1 H, J = 2.5 Hz).

β-(Diethylamino)acrylic acid methyl ester was prepared from penicillanic acid sulfone as follows. The sulfone (0.25 g, 1.1 mmol) was dissolved in methanol (5 mL) containing potassium bicarbonate (0.11 g, 1.1 mmol) and diethylamine (0.33 mL, 3.2 mmol) at 25 °C. After 4 h, the solution was diluted with water (10 mL) and extracted into ethyl acetate. The combined organic extracts were washed with aqueous sodium bicarbonate (5% w/v) and brine and then dried over $MgSO_4$. The solvent was removed by evaporation, and the product was obtained as a clear oil (0.09 g, 56%): R_f (silica) 0.69 (ethyl acetate–ethanol, 95:5 v/v); UV (methanol) λ_{max} 280 nm (ϵ 30000 M^{-1} cm^{-1}); IR (film) ν 2985, 1688, and 1615 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.12 (t, 6 H, J = 7.2 Hz), 3.16 (q, 4 H, J = 7.2 Hz), 3.62 (s, 3 H), 4.54 (d, 1 H, J = 13.1 Hz), 7.41 (d, 1 H, J = 13.1 Hz); ^{13}C NMR ($CDCl_3$) δ 13.73 (br), 45.35 (br), 50.15, (q, J = 146.4 Hz), 82.88 (d, J = 159.2 Hz), 150.76 (d, J = 164.7 Hz), 170.11 (s); mass spectrum, m/z 157 (M^+), 142, 126.

β-Lactamase was from *Escherichia coli* W3110 carrying the RP4 plasmid (Matthews & Hedges, 1976). The TEM-2 enzyme[1] was purified essentially as described earlier (Fisher et al., 1980) and had a specific activity of 4300 units/A_{280nm}. A unit of activity is that amount of enzyme that will catalyze the hydrolysis of 1 μmol of benzylpenicillin/min at 30 °C in 100 mM potassium phosphate buffer, pH 7.0. The enzyme was >95% homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Methods

Ultraviolet measurements were made on a Perkin-Elmer 554 instrument. Infrared measurements were made on a Perkin-Elmer 457 A spectrometer. NMR spectra were recorded on a Jeol FX-270 spectrometer; chemical shifts are reported relative to tetramethylsilane. Mass spectra were measured on an AEI MS-9 instrument. Radioactivity measurements were made on a Beckman LS 233 scintillation counter, in a scintillation cocktail composed of Bio-Solv–Liquifluor–toluene (10:5:85 by volume). Amino acid analyses were performed on a Beckman 121-M analyzer. Analytical thin-layer chromatography was performed with silica gel 60 glass plates (E. Merck) containing fluorescent indicator. Column chromatography was done as described by Still et al. (1978) with silica gel 60 (0.040–0.063 nm, from E. Merck).

Localization of Tritium in Penicillanic Acid Sulfone. A sample of the tritiated sulfone (25 μg, 0.11 μmol) was dissolved in 0.5 N NaOH (0.5 mL) at 30 °C. After 5 min, a solution of 2,4-dinitrophenylhydrazine (80%, 37.9 μg, 0.15 μmol) and ethanol (2.0 mL) in acetic acid (2.0 mL) was added, the resulting solution was vortexed for 5 min, and a portion (0.1 mL) was removed for radioactivity measurement. The remainder of the solution was boiled for 1 min (to effect decarboxylation), and another portion (0.1 mL) was removed for counting. The solvent from the remaining sample was evaporated under reduced pressure, water was added, and evaporation and addition of water were repeated once more. After removal of the solvent a third time, the residue was redissolved in a solution of ethanol (2.0 mL), acetic acid (2.0 mL), and water (0.3 mL), from which a portion (0.1 mL) was reserved for counting. The rest of the solution was then diluted with water and extracted into CCl_4. The 2,4-dinitrophenylhydrazone of acetaldehyde (which comigrated with an authentic sample on TLC) was isolated as described under Materials, and the entire sample was then measured for radioactivity.

Enzyme Inactivation. Tritiated penicillanic acid sulfone was used to inactivate the β-lactamase. The labeled sulfone (21 mM) in 0.1 M potassium phosphate (0.4 mL), adjusted to pH 7.0 with 0.5 M dibasic potassium phosphate, was equilibrated at 30 °C, before addition of the enzyme (77 μL of a 14 μM solution). During the incubation, the pH was maintained at 7 by the addition of 0.1 M dibasic potassium phosphate. After 12 h, less than 5% of the original enzyme activity remained. The sample was subjected to gel filtration at 4 °C on a column (1.0 × 36 cm) of Sephadex G-50 in 0.1 M potassium phosphate buffer, pH 7.0. Fractions were assayed for catalytic activity toward benzyl penicillin, for protein absorbance at 280 nm, and for radioactivity. The fractions containing the protein were pooled and dialyzed exhaustively at 4 °C against 0.1 M potassium phosphate buffer, pH 7.0. No recovery of enzymatic activity was observed after these manipulations. Portions of the sample were then measured for radioactivity. The recovery of protein following gel filtration and dialysis was checked by amino acid analysis. Samples were freeze-dried and then redissolved in 5.7 N HCl (containing phenol, 0.1% w/v); the solution was sealed in vacuo and heated at 110 °C for 24 h, prior to amino acid analysis. With native enzyme as a control, these experiments showed that the recovery of protein was >95%. Similar incubations were performed with unlabeled penicillanic acid sulfone to prepare protein samples for isoelectric focusing.

The β-lactamase (1 μM) was also inactivated with clavulanic acid (200 μM) in 0.1 M potassium phosphate buffer (1.0 mL), pH 7.0. After incubation for 3 h, when less than 2% of the initial enzymatic activity remained, the sample was dialyzed against 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.0, in preparation for isoelectric focusing.

Isoelectric Focusing of β-Lactamase. Samples (0.1–1.0 mL) of enzyme solution were prepared for isoelectric focusing by dialysis against Tris-HCl (2–10 mM) buffer, pH 7.0. The solution was then freeze-dried and the protein redissolved in a minimal amount of water (typically 80 μL). Commercial Ampholine PAGplates (pH range 4–6.5) were used and stained with Coomassie Brilliant Blue R-250 according to the manufacturer's instructions.

Attempted Radiolabeling of β-Lactamase Inactivated by Penicillanic Acid Sulfone. Protein that had been inactivated by penicillanic acid sulfone and shown to be >90% homogeneous by isoelectric focusing was used in these experiments. Inactivated β-lactamase (4 μM) was dissolved at 30 °C in 0.1 M sodium phosphate buffer, pH 7.0 (2.0 mL). A portion (20 μL) of a 0.02 N NaOH solution containing sodium boro-[^{3}H]hydride (0.55 Ci/mmol, 2 mM) was added, and further equal portions were added after 1, 3, 6, 15, and 18 h. The resulting solution was vortexed for 5 min with an equal volume

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

(2.1 mL) of 100 mM sodium phosphate buffer, pH 7.0, containing sodium dodecyl sulfate (40 mM) and applied to a column (1.0 × 36 cm) of Sephadex G-50 equilibrated at 25 °C with the same buffer containing sodium dodecyl sulfate at 20 mM. Fractions containing the protein were pooled and dialyzed for 48 h against several changes of the denaturing buffer used for gel filtration and then measured for radioactivity. Similar experiments were run in the absence of sodium dodecyl sulfate, and the samples were subjected after gel filtration and dialysis to acid hydrolysis for amino acid analysis. These samples were run either through the analyzer normally (to obtain a ninhydrin trace) or through just the ion-exchange column, after which fractions were collected for radioactivity measurement.

In another set of experiments, the inactivated enzyme (100 μL of a 22 μM solution) was added to a solution (150 μL) of 100 mM sodium phosphate buffer, pH 5.0, containing sodium dodecyl sulfate (40 μM), sodium cyanoborohydride (20 μM), and ^3H_2O (5 Ci/mL, 10 μL). Additional portions (10 μL) of a methanolic solution of sodium cyanoborohydride (0.25 mM) were added after 0.5, 1, and 2 h. The whole sample was then passed through a gel filtration column and dialyzed under denaturing conditions as described above except that the pH was maintained at 5.0. Column fractions were assayed for radioactivity and for protein absorbance at 280 nm.

Results and Discussion

On the basis of our earlier work (Brenner & Knowles, 1981; Kemal & Knowles, 1981), a chemical structure was tentatively proposed for the β-lactamase inactivated by penicillanic acid sulfone. The existence in the inactive protein of a chromophore of λ_{max} 280 nm and ϵ 16 000 suggested a β-aminoacrylate structure that could result from transimination of the acyl-enzyme imine with an enzymic lysine residue, followed by tautomerization to the conjugated system (Figure 1). This is, however, only one of a number of possibilities. One of these, for example, is that the first-formed acyl-enzyme can tautomerize to give cis and trans forms of the β-aminoacrylate and that one of the isomers is the transiently inhibited form while the other is the irreversibly inactivated species. While in simple cases it is possible to distinguish cis and trans isomers on the basis of their absorption maxima (Shad, 1955), the perturbation of the chromophore at the active site by the surrounding protein makes such an analysis dangerous in this situation.

A distinction between these and several other mechanistic possibilities is possible, however, since in the transimination pathway enzyme inactivation occurs with the loss of the portion of the molecule corresponding to the sulfinic acid of penicillamine (see Figure 1). For the isomerization pathway, in contrast, the inactivated enzyme would contain the whole penam skeleton. In principle, it should be possible to determine the amount of the penicillamine sulfinate formed relative to malonsemialdehyde, the other decomposition product from the substrate. The large (>7000) molar excess of substrate over enzyme needed to ensure the inactivation of the β-lactamase, however, makes this impractical. It was therefore necessary to label one of the two parts of the molecule with a radioactive tracer and to follow the fate of the label. On the basis of the proposed nature of the inactivation process (Figure 1) and on the basis of the previous chemical and enzymatic studies of sulfone inactivators deuterated at C-6, it seemed unlikely that a tritium label would be stable in this position. Without resorting to total synthesis or to biosynthesis of the ^{14}C-labeled penam, there was one other convenient position for the introduction of a tritium label into penicillanic acid sulfone: one of the geminal methyl groups.

Penam sulfoxides can be labeled in one of the geminal methyl groups by exchange of the methyl hydrogens with solvent protons according to the reversible sulfoxide–sulfenate rearrangement (Morin et al., 1963; Cooper, 1970). The stereospecific rearrangement incorporates solvent label into the methyl group that is *cis* to the sulfoxide oxygen. Since the methyl group is torsionally symmetric, all of its three protons may be exchanged (Usher et al., 1975; Meeschaert et al., 1976). Since the β-sulfoxide of penicillanic acid is a precursor of the sulfone, the benzyl ester of this compound was subjected to the conditions for the sulfoxide–sulfenate rearrangement in benzene containing D_2O. The recovered penam sulfoxide benzyl ester was found to contain 2.2 atoms of deuterium per molecule, exclusively located in the β-methyl group at C-2. No deuterium incorporation occurred when the esters of either penicillanic acid or of penicillanic acid sulfone were similarly treated.

Under the conditions determined for the deuteration, the benzyl ester of penicillanic acid β-sulfoxide was tritiated. The ^3H-labeled sulfoxide was oxidized to the sulfone with *m*-chloroperbenzoic acid and then hydrogenolyzed to the free acid, which was recrystallized to constant specific radioactivity (0.14 Ci/mmol). To establish that the tritium label was indeed localized in the penicillamine sulfinate portion of the molecule, the tritiated sulfone was treated with strong base, which effects a quantitative conversion to the sulfinate of penicillaminoacrylate (Kemal & Knowles, 1981). This material was then treated with 2,4-dinitrophenylhydrazine to form the phenylhydrazone of acetaldehyde (which derives from the decarboxylation of the hydrazone of malonsemialdehyde). The phenylhydrazone of acetaldehyde was isolated by extraction into carbon tetrachloride. Control experiments showed that the recovery of the phenylhydrazone was quantitative, on the basis of the amount of sulfone used. The acetaldehyde phenylhydrazone obtained from the tritiated sulfone contained *less than 0.3%* of the total radioactivity of the sample. No volatile radioactivity was detected at any stage of the chemical degradation. These experiments, taken with the deuteration results, are consistent with the tritium being confined exclusively to the penicillamine sulfinate portion of the penam sulfone, as expected.

The tritiated sulfone was then used to inactivate the β-lactamase. When the enzyme was incubated with a 7000-fold molar excess of the labeled sulfone for 12 h (Brenner & Knowles, 1981) and the completely inactivated protein was isolated free from excess reagent and its decomposition products, *less than 6 mol %* of label was found per mol of protein. The recovery of protein after inactivation and reisolation was checked by amino acid analysis. This result demonstrates that *the inactivated enzyme does not contain the penicillamine portion of the original sulfone* and is consistent with the transimination process proposed in Figure 1. It may be noted, in contrast, that when the *Bacillus cereus* I β-lactamase is inactivated with similarly tritiated 6β-[[(trifluoromethyl)sulfonyl]amido]penicillanic acid sulfone, the inactivated enzyme retains 0.82 mol of label/mol (Mezes et al., 1982).

The β-lactamase can be analzyed by isoelectric focusing on nondenaturing polyacrylamide gels in the pH range between 4 and 6.5. When the enzyme is completely inactivated by penicillanic acid sulfone by incubation with a 7000-fold molar excess of the sulfone at 30 °C, a multitude of bands is seen on the gel (Figure 2, lane b). These bands are all more acidic than that of the native protein. By using less than the

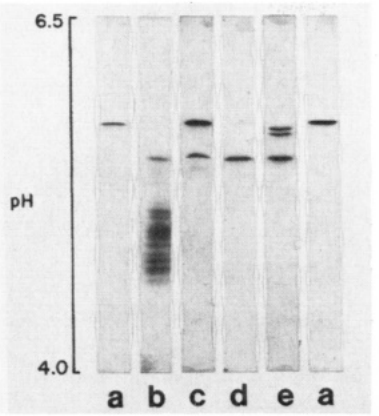


FIGURE 2: Isoelectric focusing gels of (a) native β-lactamase and β-lactamase treated with (b) an 8000-fold molar excess of penicillanic acid sulfone at 25 °C for 8 h, (c) an 800-fold molar excess of sulfone at 25 °C for 8 h, (d) a 3000-fold molar excess of sulfone at 4 °C in a stepwise fashion (see text), and (e) a 200-fold molar excess of clavulanic acid at 25 °C for 5 h.

7000-fold molar excess of substrate that is necessary to inactivate the protein completely (Figure 2, lane c) or by lowering the temperature of the incubation to 4 °C, the protein focuses predominantly as one band having a p*I* somewhat lower than that of the native protein. The origin of the multitude of bands observed from inactivation reactions at 30 °C seems likely to derive from the relatively large amount of malonsemialdehyde oligomers formed from enzyme-catalyzed hydrolysis of the sulfone (Brenner & Knowles, 1981), which may react nonspecifically with the protein in a way similar to glutaraldehyde (Richards & Knowles, 1968). The distribution of the bands (Figure 2, lane b) is consistent with many protein species that differ in total charge depending upon the number of surface lysine residues that have reacted with malonsemialdehyde oligomers.

It could be argued that the reaction of the enzyme with malonsemialdehyde (or compounds derived therefrom) is responsible for the observed time-dependent inactivation. This possibility has been ruled out by incubation of the enzyme with a large excess of malonsemialdehyde [prepared synthetically from ethyl β,β-diethoxypropionate (Den et al., 1959) or from the chemical hydrolysis of penicillanic acid sulfone]. No change in the catalytic activity of the enzyme was detected, even in the presence of a 10000-fold molar excess of malonsemialdehyde. Moreover, inactivation by malonsemialdehyde should be dependent on its concentration, yet the rate constant for the inactivation of the enzyme by penicillanic acid sulfone (for a given molar ratio of substrate to enzyme) is unchanged over a range of initial substrate concentrations. The inactivation of the β-lactamase must therefore be a mechanism-based process (Abeles & Maycock, 1976) of the kind depicted in Figure 1. The data are best accommodated by the notion that the hydrolysis products of penicillanic acid sulfone accumulate in the incubation solution and cause further chemical modification of the inactivated protein (and undoubtedly of the native enzyme as well, though without significant loss of catalytic activity). This conclusion is supported by the results of an experiment in which the protein is inactivated in a stepwise fashion. A sample of the β-lactamase was subjected to cycles of incubation with a 3000-fold molar excess of sulfone at 4 °C for 6 h followed by dialysis of the sample to remove the reaction products. This treatment was repeated until less than 5% of the catalytic activity remained. The inactivated enzyme from this experiment had an ultraviolet absorption spectrum identical with that produced from enzyme inactivated in a single incubation at 30 °C by a large molar excess of sulfone. Isoelectric focusing of the protein produced by the stepwise inactivation protocol gave predominantly (>90%) a single band (Figure 2, lane d). It appears that only one inactivated complex is formed but that, in the presence of malonsemialdehyde, this complex may be transformed into a number of other nonspecifically modified forms. Although the inactivation of β-lactamase with tritiated sulfone was performed under the single incubation conditions, the conclusion drawn from the experiment is valid, since the *absence* of protein-bound radioactivity is observed.

When a sample of β-lactamase that had been inactivated in a stepwise fashion by the sulfone is compared on an isoelectric focusing gel to β-lactamase inactivated by clavulanic acid, the single band of protein from the sulfone incubation has a p*I* identical with that of the most acidic band of the three formed when the enzyme is inactivated by clavulanate (Figure 1, lane e) (Charnas et al., 1978; Charnas & Knowles, 1981). This result suggests that these bands represent the same protein species. Inspection of the structures of the two inactivators reveals that there is only one possible common fragment from the two inactivators, which originates from carbons 5, 6, and 7 of each structure. This fragment is that portion of penicillanic acid sulfone postulated to cross-link the active site as a β-aminoacrylate ester (Figure 1), and it is most likely that the same species arises when the enzyme is inactivated by clavulanate, also.

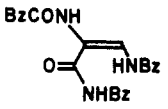
To probe further into the nature of the β-lactamase inactivated by penicillanic acid sulfone, an attempt was made to isolate a labeled peptide fragment from the modified enzyme. Since it has been argued that incubation with penicillanic acid sulfone results in the formation of a cross-linked β-aminoacrylate structure with the β-lactamase, this chromophore should permit, in principle, the isolation and identification of active site peptides. Previous studies, however, had shown that β-penicillaminoacrylate sulfinate has a half-life of only 8.5 min at pH 9.6 (Kemal & Knowles, 1981), and it was therefore necessary to stabilize the inactivated enzyme prior to any attempt at proteolytic digestion and peptide isolation. The β-aminoacrylate should be in equilibrium with its imine tautomer, and it seemed possible that the fraction of the equilibrium mixture that exists as the imine (even if small) would be susceptible to hydride reduction. In this way, the inactivated material might be transformed into a species (a β-amino ester) that would be stable even under protein denaturing conditions. Additionally, by use of tritiated reagents, a radioactive marker could be positioned on the covalent linkage. Two possibilities were envisioned for incorporation of a tritium label. The first was to use a tritiated hydride source that would incorporate the tritium at the imine carbon. The second possibility relied on the fact that in the tautomerization of the β-aminoacrylate a proton must be picked up by the carbon β to the imine of the 3-carbon fragment (what was C-6 in the original penam). By incubating the inactivated enzyme in tritiated water, label would be incorporated at this position, and with a nonradioactive hydride source, the label would be secured on the propionate fragment.

Both of the above approaches were investigated experimentally. A sample of inactivated enzyme that was more than 90% homogeneous by isoelectric focusing was used for these experiments. Reduction was performed either with boro-[^{3}H]hydride in buffer or with unlabeled cyanoborohydride in buffer containing tritiated water. Following reduction, protein samples were subjected to gel filtration and/or to exhaustive dialysis, under denaturing conditions. Gel filtration column

profiles for samples of inactivated and of native protein similarly treated (that is, exposure to hydride followed by purification) showed that the levels of radioactivity attached to the inactivated protein and to the native enzyme were indistinguishable. Moreover, when a sample that had been treated with boro[^{3}H]hydride was further subjected to amino acid analysis, radioactivity was found associated with a number of ninhydrin-positive peaks in a nonspecific fashion. Since borohydride has been shown by several workers to cause considerable amide bond reduction (to yield C-terminal β-amino alcohols)(Crestfield et al., 1963), this finding is not unexpected. In other experiments using tritiated water with cyanoborohydride, no specific labeling of the inactivated protein could be detected. Unfortunately, due to the relatively large amount of sulfone that is required for enzyme inactivation, any attempt to use ^{14}C-labeled sulfone (labeled at C-5, C-6, or C-7) would be very costly, and was not attempted.

Finally, to see if the proposed inactivation pathway is chemically reasonable, model experiments were performed on penicillanic acid sulfone. The suggested inactivation pathway proceeds by attack of the hydroxyl group of an enzyme serine residue upon the β-lactam carbonyl group. The collapse of the tetrahedral intermediate results (probably in a stepwise fashion) in the expulsion of the sulfinic acid and the formation of an imine (Brenner & Knowles, 1981; Kemal & Knowles, 1981). It has been proposed that this imine may then undergo transimination with a neighboring lysine residue. The lysyl imine then tautomerizes to a conjugated enamine, which seals the fate of the enzyme.

This sequence is not restricted to the reaction with the β-lactamase nor to this particular imine, and there is good chemical precedent for the reactions proposed. In the presence of benzylamine, both the sulfoxide and the sulfone of benzylpenicillin methyl ester are converted to the benzylamide of β-(benzylamino)-α-(phenylacetamido)acrylate (**3**) at room



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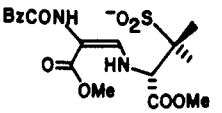

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temperature (Peck & Folkers, 1949). The reaction presumably proceeds by aminoysis of the β-lactam to give an imine corresponding to the acyl-enzyme (see Figure 1). This imine then reacts further with free amine to produce a second imine that subsequently tautomerizes to the more stable vinylogous urea, **3** (λ_{max} 283 nm; ϵ 23 940 M^{-1} cm^{-1}). The sulfone of benzylpenicillin methyl ester also reacts with methanol and, in the presence of *N*-ethylpiperidine, forms the chromophoric species **4** (λ_{max} 278 nm; ϵ 15 500 M^{-1} cm^{-1})(Peck & Folkers, 1949). When penicillanic acid sulfone was treated with methanol and diethylamine at ambient temperature, the methyl β-(diethylamino)acrylate (**6**, Figure 3) is formed. The reaction presumably proceeds through the intermediate imine **5**, followed by transimination and tautomerization. The formation of **5** is completely analogous to the enzymatic acylation of the sulfone to give the acyl-enzyme shown in Figure 1. The transimination reaction then substitutes diethylamine for the sulfinate of penicillamine. Finally, the transiminated species **5** tautomerizes to the more stable enamine **6**, analogous to the inactivated enzyme in Figure 1 (see also **8**, Figure 3).

Recently, a study of the reaction of clavulanic acid with methanol has been reported (Davies & Howarth, 1982). It was found that brief heating of clavulanate in methanol gave a methyl β-aminoacrylate (**7**, Figure 3) in high yield (70%).

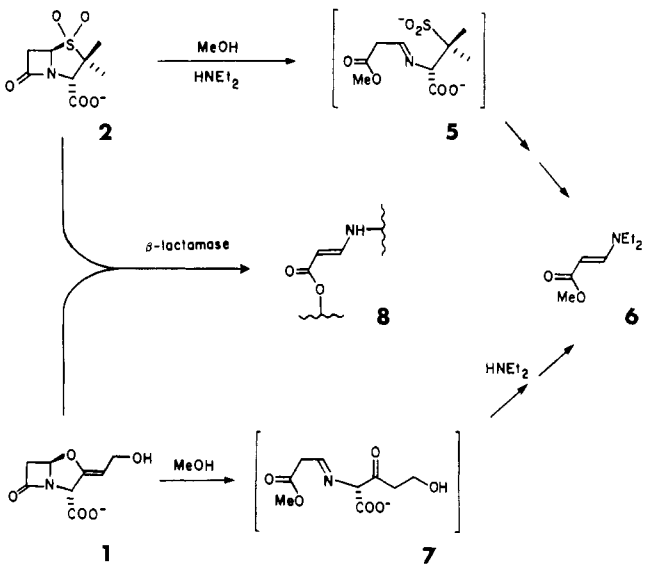

FIGURE 3: Formation of the β-aminoacrylate **6** from penicillanic acid sulfone (**2**) and from clavulanic acid (**1**) and of the analogous structure (**8**) at the active site of the β-lactamase.

The formation of **7** presumably occurs by a pathway analogous to that for the production of **5** from the sulfone (though with clavulanate the decarboxylation of the initial β-keto acid is also observed). If, however, clavulanate is treated with methanol and diethylamine at room temperature, the reaction product is **6**, identical with that formed from the sulfone. This last finding is important since the β-lactamase inactivated by clavulanic acid shares a common species with that formed by the sulfone. The species **6** is also a β-aminoacrylate ester, and it is therefore appropriate to draw an analogy between the chemical reaction (with methanol and amine) and the inactivation of the β-lactamase to **8** (Figure 3).

Although the inactivation of β-lactamase by clavulanic acid and by penicillanic acid sulfone appears to involve a common mechanism, the reactions differ in two ways. The first is that clavulanic acid also produces two other forms of inactive enzyme (Charnas et al., 1978; Charnas & Knowles, 1981). While no chemistry has been elucidated for these two additional complexes, many structures have been proposed. Clavulanic acid itself undergoes a wider variety of chemical transformations in solution than does the sulfone (Cherry & Newall, 1982), and this may account for its behavior with the enzyme as well. The second difference between the actions of clavulanic acid and of penicillanic acid sulfone with the enzyme is kinetic. Judged by the molar excess of inactivator needed to inactivate the RTEM β-lactamase completely, clavulanate is more efficient than penicillanic acid sulfone by a factor of nearly 70-fold (Fisher et al., 1978; Fisher & Knowles, 1980). Although the inactivation is in neither case a strictly first-order process, the half-lives for inactivation also differ by a factor of about 70. Additionally, the transiently inhibited enzyme complex from clavulanic acid exhibits a reactivation rate constant about 40 times smaller than that of the corresponding sulfone-derived species. These kinetic features must arise from structural differences between the acyl-enzymes formed from the two inactivators, but it is clear that slight perturbations in the structure of the β-aminoacrylate acyl-enzyme can dramatically affect its fate and the efficacy of the β-lactam derivative as an enzyme inactivator.

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6-(Methoxymethylene)penicillanic Acid: Inactivator of RTEM β-Lactamase from *Escherichia coli*†

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ABSTRACT: The *Z* and *E* isomers of 6-(methoxymethylene)-penicillanic acid have been synthesized, and their interaction with the RTEM β-lactamase has been studied. The *Z* isomer is an inhibitor and an inactivator of the enzyme, and there is some similarity between its behavior and that of other mechanism-based inactivators such as clavulanic acid and the penam sulfones. Kinetic analysis of the interaction of the enzyme with the *Z* isomer has allowed a detailed evaluation of the factors that are important in the design of anti-β-lactamase agents. In contrast to the *Z* compound, the *E* isomer of 6-(methoxymethylene)penicillanic acid is not a substrate, an inhibitor, or an inactivator of the enzyme.

The most common basis for bacterial resistance to β-lactam antibiotics is the existence of an enzyme, the β-lactamase, that catalyzes the hydrolysis of the penicillin to the corresponding penicilloic acid. In the past 7 years, a number of naturally occurring and semisynthetic β-lactam derivatives have been reported that inhibit the β-lactamase and thus have the potential of preventing the enzyme from destroying hydrolytically sensitive but otherwise potent antibiotics. The mechanisms of the interaction of several of these inhibitors with isolated β-lactamases have been studied, and the characteristics of the inhibitory processes have been delineated (Knowles, 1983). Nearly all of the inhibitors belong to the class of "mechanism-based" or "suicide" reagents, in which the hydrolytic reaction is diverted from its normal course at an intermediate stage. For the two compounds most thoroughly investigated, clavulanic acid (Fisher et al., 1978; Charnas et al., 1978; Labia & Peduzzi, 1978; Cartwright & Coulson, 1979) and penicillanic acid sulfone (English et al., 1978; Fisher & Knowles, 1980; Brenner & Knowles, 1981, 1984; Kemal & Knowles, 1981), it appears that the enzyme recognizes and

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 19, 1984.* This work was supported by the National Institutes of Health.

0006-2960/84/0423-5839$01.50/0