Inactivation of *Bacillus cereus* β-Lactamase I by 6β-Bromopencillanic Acid: Mechanism†

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ABSTRACT: The mechanism of the inactivation of *Bacillus cereus* β-lactamase I by 6β-bromopenicillanic acid, a probable suicide substrate [see Loosemore, M. J., Cohen, S. A., & Pratt, R. F. (1980) *Biochemistry* (preceding paper in this issue)], is described. Inactivation is accompanied by covalent modification of the protein with the appearance of a characteristic chromophore at 326 nm. Ultraviolet (UV) absorption, nuclear magnetic resonance (NMR), and circular dichroic (CD) spectra of the modified protein, of a modified peptide derived from the protein by enzymatic digestion, and of relevant model compounds suggest that acylation of the enzyme by 6β-bromopenicillanic acid is accompanied by rearrangement and cyclization of the inhibitor to a 2,3-dihydro-1,4-thiazine-3,6-dicarboxylic acid derivative, which is the observed chromophore. The acylated residue is shown to be Ser-70. The mechanism of action of β-lactamase inhibitors is discussed.

The preceding paper (Loosemore et al., 1980) describes our investigation of the kinetics of the reaction between the β-lactamase I (EC 3.5.2.6) of *Bacillus cereus* (BCI)[1] and 6β-bromopenicillanic acid (β-BPA). The latter compound has been shown to be a specific, irreversible inhibitor of a number of β-lactamases (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979). The kinetic results indicate that β-BPA reacts with the enzyme, probably at the active site, and inactivates it in a single-phase reaction, whose rate-determining step may be acylation of the enzyme by β-BPA as would occur in normal turnover. In the present paper the results of experiments designed to probe the mechanism of the inactivation process and the structure of the inhibited enzyme are described.

Experimental Procedures

Materials. The enzyme and β-BPA were prepared as described in the preceding paper (Loosemore et al., 1980). Sephadex chromatography resins were obtained from Pharmacia Fine Chemicals, Inc., and Bio-Gel P-4 was the product of Bio-Rad Laboratories. Pronase (type VI, the protease from *Streptomyces griseus*), bovine carboxypeptidase A, leucine aminopeptidase (type IV-5), sodium thioglycolate, and deuterium oxide (99.8 and 99.996 atom %) were purchased from Sigma Chemical Co. Guanidine hydrochloride (Ultra Pure grade) was obtained from Schwarz/Mann. All buffer components were reagent grade.

Analytical Methods. Determinations of enzyme concentration, β-BPA concentration, and enzyme activity were performed as described in the preceding paper (Loosemore et al., 1980).

Enzyme Inactivation. BCI (15 mg, 535 nmol) in 0.05 M phosphate buffer, pH 7.5 (25 mL), was inactivated at room temperature by the addition of a twofold molar excess of β-BPA. After 5 min the essentially inactive (>99.8%) enzyme was separated from excess reagent by exhaustive dialysis against 0.1 M ammonium bicarbonate at pH 8.0 ("bicarbonate buffer"). At this stage the absorption spectrum of the inactivated enzyme was as shown in Figure 4 of the preceding paper (Loosemore et al., 1980), i.e., containing the characteristic 326-nm chromophore.

Preparation and Purification of the Modified (Chromophore-Containing) Peptides. Inactivated enzyme (BPA–BCI) was denatured by heat until precipitation was complete, and Pronase (1% w/w) was added to the mixture when it had cooled to room temperature. Proteolytic digestion was then allowed to proceed for 2 h, after which the mixture was lyophilized. The residue was taken up in the minimum volume of bicarbonate buffer and applied to a Bio-Gel P-4 column (1.2 × 45 cm) equilibrated in the same buffer. The column was developed again in the same buffer; 1.0-mL fractions were collected at 20 mL/h and monitored for absorbance at 280 and 315 nm. Those containing the chromophore (and beyond the void volume peak, which contained a small amount of the chromophore in presumably undigested material) were pooled and lyophilized. The dried material was redissolved in bicarbonate buffer and chromatographed on a column (0.6 × 15 cm) of Sephadex QAE-25 equilibrated in the same buffer. After the column had been washed with 20 mL of the initial buffer, it was eluted with a 200-mL linear gradient (0.1–1.0 M) of ammonium bicarbonate at pH 8.0. Two-milliliter fractions were collected at a flow rate of 30 mL/h.

The fractions comprising the major peak of the chromophore (now absorbing maximally at 315 nm) were pooled (designated Q_2) and lyophilized. The chromophoric peptide mixture was redissolved in 0.4 mL of 10 mM ammonium acetate at pH 5.0, at 4 °C, and applied to a column (1.2 × 13 cm) of Sephadex SP-25 equilibrated in the same buffer at 4 °C. Chromatography was carried out at 4 °C, and the column was eluted with the above-mentioned acetate buffer. Fractions of 1.0 mL were collected at 10 mL/h and monitored for absorbance at 280 and 315 nm. The 315-nm chromophore-containing fractions were pooled and lyophilized. The residue was taken up in 0.5 mL of the acetate buffer and allowed to stand at 25 °C for 3 h. The mixture was then rechromatographed on the same Sephadex SP-25 column and under the same conditions as above. Fractions were monitored at 280 and 305 nm and those with absorbance at 305 nm were pooled and lyophilized. Residual acetate was removed by chromatography on a small column of Sephadex QAE-25. The peptide was applied in the bicarbonate buffer and eluted with 1 M ammonium bicarbonate. The resulting peptide was rendered salt free by repeated lyophilization.

Isolation of the Chromophore from the Inactive Enzyme. BPA–BCI (8 mg, 286 nmol) was prepared as described above. The dialyzed material was concentrated to 1.6 mL by ultrafiltration through an Amicon PM-10 membrane and dia-

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[1] Abbreviations used: BCI, *Bacillus cereus* β-lactamase I; β-BPA, 6β-bromopenicillanic acid; α-BPA, 6α-bromopenicillanic acid; BPA–BCI, β-BPA inactivated BCI.

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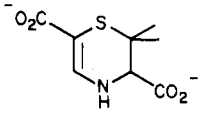
Table I: Absorption Spectral Data for the Chromophore and Models

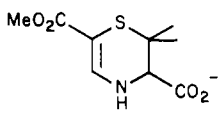
compd	λ_{max} (nm)	ε
I	306	8 200
II	314	10 000
III	304	9 200
peptide Q_2	315	10 600
peptide Q_2 after rearrangement at pH 5	305	9 100
BPA-BCI, pH 7.5	326	12 300
BPA-BCI, 6 M Gdn·HCl, pH 7.3	315	10 800

lyzed against 10 mM ammonium bicarbonate at pH 7.9. The resulting solution was made 0.2 M in hydroxide ion by the addition of the calculated volume of 1 M NaOH and then allowed to stand at 25 °C for 1 h. After reneutralization to pH 7.8 with 1 M HCl, the solution was applied to a Sephadex CM-50 column, equilibrated in 10 mM ammonium bicarbonate, and eluted with the same buffer. Fractions corresponding to a single chromophoric band, absorbing at 306 nm, were pooled and lyophilized twice.

2,3-Dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate (I). This compound was prepared by the treatment of α-BPA



I



II

with aqueous alkali in a reaction analogous to that described by McMillan & Stoodley (1966, 1968). The dibenzylethylenediamine salt of α-BPA (Loosemore & Pratt, 1978), 50 mg, was added with stirring to 1 mL of 0.1 M NaOD in D_2O. After 15 min the reaction mixture was extracted three times with 1-mL portions of chloroform to remove the diamine, and the aqueous layer was titrated to pH 7.3 with 1 M DCl. The product, I, is identified by its UV absorbance (Table I) and NMR (TableII) spectra.

2,3-Dihydro-2,2-dimethyl-6-methoxycarbonyl-1,4-thiazine-3-carboxylate (II). The α-BPA dibenzylethylenediamine salt (100 mg) was dissolved in 2 mL of methanol containing a 1.5 molar excess of sodium methoxide. After 30 min at room temperature the reaction mixture was brought to dryness by rotary evaporation. The residue was taken into 2 mL of 10 mM ammonium bicarbonate, extracted three times with chloroform, and then neutralized with 1 M HCl; for NMR samples deuterated water was used.

The characteristic UV absorbance (Table I) and NMR (Table II) spectra are essentially identical with those described by McMillan & Stoodley (1966, 1968).

^1H NMR Spectra. These were recorded either on a Varian HA-60 spectrometer or on the Bruker 270-MHz instrument at the Southern New England High-Field NMR Facility at Yale University, New Haven, CT. Model compounds were dissolved in 99.5% D_2O, while for the chromophoric peptide and the protein-derived chromophore 99.996% D_2O was used. Sodium 3-trimethylsilyl-1-propanesulfonate (DSS) was used as a reference.

CD Spectra. A sample of BPA–BCI (0.56 mg/mL) in bicarbonate buffer was made 6 M in guanidine hydrochloride by addition of the solid hydrochloride. The final protein concentration was then 0.36 mg/mL. A solution of II in 6 M guanidine hydrochloride was prepared analogously. The concentration of the latter was estimated using the literature extinction coefficient of 10 600 in methanol (McMillan & Stoodley, 1968) and the measured extinction coefficient change between solutions in methanol and in aqueous buffer. The pH of the final solutions was 7.3 in each case. CD spectra were recorded on a Cary 60 spectrophotometer at 25 °C.

Magnetic CD Spectra. Solutions of known concentrations of BCI and BPA–BCI were prepared in 0.05 M phosphate buffer at pH 7.5. CD spectra were run in the presence and absence of a 40-kG magnetic field using a Cary 61 CD spectrophotometer equipped with a Varian Model V_{4145} super-conducting magnet. The magnetic CD intensity was calibrated using the intensity of the positive MCD peak near 290 nm of *N*-acetyltryptophan (Holmquist & Vallee, 1973).

Amino Acid Analyses. Peptides were hydrolyzed in evacuated tubes in 6 M HCl containing 1% phenol (v/v) at 105 °C for 22 h. Amino acid analyses were then performed by Dr. Paul Fletcher at Yale University, New Haven, CT, using a Durrum D-500 instrument.

Peptide Maps. Heat-denatured samples of BCI and BPA–BCI in ammonium bicarbonate buffer were digested with 1% (w/w) each of Pronase and chymotrypsin for 4 h. The resulting peptide mixture was subjected first to electrophoresis on Whatman No. 3MM paper in pyridine/acetic acid/water (1:10:289) buffer, pH 3.6, and then to descending chromatography in butanol/pyridine/acetic acid/water (15:10:3:12) in a direction perpendicular to the electrophoresis. After drying, the peptides were located by the use of a ninhydrin in acetone spray and the tryptophan peptides were located with Ehrlich's reagent (Scoffone & Fontana, 1970).

Results

Tryptophan Content of BPA–BCI. Although tryptophan analysis by the method of Abrams et al. (1975) initially suggested that one tryptophan may be lost on β-BPA inactivation of BCI, a comparison of peptide maps, employing the Ehrlich reagent to detect tryptophan peptides, did not support this conclusion. The resulting dilemma was resolved by the use of what is probably the best method of tryptophan determination in intact proteins, magnetic circular dichroism, which indicated no (±5%) loss of tryptophan in the modified

Table II: ^1H NMR Data for the Chromophore and Models

compd	solvent	chem shifts (δ, ppm) 2-CH_3	2'-CH_3	3-H	5-H	6-CO_2CH_3	3-OCH_3
I	D_2O	1.25	1.51	3.77	7.52	-	-
II	D_2O	1.24	1.48	3.86	7.87	3.76	-
III	D_2O	1.14	1.31	[a]	7.77	3.77	-
III	$CDCl_3$	1.20	1.34	4.34	7.62	3.79	-
IV	$CDCl_3$	1.20	1.36	4.07	7.61	3.77	3.47
IV[d]	$CDCl_3$	1.19	1.37	4.09	7.60	3.77	3.44
alkali-released chromophore	D_2O	1.27	1.51	3.78[b]	7.54	-	-
Q_2 chromophore	D_2O	1.15	1.35	[c]	7.63	-	-

[a] Obscured by DOH peak. [b] Seen as a shoulder on a large contaminant peak at δ 3.77. [c] Obscured by peptide resonances and DOH side bands. [d] Stoodley (1968).

Table III: Purification of Chromophoric Peptides from BPA-BCI

purifcn step		amt isolated (nmol)	% yield step	% yield overall
initial		535	100	100
inactivation and dialysis		508	95	95
P-4 column		497[a]	98	93
QAE-25 column:	total	387[a]	78	73
	Q_1	101	20	19
	Q_2	208	42	39
	Q_3	70	14	13
first SP-25 column (Q_2)		200[a]	96	37
second SP-25 column (Q_2)		136[b]	68	25

[a] Determination based on an extinction coefficient of 10 600 at 315 nm. [b] Determination based on an extinction coefficient of 9100 at 305 nm.

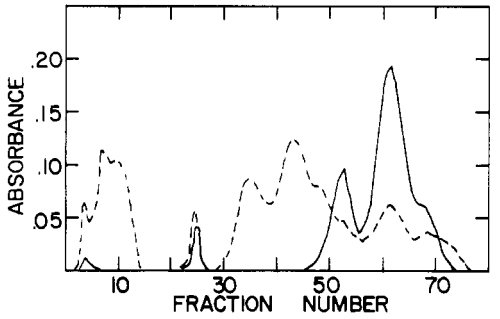


FIGURE 1: Elution profile of the chromophoric peptides on Sephadex QAE-25. Fractions were monitored for absorbance at 315 (—) and 280 (---) nm.

enzyme. The original positive result presumably arose due to loss of tryptophan during the sample preparation procedure.

Isolation of the Chromophore-Containing Peptide. This was achieved by chromatography of a proteolytic digest of the inactivated enzyme as described under Experimental Procedures and monitored by the absorption maximum of the chromophore. The results are summarized in Table III. Proteolysis of BPA–BCI led to a change in the absorption maximum of the chromophore from 326 to 315 nm, where the latter is very similar to that observed in the intact protein dissolved in 6 M guanidine hydrochloride (Table I). After initial Bio-Gel P-4 chromatography (profile not shown), where all of the chromophore-containing material, apart from undigested protein, was pooled, anion exchange chromatography on QAE-25 resulted in the resolution of chromophoric material shown in Figure 1. Clearly the chromophore is concentrated in two main peaks, designated Q_1 (fractions 47–56 pooled), containing 24% of the chromophore, and Q_2 (fractions 57–65 pooled), containing 56% of the chromophore. Most of the remaining chromophore (18%) occurs in Q_3 (fractions 66–74), but much of this, it is clear from Figure 1, must also be the material of Q_2.

Complete separation of Q_1 and Q_2 could be achieved by employment of a shallower ammonium bicarbonate gradient in the region of overlap of the two (not shown). When Q_2, dissolved in 10 mM ammonium acetate at pH 5.0, was immediately chromatographed in Sephadex SP-25 at 4 °C and eluted with the same buffer, the chromophoric material eluted in the void volume (Figure 2a). However, the eluted chromophoric material, now separated from some non-chromophore-containing material, after standing at ambient temperature for 3 h no longer eluted in the void volume of the SP-25 column, but only after 2 bed volumes of the ammonium acetate buffer (Figure 2b). Further purification of the chromophoric material has clearly also been achieved. Apparently some reaction of the chromophoric peptide(s) has

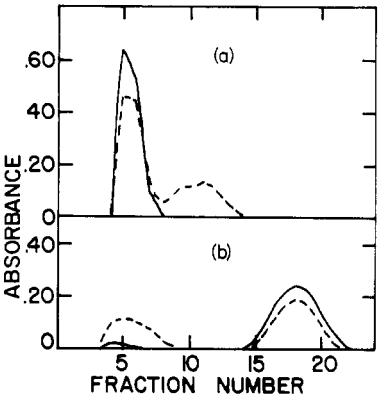


FIGURE 2: Chromatography of Q_2 on Sephadex SP-25. (a) Before the reaction at pH 5. Fractions were monitored for absorbance at 315 (—) and 280 (---) nm. (b) After the reaction at pH 5. Fractions were monitored for absorbance at 305 (—) and 280 (---) nm.

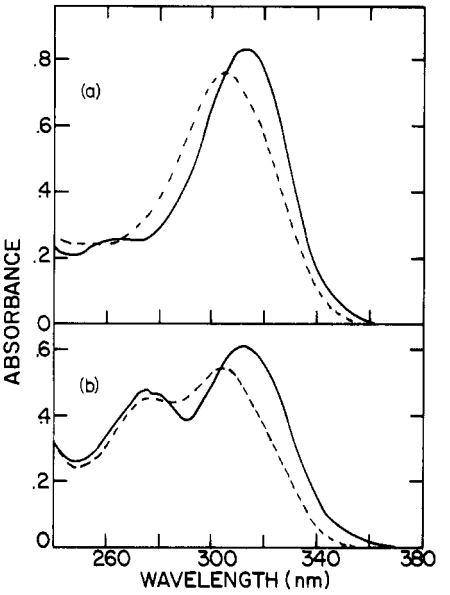


FIGURE 3: (a) Absorption spectra of II before (—) and after (---) incubation at pH 4.0. (b) Absorption spectra of Q_2 before (—) and after (---) incubation at pH 5.0.

occurred at low pH, leading to an increase in its affinity for the SP-25 column. The reaction, discussed further below, involves the chromophore itself, since the absorption maximum of the latter decreases from 315 to 305 nm during the room temperature incubation (Figure 3b). It does lead, however, to a method of specific purification of the chromophoric peptides, since nonchromophoric peptides with affinity for the SP-25 column are removed on the first passage, while nonchromophoric peptides without affinity are removed in the second. Q_1 was treated in an analogous fashion with analogous response.

Amino Acid Analysis of the Chromophoric Peptides. Analysis of Q_2, purified as described above, indicated the presence of only four amino acids, alanine, threonine, serine, and tyrosine, in significant and equimolar amounts (Table IV) and with only minor contamination. The isolation and analysis of Q_2 were reproducible. Analysis of Q_1 yielded only alanine, threonine, and serine (Table IV).

Carboxypeptidase A (3%) and leucine aminopeptidase (5%) treatment (24 h, pH 7.5, 37 °C) of Q_2 yielded 1 equiv of tyrosine and alanine, respectively, establishing the C and N termini of the peptide. No further cleavage occurred.

Identification of the Modified Amino Acid Residue. After a solution of Q_2 (0.25 mL, 10 nmol) was made 10 mM in

Table IV: Amino Acid Analyses of the Peptides Q_1 and Q_2

amino acid	peptide[a] Q_1	peptide[a] Q_2	peptide[a] Q_2 (thioglycolate)[b]
CMCys	0 (0)	0 (0)	7.5 (1.0)
Asp	0.3 (0.05)	0.6 (0.1)	0
Thr	5.9 (1.0)	8.0 (1.0)	8.1 (1.0)
Ser	6.2 (1.0)	8.9 (1.1)	0
Glu	0.4 (0.05)	1.3 (0.15)	0
Pro	0.1 (0)	0.1 (0)	0
Gly	0.6 (0.1)	1.7 (0.2)	0
Ala	6.0 (1.0)	7.9 (1.0)	7.8 (1.0)
Val	0.2 (0)	0.4 (0.05)	0.1
Met	0.2 (0)	0.2 (0)	0
Ile	0.2 (0)	0.2 (0)	0
Leu	0.2 (0)	0.3 (0.05)	0.1
Tyr	0.2 (0)	6.2 (0.8)	8.1 (1.0)
Phe	0.6 (0.1)	0.5 (0.05)	0.6 (0.1)
His	0.2 (0)	0.5 (0.05)	0
Lys	0.2 (0)	0.3 (0.05)	0
Arg	0.2 (0)	0.2 (0)	0.1

[a] Amounts of each amino acid in nanomoles. Numbers in parentheses are the numbers of each residue based on Ala as 1.0. [b] Thioglycolate background (<1 residue of each amino acid) has been subtracted.

sodium thioglycollate by addition of the solid, it was purged with nitrogen and the pH was raised to 9.5 by KOH addition. The mixture was incubated at 37 °C for 24 h and then evaporated and acid hydrolyzed for amino acid analysis. The analysis showed no change in alanine, threonine, or tyrosine content but complete loss of serine and the appearance of 1 equiv of carboxymethylcysteine (Table IV); treatment of serine itself with thioglycolate led to no carboxymethylcysteine. Treatment of Q_2 with 0.2 M sodium hydroxide to remove the chromophore (see below), followed by acid hydrolysis, yielded unchanged serine by amino acid analysis.

Structure of the Protein-Bound Chromophore. The most direct evidence on this point results from isolation of the chromophore after its release from the protein by alkali. Treatment of BPA–BCI with 0.2 M sodium hydroxide as described under Experimental Procedures resulted in a rapid (complete in less than 30 s) bathochromic shift of the protein absorption maximum from 280 to 290 nm, arising presumably from ionization of the tyrosine residues and a hypsochromic shift of the 326-nm chromophore to a position below 320 nm (as a shoulder on the side of the protein absorption). In view of the spectral effects of guanidine hydrochloride and proteolysis on the modified protein, the latter shift on alkali treatment probably reflects at least partial denaturation of the protein under these conditions. Subsequently and more slowly (with a half-time of ~5 min), the shoulder of the chromophore disappeared. After complete reaction, neutralization gave a solution whose absorption spectrum still contained a chromophore other than that of the protein, but with an absorption maximum now below 310 nm. That the resultant chromophore had been cleaved from the protein was demonstrated by passage of the reaction mixture through a small CM-50 column at pH 8. Here the chromophore eluted in the void volume, and is thus likely anionic at that pH; it was found to have an absorption maximum at 306 nm, while the protein, cationic at this pH, remained bound to the column. Recovery of the chromophore was essentially quantitative. The pH of the pooled chromophore fractions was raised to 12.0 with 1 M sodium hydroxide, and the solution was then lyophilized. At lower pH the chromophore was unstable to lyophilization. The NMR spectrum of the chromophore thus isolated is given in Table II.

Spectral Properties of the Bound Chromophore. The absorption spectra of the peptide and protein-bound chromophore have been described above and are summarized in Table I, along with those of a variety of reference compounds whose relevance will be discussed below. The circular dichroic spectrum of BPA–BCI in 6 M guanidine hydrochloride is very similar to that of II in the same solvent. Both contain strong minima ($[\theta]$ = −12 100 and −12 500 deg cm^2 dmol^{-1}, respectively) at wavelengths corresponding to their absorption maxima (Table I). Note that in the absorption spectra of Table II and the circular dichroic spectrum described above the extinction coefficients and molar ellipticity, respectively, are calculated assuming 1 molar equiv of chromophore per mol of enzyme.

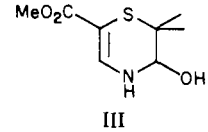
Since the method of purification of the chromophoric peptides derived from BCI–BPA included rearrangement of the chromophore, as described above, it was not possible to obtain NMR spectra of peptides containing the original chromophore. However, an NMR spectrum of the rearranged Q_2 was obtained, and peaks in it not attributable to peptide resonances are reported with NMR data for model compounds in Table II. The expected peptide resonances were also present, including doublets at δ 1.38 (J = 7.3 Hz) and 1.21 (J = 6.8 Hz) (Ala and Thr methyl resonances) and at 7.15 and 6.84 (J = 7.3 Hz for each) (Tyr aromatic resonances). Spectra were taken on samples from two separate preparations of Q_2, each having the reported features. In neither case, however, was it possible to usefully assign resonances in the δ 3.5–4.5 region of the spectrum because of a combination of factors, the residual solvent resonance, the complexity of the peptide resonances in this region due to coupling, and the small quantities of peptide available (ca. 120 nmol).

Reactions of the Model Compound II. In view of the curious rearrangement of the chromophoric peptide under mildly acidic conditions, it was useful to subject model compounds to the same treatment; below the results of such an examination of II are described.

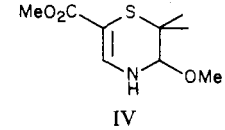
Treatment of II in dilute solution (<0.15 mM) with mild acid (ca. 0.1 M acetate buffer at pH 4.0) for 2 h resulted in a shift of its absorption maximum from 314 to 304 nm (Figure 3a) in a manner very similar to that observed with the peptide-bound chromophore (Figure 3b). Following extraction of the product of the former reaction into chloroform and evaporation of the dried chloroform extracts to dryness, an NMR spectrum of the product III was recorded (Table II). An infrared spectrum of III contained broad absorption at 3300 cm^{-1} (N–H, O–H) and sharp peaks at 1680 (C=O) and 1610 (C=C) cm^{-1}. The mass spectrum of III contains significant peaks at m/e 203, 185, and 170.

In a further reaction to assist identification, III was dissolved in an excess of dry methanol containing 0.003% acetic acid. After standing at room temperature for 48 h, the mixture was evaporated to dryness, resulting in compound IV, which had the following spectral properties: NMR ($CDCl_3$) Table II; infrared, 3320 (N–H), 1670 (C=O), and 1610 (C=C) cm^{-1}; ultraviolet, 340 nm; mass spectrum, m/e 217, 185, 170.

The above spectral data are sufficient to identify III and IV as the compounds whose structures are drawn below. IV



III



IV

has been prepared previously by Stoodley (1968), whose reported spectral data are essentially identical with those of IV.

The spectra of III and its mode of conversion to IV argue strongly for the structure proposed.

Discussion

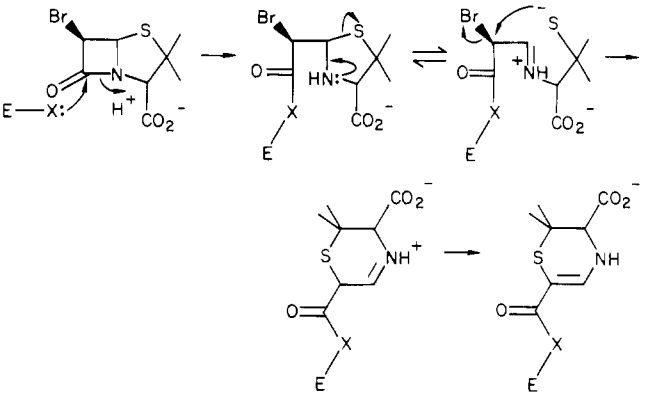
The inactivation of β-lactamases and in particular that of BCI by β-BPA has been shown to be rapid and effective (Pratt & Loosemore, 1978; Loosemore et al., 1980). We have shown that the inhibition produced by β-BPA is not easily reversible and is active-site directed. The inactivation process is characterized by a decrease in protein fluorescence intensity, the appearance of a new chromophore at 326 nm, the loss of tritium from 6-α-[^{3}H]-β-BPA, and by the fact that all of these processes probably have the same rate-determining step. The present paper bears on two points. First, since the inhibitor is active-site directed and the inhibition apparently takes place in a single-phase reaction, there is clearly an opportunity here to identify an active-site amino acid residue. Second, particularly in view of the novel chromophore generated, the structure of the covalently bound inhibitor in the inactivated enzyme has been determined.

The appearance of the protein-bound chromophore at 326 nm argues strongly for covalent modification of the protein. Since the appearance of the chromophore was associated with partial quenching of the protein fluorescence, there was a possibility that both observations might arise from covalent modification of tryptophan in the protein. This idea was reinforced by the well-known sensitivity of BCI to inactivation by tryptophan oxidants (Ogawara & Umezawa, 1975) and by the reasonable chance, on paper at least, of generating positive bromine from β-BPA. Peptide maps, however, gave no support to a mechanism involving tryptophan destruction, and the latter conclusion was confirmed by the magnetic circular dichroism results, which clearly show that no tryptophan is lost on inactivation. A further indication here was provided later by the product of β-BPA inactivation of the *Staphylococcus aureus* β-lactamase, which contains the same novel chromophore. The *S. aureus* enzyme does not contain tryptophan (Ambler, 1975). The diminished fluorescence emission of the inactivated BCI then presumably must arise from a substantial protein conformational change accompanying the inactivation reaction.

After Pronase/chymotrypsin digestion of BPA–BCI the major chromophoric peptide, Q_2, could be isolated by classical peptide procedures (see Results). The unexpected feature of this sequence was the reaction of the chromophore at low (<6) pH under the conditions used for cation exchange chromatography on Sephadex SP-25. This rearrangement, which apparently converted an anionic peptide with little affinity for the anionic column into a neutral peptide with moderate affinity, had both positive and negative aspects. On the positive side it enabled a rapid purification of the rearranged peptide (Figure 2), but on the negative it meant that the isolated peptide was altered from the original in some significant way. The rearrangement is characterized (Figure 3) by a change in the absorption maximum of the chromophore from 315 to 305 nm. What is probably the same rearrangement was noted by Knott-Hunziker et al. (1979) in a tryptic hexapeptide, whose electrophoretic behavior changed from that corresponding to a neutral peptide at pH 6.5 to that of a cation.

Amino acid analysis of the rearranged Q_2 (Table IV) gave its composition and thus, by comparison with the published amino acid sequence of BCI (Thatcher, 1975), its sequence as Ala-Ser-Thr-Tyr, comprising residues 69 through 72 of the sequence, using the numbering system of Ambler (1979). This is supported by the determination of the N- and C-terminal amino acids of Q_2 as alanine and tyrosine, respectively. The

Scheme I

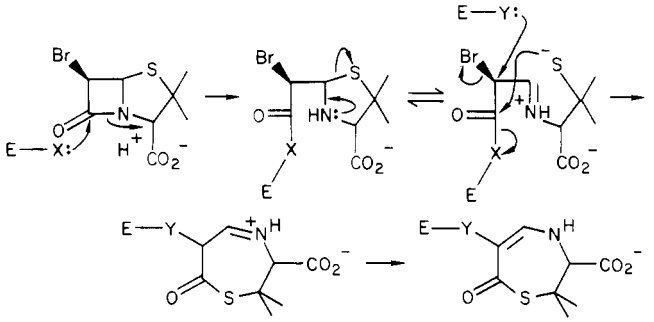


chromophore must then be attached to an aliphatic hydroxyl group, either that of serine or of threonine. Modification of one of these residues is also suggested by the inability of carboxypeptidase A and leucine aminipeptidase to cleave the Ser-Thr peptide bond under the conditions described under Results. The quantitative conversion of the serine of Q_2 into carboxymethylcysteine on treatment with thioglycollate conclusively identifies the serine (Ser-70 of BCI) as the point of attachment of the chromophore. Knott-Hunziker et al. (1979), who were apparently able to cleave the Ser-Thr linkage of their modified peptide with leucine aminopeptidase, also reached this conclusion.

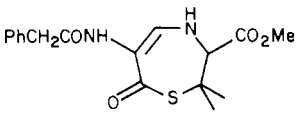
The amino acid analysis of Q_1 (alanine, serine, and threonine) suggests that modification of the same residue is involved as in Q_2. Thus, the data reported (Table IV, Figure 2) suggest that at least 88% of the protein-bound chromophore is attached to Ser-70. This figure is achieved by assuming that all of Q_1 and Q_2 and half of Q_3 (that part assumed to be Q_2) contain the label in this position. The unaccounted chromophore could reside on the same residue, but this has not been proven. With a number of other active-site directed β-lactamase inhibitors, for example, clavulanic acid (Charnas et al., 1978) and quinacillin (Virden et al., 1978), it is found that the migration of the bound inhibitor to several residues occurs after initial interaction between inhibitor and enzyme. If migration of this type did occur in the case of β-BPA, it would appear to be very close to specific migration to Ser-70. Certainly the kinetic studies of the β-BPA inactivation (Loosemore et al., 1980) give no indication of a second phase to the inactivation and chromophore formation reactions. There is then no evidence for any migration of β-BPA on the enzyme from its site of initial interaction. It might be suggested, however, that such a migration could have occurred during the acid-catalyzed reaction of the chromophore observed during isolation of the labeled peptide and described above. Consequently, it is important to determine the chemical nature of the bound chromophore and the rearrangement reaction.

The absorption maximum of the protein-bound chromophore at 315 nm in 6 M guanidine hydrochloride or on the peptide Q_2 is suggestive of that of the products of two well-documented rearrangements, one of penicillins in general, the other of halopenicillanic acids in particular. Schemes I and II show these in the context of β-lactamase inactivation, assuming in each case, as proposed in the accompanying paper (Loosemore et al., 1980), that the inactivation proceeds by way of the same covalent acyl-enzyme intermediate as does normal turnover. In each case the actual order of events is speculative, but the products are those of the known rearrangements. Scheme I derives from the base-catalyzed reaction of 6-halopenicillanic acid described by McMillan & Stoodley (1966, 1968), which

Scheme II



was used in this work (Loosemore et al., 1980) as an assay method for 6-bromopenicillanic acids. A relevant model here would be the ester II. Scheme II derives from the base-catalyzed rearrangement of penicillins first described by Kovacs et al. (1969) and subsequently investigated by them in some detail (Kovacs et al., 1973). The best model compound available here would probably be 2,2-dimethyl-3(*S*)-methoxycarbonyl-6-phenylacetamido-7-oxo-2,3,4,7-tetrahydro-1,4-thiazepine (V), which is reported (Kovacs et al., 1973) to



V

have maximal absorbance in methanol at 315 nm (ε 9600).

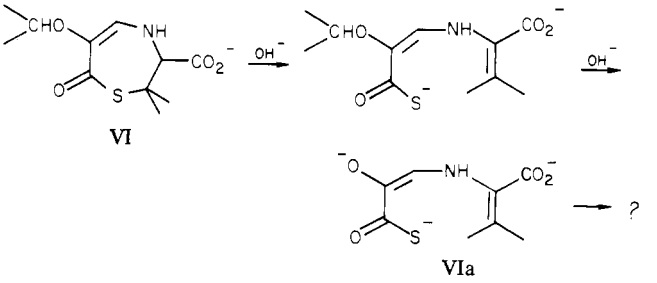
Both of these models could explain certain of the properties of the enzyme-bound chromophore, for example, its absorption spectrum and its stability to nucleophiles, since both are vinylogous carbamates. The thiazepine ring system in V, for example, is apparently stable to hydrazine treatment (Kovacs et al., 1973). These schemes also explain the loss of tritium from 6-α-[^{3}H]-β-BPA on its reaction with the enzyme (Loosemore et al., 1980).

Distinction between these two possibilities is clearly important, however, because in one case (Scheme I) the nucleophile modified in the isolated product would likely be the primary nucleophile participating in normal turnover, while in the other (Scheme II) it would be an adjacent nucleophile.

Our evidence for the mechanism of Scheme I over Scheme II derives from a detailed comparison of the properties of the enzyme-bound chromophore with those of the model compound II; II would be particularly appropriate as a model of course if an enzyme–oxygen nucleophile were involved, as is suggested by the finding discussed above of the modified serine residue. The absorption spectrum of the protein-bound chromophore and that of II are very similar (Table I), as are their circular dichroic spectra (Results) under the same conditions (6 M guanidine hydrochloride). The absorption spectrum of the chromophore in the peptide Q_2 in aqueous solution is also almost identical with that of II under the same conditions (Table I).

Further very strong evidence is available from the properties of the chromophore released from the protein by base treatment. Both the absorption spectrum (Results) and the NMR spectrum (Table II) of the isolated chromophore are identical with those of the dihydrothiazine I. In base, VI, the product of Scheme II, should undergo facile elimination reactions removing the chromophore from the enzyme and yielding VIa or further hydrolysis products (Scheme III). This route can be discounted first because such a sequence would leave a dehydroalanine residue on the protein, which on acid hydrolysis

Scheme III



would yield pyruvate rather than the observed serine. Secondly, the absorption and NMR spectra of VIa certainly should not be identical with that of I. (For example, the chemical shifts of the methyl group protons should be lower in VIa than observed, and the olefinic proton, under the basic conditions used to obtain the NMR spectra, should have exchanged with solvent deuterium; the latter should also be true of any hydrolysis products of VIa.)

Finally, the dihydrothiazine ester II is observed to undergo a reaction at low pH analogous to that of the peptide-bound chromophore (Figure 3). The product of this reaction of II is shown (Results) to be III. A reaction of this type, an oxidative decarboxylation, has been described by Stoodley (1968) to occur with these compounds, although its occurrence under the conditions used here is unexpected; adventitious metal ion catalysis seems a likely possibility. Compound III has also been suggested by Orlek et al. (1979), on the basis of a comparison of absorption maxima, to be the product of the rearrangement. That the rearrangement undergone by the peptide does yield a product analogous to III is demonstrated by the close similarity of the absorption spectra (Table I) and the NMR spectra (Table II) of III and the rearranged peptide.

In summary then, the spectral properties and chemistry of the dihydrothiazine ester II seem in very close accord with those of the enzyme-bound chromophore after its release from the environment of the protein; particularly telling of course is the spectral identity of I and the compound released from the inhibited enzyme by alkali. Although model compounds relevant to Scheme II are not available, it seems unlikely that they could fit the requirements as closely. Consequently, it seems likely from this work that Scheme I is a better representation of the course of inactivation of BCI by β-BPA than Scheme II.

We are also now in a position to summarize the evidence for the stoichiometry of the inactivation, which we believe to be 1:1. First, in previous work (Pratt & Loosemore, 1978; Loosemore et al., 1980) it is shown that BCI is inhibited by very close to 1 molar equiv of β-BPA (≤1.5) and that the enzyme is covalently modified. Second, both the extinction coefficient and molar ellipticity of the protein or peptide-bound chromophore are identical with those of relevant models, assuming 1 molar equiv of chromophore per mol of enzyme. Third, 1 equiv of a material identical in spectral properties with the model chromophore I is released from the enzyme on base treatment. Finally, at least 88% of the chromophore is present on a single amino acid residue. Together these points argue decisively for 1:1 stoichiometry.

Since the acid-catalyzed reaction of the peptide-bound chromophore appears to involve only the chromophore itself, there is little reason to doubt that Ser-70 must be the original and sole point of attachment of β-BPA to the enzyme and, according to Scheme II, the primary nucleophile of the BCI active site. That serine is in fact the labeled residue can be taken, in itself, as evidence that a covalent acyl-enzyme in-

termediate, an acyl-serine, is involved in normal β-lactamase turnover, as is suggested by Scheme I and in the accompanying paper (Loosemore et al., 1980), since it seems unlikely that a serine that was not specifically activated for such a purpose (cf. the serine proteases) would otherwise appear as the primary nucleophile toward β-BPA.

These conclusions are well in accord with other recent findings. A preliminary communication of independent work by Knott-Hunziker et al. (1979) also identifies Ser-70 as the site of β-BPA attachment in BCI. Fisher et al. (1980) have reported evidence for the existence of a covalent acyl-enzyme intermediate, which has the physical and chemical characteristics of an ester, in the interaction of cefoxitin with the *Escherichia coli* β-lactamase. With the same enzyme they have shown that the inhibitor quinacillin sulfone becomes covalently bound to a peptide homologous to the present one. A homologous peptide in the *S. aureus* β-lactamase is also labeled by 6-chloropenicillanic acid sulfone (S. J. Cartwright and A. F. W. Coulson, presented at the β-lactamase Workshop, Newcastle-upon-Tyne, April 27-29, 1979).

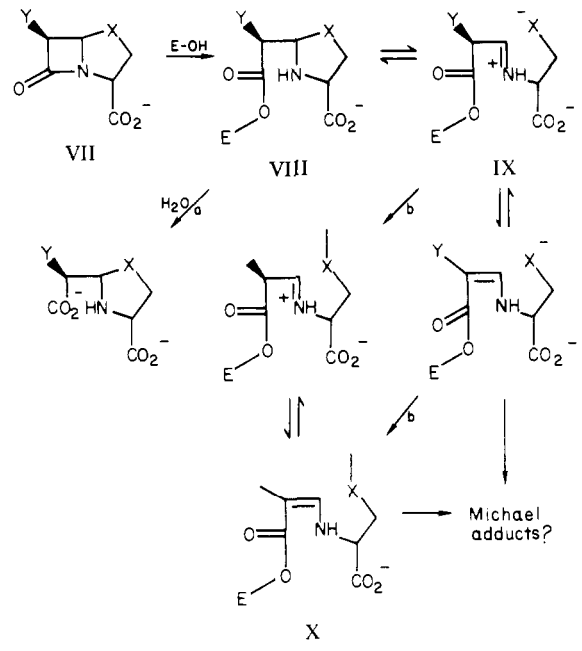
Recently also Yocum et al. (1979) have reported that both substrates and penicillin inhibitors acylate a serine residue at the active sites of two bacterial D-alanine carboxypeptidases and, furthermore, that the amino acid sequences about these serine residues are homologous to those about serine-70 of the β-lactamases mentioned above. This seems to confirm the previously suggested (Tipper & Strominger, 1965) evolutionary relationship between the D-alanine carboxypeptidases and the β-lactamases. In view of this relationship, and to probe it further, it would clearly be of interest to compare the interactions of β-lactamase inhibitors with the D-alanine carboxypeptidase active sites and vice versa. In preliminary work we have found that β-BPA does not interact strongly with the *Streptomyces* R-61 D-alanine carboxypeptidase/transpeptidase and is not an effective inhibitor. This presumably reflects the poor affinity of penicillins with small C-6 substituents for the R-61 carboxypeptidase active site (Ghuysen et al., 1979). With regard to the latter, however, it might be noted that we did obtain evidence for a strong interaction between clavulanic acid, which has no C-6 substituent, and the R-61 enzyme.

Finally, it is useful to examine the structure of β-BPA in light of the proposed mechanism of inhibition (Scheme I) to attempt to identify those elements of its structure that are essential to its inhibitory power. In this regard it is useful also to consider the structures of the other well-characterized β-lactamase inhibitors, clavulanic acid (Fisher et al., 1978; Charnas et al., 1978), and the various penicillin sulfones (English et al., 1978; Cartwright & Coulson, 1979; Fisher et al., 1980). Scheme IV gives a general mechanism for an inhibitor of this type, represented by VII.

The first requirement of such inhibitors appears to be that they be poor β-lactamase substrates: substrates so that the acyl-enzyme intermediate VIII can be achieved and poor so that hydrolysis of the acyl-enzyme intermediate (path a) is not so fast to preclude the steps (paths b) leading to inhibition. In the case of β-BPA the small C-6 side chain probably ensures that it is a poor enough substrate.

The main basis for the inhibition (omitting for the moment further possible reactions such as the Michael additions also shown in Scheme IV) is the formation of an acyl-enzyme derivative, which is much less readily hydrolyzed than the normal penicilloyl-enzyme VIII. This is accomplished in each of the above cases by rearrangement (paths b) of the penicilloyl-enzyme through abnormal functionality built into the penicillin (these inhibitors are suicide substrates (Abeles & Maycock, 1976) or Trojan horse substrates (Miesowitz & Bloch, 1979)). This can be seen here as occurring through opening of the 3-heteroazolidine ring to IX, followed by irreversible trapping of the leaving group X^-, thus allowing rearrangement into the hydrolytically inert vinylogous carbamate system X. In the case of β-BPA, X^- (S^-) is trapped by its facile displacement of bromide ion and formation of the stable dihydrothiazine system as described above; in the case of clavulanic acid, X^- (O^-) is trapped by ketonization of the enolate resulting from ring opening; and in the case of the sulfones, X^- (SO_2^-) is trapped as itself. In contrast to thiazolidines, where the VIII ⇌ IX equilibrium lies to the left (otherwise all penicillins would be inhibitors!), in thiazolidine sulfones, which are not known as stable species without N-acylation, the equilibrium lies far to the right (Cook & Heilbron, 1949). Structures analogous to X have been suggested to be intermediates in the inhibition pathway of clavulanic acid by Fisher et al. (1978) and by Reading & Hepburn (1979) and of the sulfones by Fisher et al. (1980).

Scheme IV



Knowles and co-workers have suggested (Fisher et al., 1980) that an enzymatic base is present, which stereospecifically assists proton removal from the 6-α position in all of the above inhibitors, and that this is the driving force for a trans elimination across the C-5 and C-6 positions, directly yielding products corresponding to X. Although it is tempting to propose the presence of a base in the β-lactamase active site for activation of the serine nucleophile (cf. serine proteases), it seems there is no absolute requirement for such a base to explain the action of these inhibitors, as indicated in Scheme IV. After β-lactam ring opening, the remaining heterocyclic rings are well known to open spontaneously, generating the malonate aldimine system, which should be acidic enough not to require a specific base on the enzyme for proton removal. Indeed, initial elimination between C-5 and C-6 would appear to hinder formation of the dihydrothiazine in the case of β-BPA, since this would require regression from the double-bond conjugated state X back to IX before cyclization could occur, followed by reversion of the double bond to the conjugated state. The tautomerization back to IX might well be a slow step at neutral pH, for which there is no evidence (Loosemore et al., 1980).

Acknowledgments

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