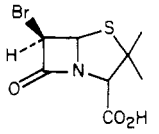
Inactivation of *Bacillus cereus* β-Lactamase I by 6β-Bromopenicillanic Acid: Kinetics†

M. J. Loosemore, S. A. Cohen, and R. F. Pratt*

ABSTRACT: The kinetics of the inactivation of *Bacillus cereus* β-lactamase I by 6β-bromopenicillanic acid are described. Loss of β-lactamase activity is accompanied by a decrease in protein fluorescence, by the appearance of a protein-bound chromophore at 326 nm, and by loss of tritium from 6α-[^{3}H]-6β-bromopenicillanic acid. It is shown that all of the above changes probably have the same rate-determining step. The inactivation reaction is competitively inhibited by cephalosporin C, a competitive inhibitor of this enzyme, and by covalently bound clavulanic acid, suggesting that 6β-bromopenicillanic acid reacts directly with the β-lactamase active site. It is proposed that this inhibitor reacts initially as a normal substrate and that the rate-determining step of the inactivation is acylation of the enzyme. A rapid irreversible inactivation reaction rather than normal hydrolysis of the acyl-enzyme then follows acylation; 6β-bromopenicillanic acid is thus a suicide substrate.

There has been considerable interest recently in the development and mechanism of action of β-lactamase (EC 3.5.2.6) inhibitors. This interest should lead ultimately to a wide variety of potent β-lactamase inhibitors, some of which may be clinically useful, and to a fuller knowledge of the mechanism of action of these enzymes. It has been previously demonstrated (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a) that 6β-bromopenicillanic acid (β-BPA)[1] (I) is a



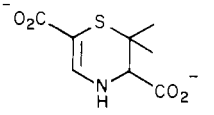
I

specific, powerful, and irreversible inhibitor of a variety of β-lactamases. In the present paper and the following one, we present further data concerning the details of the inhibition process with a view toward determination of the mechanism of action of this inhibitor. In this paper the kinetics of the inactivation process are presented, while in the next the chemical nature of the inactivation is explored.

Experimental Procedures

Materials. The β-lactamases (a mixture of I and II) of *Bacillus cereus* (strain 569/H/9) were purchased from the Microbiological Research Establishment at Porton Down, England, and were separated and purified by the method of Davies et al. (1974). The *B. cereus* β-lactamase I (BCI) was demonstrated to be pure by polyacrylamide gel electrophoresis (Laemmli, 1970) and had a specific activity of 3500 units/mg of protein at 25 °C. The sodium salt of β-BPA was prepared as a 28 ± 5% (analysis by NMR spectroscopy) mixture with the sodium salt of α-BPA as previously described (Loosemore & Pratt, 1978). [^{3}H]Water was purchased from ICN. Cephalosporin C was the generous gift of Eli Lilly and Co., sodium clavulanate was a gift of Beecham Pharmaceuticals, and nitrocefin was a gift of Glaxo Research.

Analytical Methods. Absorption spectra were recorded on a Cary 14 spectrophotometer and fluorescence spectra on a Perkin-Elmer MPF-44A spectrofluorometer. The rapid (half-times ≤ 5 s) rates of the fluorescence and absorbance changes occurring on inactivation of BCI with higher concentrations of β-BPA were obtained from a Durrum D-10 stopped-flow spectrophotometer. The fate of the radioactively labeled inhibitor was followed by scintillation counting in Scintisol (Isolab Inc.) in a Packard Model 3324 liquid scintillation counter. β-Lactamase activity was routinely estimated in 0.1 M potassium phosphate buffer, pH 7.5 (μ = 0.5 with KCl), at 30 °C against benzylpenicillin by the spectrophotometric method of Waley (1974). The chromophoric cephalosporin nitrocefin (O'Callaghan et al., 1972) was used for activity assays in cases where the benzylpenicillin absorption at low wavelength was obscured by strongly absorbing species such as cephalosporin C. BCI concentrations were determined spectrophotometrically assuming $A_{280}^{1\%}$ = 10.0 (Imsande et al., 1970). The estimation of total BPA in solutions was conveniently carried out spectrophotometrically by following at 306 nm either the rate or the extent of formation of II in 0.1



II

M KOH at 30 °C (McMillan & Stoodley, 1966, 1968). The method was calibrated with solutions prepared from analytically pure samples of the *N,N'*-dibenzylethylenediamine salt of α-BPA (Loosemore & Pratt, 1978).

Preparation of Tritiated BPA. Tritiation of BPA was achieved by incubation of α-BPA (0.3 M) in aqueous solution containing [^{3}H]water (5 Ci/mL) under conditions previously demonstrated (Loosemore & Pratt, 1978) to lead to both exchange and epimerization at the 6 position of BPA (viz., 20 mM sodium pyrophosphate, pH 9.2, 30 °C, 3 days). Workup in the previously described manner then yielded the tritiated BPA (30 Ci/mol). For the enzyme studies described below, this material was diluted ca. 1/200 with the cold 28% β-BPA mixture. That β-BPA had in fact been labeled by this procedure was demonstrated by the following experiments.

A mixture of α- and β-BPA methyl esters was prepared by methylation of the 28% sodium salt mixture by the method

† From the Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457. *Received December 28, 1979.* This work was supported by National Institutes of Health Grants AI 12942 and AI 15077.

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

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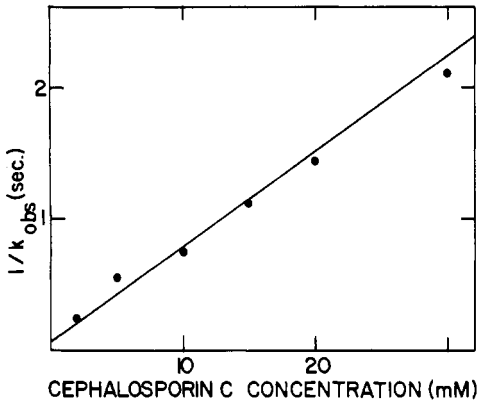

FIGURE 1: Effect of various cephalosporin C concentrations on the first-order rate constant, k_{obsd}, of inactivation of 0.15 μM BCI by 1.0 μM β-BPA.

of Bamberg et al. (1967). The resulting mixture of epimeric esters could be separated analytically by thin-layer chromatography (silica gel/benzene, where α-BPA methyl ester has an R_f of 0.52 and the β-BPA ester has an R_f of 0.48) or preparatively by elution with benzene from a silica gel column. The pure methyl esters are characterized by their NMR spectra: α-BPA methyl ester, τ ($CDCl_3$) 1.47 (3 H, s, CH_3), 1.63 (3 H, s, CH_3), 3.72 (3 H, s, OCH_3), 4.48 (1 H, s, 3-H), 4.73 (1 H, d, J = 1.5 Hz, 6-H), and 5.33 (1 H, d, J = 1.5 Hz, 5-H); β-BPA methyl ester, τ ($CDCl_3$) 1.48 (3 H, s, CH_3), 1.68 (3 H, s, CH_3), 3.80 (3 H, s, OCH_3), 4.53 (1 H, s, 3-H), 5.33 (1 H, d, J = 4.0 Hz, 6-H), and 5.60 (1 H, d, J = 4.0 Hz, 5-H).

A sample of the diluted tritiated sodium salt mixture was methylated in the same way. Thin-layer chromatography (silica gel/benzene) of the product mixture yielded two radioactive spots containing 90% of the total radioactivity on the plate and coincident with the iodine-visualized α-BPA and β-BPA spots. This demonstrates clearly that the tritiation procedure described above produced a mixture of tritiated α-BPA and β-BPA. The tritium is presumably in the 6 position. From the relative amounts of radioactivity in the two spots, it seemed that the α and β epimers had been tritiated to approximately equal extents. Exact quantitation was not possible because some tritium was lost during the methylation reaction.

Results

Inhibition of BCI by β-BPA in the Presence of Cephalosporin C. A study of the rate of inhibition of BCI by β-BPA in the presence of a competitive inhibitor/very poor substrate of BCI (cephalosporin C) gave the results plotted in Figure 1. In these experiments 0.15 μM BCI was incubated with 1.0 μM β-BPA in the presence of cephalosporin C at various concentrations (0–30 mM), and the rate of inactivation at each cephalosporin C concentration, followed by β-lactamase assay of aliquots of the reaction mixture, was taken at appropriate times.

Inhibition of BCI by β-BPA in the Presence of Sodium Clavulanate. BCI (0.2 μM) was incubated with 0.24 mM sodium clavulanate in 0.1 M phosphate buffer, pH 7.5, at 30 °C for 15 min. Dilution of a 10-μL aliquot of this mixture into 3 mL of the normal benzylpenicillin assay mixture showed that the enzyme was initially some 80% inactivated but that about 50% of the lost activity was recovered after 5 min in the assay medium. β-BPA (1.0 μM) was added to the original (15 min) incubation mixture. After a further 5-min dilution of a 10-μL aliquot into the assay mixture as above showed that the enzyme was close to completely inactivated initially, but after 5 min some 40% of its activity was recovered.

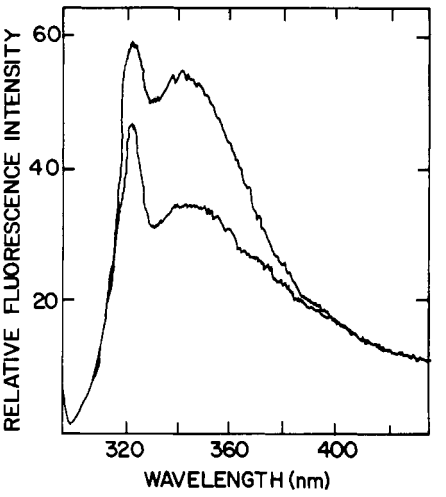

FIGURE 2: Fluorescence emission spectra of BCI (0.05 μM) before (upper curve) and 20 min after (lower curve) addition of β-BPA (0.05 μM). The excitation wavelength was 288 nm. The sharp spike at low wavelength is the Raman scattering peak.

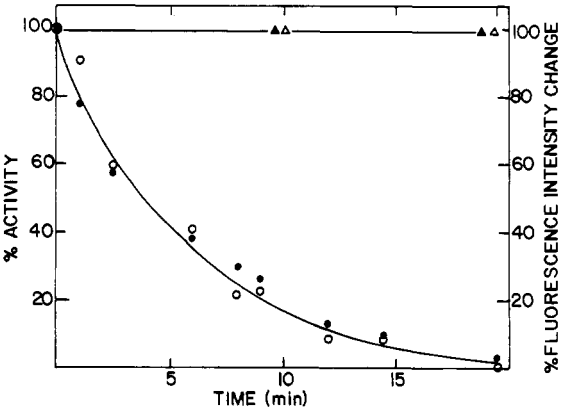

FIGURE 3: Correlation between loss of β-lactamase activity (●) and quenching of protein fluorescence (○) on reaction of 0.05 μM β-BPA with 0.05 μM BCI. The effect of α-BPA under the same conditions on the activity (▲) and fluorescence (△) of the enzyme is also shown.

Effect of Inactivation of BCI by β-BPA on the Fluorescence Spectrum of BCI. Figure 2 shows the fluorescence spectrum of 0.05 μM BCI in 0.1 M phosphate buffer at pH 7.5 and at 30 °C, obtained by excitation at 288 nm, both before and after inactivation of the enzyme with 0.05 μM β-BPA. Further addition of β-BPA (up to 0.1 mM) had no effect on the final spectrum. α-BPA, up to 80 μM, had no effect on the original BCI spectrum.

Figure 3 shows the correlation between the fluorescence change brought about by β-BPA and the activity of the enzyme as a function of time under the same conditions as in the preceding paragraph. Within experimental uncertainty the rate of loss of β-lactamase activity is the same as the rate of the fluorescence change. Equally good correlations were obtained at 0.1 and 0.2 μM β-BPA. Although the rapid rates of inactivation of the enzyme at high β-BPA concentrations precluded inactivation rate measurements, the rate of the fluorescence change could be measured under these conditions using the stopped-flow method. The enzyme concentration was 0.05 μM, while β-BPA concentrations ranged up to 2 mM. Higher concentrations of β-BPA were precluded by background absorption. Good first-order plots were obtained for decay of the fluorescence intensity with time, and the first-order rate constants thus obtained were linear with β-BPA concentration up to 2 mM (not shown).

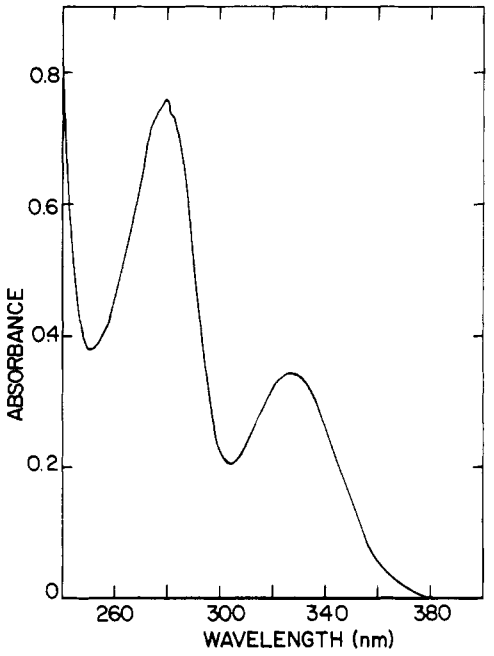

FIGURE 4: Absorption spectrum of BCI (27 μM) after inactivation with β-BPA. The inactivated enzyme was separated from small molecules by P-4 gel chromatography.

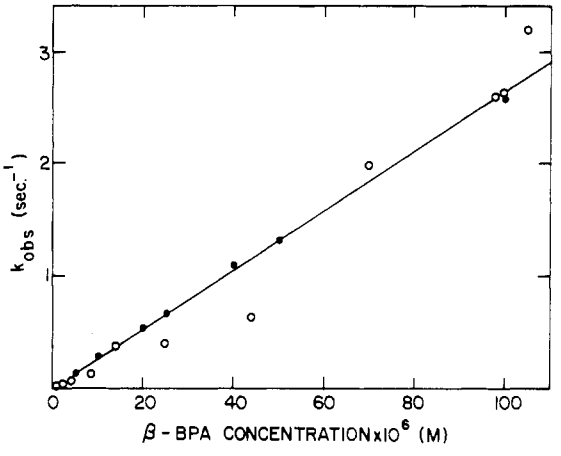

FIGURE 5: Effect of β-BPA concentration on the rate of formation of the 326-nm chromophore (●) on inactivation of BCI (0.6 μM) with β-BPA. The fluorescence rates (○) over the same concentration range are included for comparison.

Absorption Spectrum of the Inactivated Enzyme. An absorption spectrum of the β-BPA inactivated enzyme after its separation from small molecules by passage down a Bio-Gel P-4 column is given in Figure 4. The distinctive feature is of course the presence of an intensely absorbing ($\epsilon \sim 12600$) chromophore at 326 nm. A similar chromophore is seen in the inactivated *Escherichia coli* and *Staphylococcus aureus* β-lactamases. This chromophore has also been reported in a recent communication by Knott-Hunziker et al. (1979b). The time course of appearance of the 326-nm chromophore was investigated using the stopped-flow method. The enzyme and β-BPA concentrations used were 0.6 μM and up to 0.1 mM, respectively. The results of these experiments are shown in Figure 5. It was important in these experiments to remove trace quantities of β-lactamase II activity (<1%) from the BCI preparations by dialysis overnight against phosphate buffer at pH 7.5 containing 0.05 M ethylenediaminetetraacetic acid (Davies & Abraham, 1974). Since both α- and more particularly β-BPA appear to be quite good substrates of the β-lactamase II, and the product of their hydrolysis is, judging

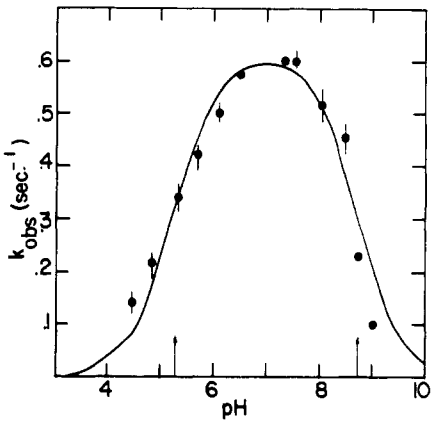

FIGURE 6: pH–rate profile for the appearance of the 326-nm chromophore on reaction of BCI (0.6 μM) with β-BPA (ca. 20 μM). The points shown (●) represent the mean of three or four determinations, while the vertical lines through them represent the spread of the rate constants obtained. The solid line represents a classical bell curve with pK_a values of 5.25 and 8.7.

from the spectra, the thiazine (II), absorbing maximally at 306 nm, any traces of the β-lactamase II activity distorted measurements of the appearance of the 326-nm chromophore on BCI. However, in the absence of β-lactamase II activity, good first-order plots were obtained for the rate of appearance of the 326-nm chromophore. At a particular enzyme concentration, the amplitude of the 326-nm absorbance change did not vary with β-BPA concentration although the rate of its formation did, as indicated in Figure 5. The number of turnovers prior to inactivation is estimated from the spectral data to be less than 0.5 per enzyme molecule.

It is also possible to demonstrate by this method that no significant release of inhibitor into solution occurred on interaction of BCI with excess β-BPA. The fluorescence of a 0.03 μM solution of BCI after addition of 1.0 μM β-BPA was followed to completion in a first-order process as described above. A further aliquot of concentrated enzyme equal to the original was then added. The fluorescence decay occurred with the same amplitude and first-order rate constant as at first; the inhibition process was thus the same.

The rate of appearance of the 326-nm chromophore varied with pH, and the results of experiments designed to assess this variation are shown in Figure 6 as a pH–rate profile. The buffers used were 0.1 M acetate (pH 4–6), 0.1 M phosphate (pH 6–7.5), and 0.05 M pyrophosphate (pH 8–9), where the ionic strength was adjusted to 0.5 with potassium chloride in each case.

Inactivation of BCI with [^{3}H]-β-BPA. Approximately 2 × 10^{-5} M BCI (0.5–1 mg/mL) was inactivated with 5 × 10^{-4} M [^{3}H]-β-BPA in 0.1 M phosphate buffer at pH 7.5 and at 25 °C, and, after a 5-min incubation period, the protein was separated from small molecules by passage of the reaction mixture through a 45 × 1 cm Bio-Gel P-4 column previously equilibrated in 0.1 M phosphate buffer at 4 °C. This separation took approximately 25 min. Scintillation counting at this stage showed in three separate experiments that no tritium was associated with the protein and that all the initial radioactivity appeared in the small molecule fraction.

In other experiments, samples inactivated under the same conditions as above were rapidly frozen at −80 °C 10 s after addition of the BPA. Control experiments showed the enzyme was completely inactivated at that time. On freeze-drying of these samples about 0.5 mol of volatile tritium/mol of enzyme was obtained after correction for the amount of tritium lost spontaneously from the BPA (0.1–0.2 mol/mol of enzyme).

The less than whole number obtained here probably reflects the uncertainty as to the exact specific activity of β-BPA.

Similar results were obtained when inactivation mixtures were passed through small Dowex-50W-8X anion exchange columns on which the BPA was retained, which took approximately 2 min to run; the eluted tritium was volatile.

Discussion

Previously, we have presented evidence that β-BPA is a specific inhibitor of a number of β-lactamases (Pratt & Loosemore, 1978). Although pure β-BPA was not isolated, a mixture of the α and β epimers could be used since the α epimer appeared to be neither an inhibitor nor a substrate of most β-lactamases. No separation of either the acids or their *p*-bromophenacyl esters (Loosemore & Pratt, 1978) was achieved. In this paper, we first show that the epimers can be separated as their methyl esters by chromatography on silica gel yielding two compounds with the expected properties of epimeric 6-bromopenicillanic acid esters and thus complete the characterization of the epimer mixture used for the enzyme inhibition studies.

The *B. cereus* β-lactamase I is inhibited by close to 1 molar equiv of β-BPA in a manner not reversible by dialysis or treatment with strong nucleophiles (Pratt & Loosemore, 1978). In the latter work, we have also shown that the rate of inhibition of BCI by β-BPA is significantly decreased in the presence of a good BCI substrate such as benzylpenicillin. This effect is demonstrated more quantitatively here by the data of Figure 1, where the ability of cephalosporin C, a competitive inhibitor of substrates at the active site, to inhibit the β-BPA inactivation reaction is demonstrated. The linear fit of the data to the plot of Figure 1 is consistent with (but does not prove) the proposition that β-BPA directly competes with cephalosporin C for the active site of the enzyme.

To obtain further evidence on this point, the possibility of competition between β-BPA and clavulanic acid was tested. It is believed that clavulanic acid is an active site directed inhibitor of β-lactamases. We and others (Fisher et al., 1978; Charnas et al., 1978; Durkin & Viswanatha, 1978) have observed a slowly reversible phase in the interaction between clavulanic acid and β-lactamases whereby a substantial amount of the enzyme activity can be restored by dilution of the enzyme/clavulanate mixture. The amount that can be restored, however, decreases as the time of incubation of the enzyme with clavulanate increases. One possibility is that the reversible enzyme/clavulanate complex is an acyl-enzyme species where the acyl group is clavulanate derived and the acyl acceptor is the primary nucleophile of the active site for normal turnover (if one exists). Certainly, the primary interaction of clavulanate with β-lactamases is thought to be at the active site.

Our results show the enzyme/clavulanate complex, at least in the slowly reversible form, is inert to β-BPA inactivation, since on dilution of an enzyme/clavulanate/β-BPA mixture the same return of activity is observed as in the absence of β-BPA. This strongly suggests competition between the two inhibitors for the same site.

Accompanying inactivation of BCI by β-BPA, spectral changes (Figures 2 and 4) occur. The quenching of fluorescence (Figure 2) could represent either covalent or noncovalent modification of the protein (covalent modification of tryptophan or simply a change in its physical environment), but the change in the absorption spectrum, where a distinct new chromophore at 326 nm is produced, certainly does argue for covalent modification.

The above evidence, taken together, suggests that β-BPA is an active-site directed inhibitor, acting by covalent modification of the protein close to the β-lactamase active site.

In Figure 3 the relationship between the fluorescence change induced by β-BPA and the loss of enzyme activity under the same conditions is shown. There is clearly a direct correlation between the two processes such that one very real possibility is that the same molecular event leads to both phenomena. With the assumption that this correlation extends to higher β-BPA concentrations, it then becomes possible to measure rates of inactivation of the enzyme at high β-BPA concentrations, where this is not possible through direct assay, by measurement of the rates of the fluorescence change. The relationship between the first-order rate constants for fluorescence quenching and β-BPA concentration is, within experimental uncertainty, quite linear up to 2 mM β-BPA, and yields a second-order rate constant of 1.8×10^4 s^{-1} M^{-1} for the enzyme/β-BPA reaction.

Also linear (Figure 5) and yielding a second-order rate constant of 2.6×10^4 s^{-1} M^{-1} is the variation of the rate constants of formation of the 326-nm chromophore with β-BPA concentration. The similarity of the second-order rate constants here (and taking into account the fluorescence rates over the same concentration range, also shown in Figure 5) suggests that the production of the chromophore and the fluorescence quenching are correlated and, thus, both of these are directly correlated with the loss of β-lactamase activity.

Loss of tritium from the [^{3}H]-β-BPA also occurs rapidly on interaction of the latter with the enzyme. The experiments described suggest that under conditions whereby activity should be lost with a half-time of around 1 s, essentially all of the tritium is in the water after 10 s.

The experiments described above indicate a single rate-determining step leading to the inactive enzyme, i.e., with no evidence of an accumulation of intermediate species (cf. clavulanic acid inactivations). The following kinetic scheme (eq 1) can then be considered. The reasonable assumption is made

$$\mathrm{E} + \mathrm{B} \underset{K}{\rightleftharpoons} \mathrm{E{\cdot}B} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E\text{-}B'} \xrightarrow{k_3} \mathrm{E\text{-}B''} \qquad (1)$$

$$\mathrm{E\text{-}B'} \xrightarrow{k_4} \mathrm{E} + \mathrm{P}$$

that β-BPA behaves, initially at least, as a normal substrate. The latter is certainly suggested by its structure and by the specificity of the inactivation with respect to stereochemistry at the 6 position. In eq 1, B represents β-BPA, E·B represents the Michaelis complex with B, and E–B′ represents an acyl-enzyme intermediate that can proceed further, either to the normal penicilloate product P or to the inactive enzyme/β-BPA complex E–B″.

The spectral experiments, where the production of chromophore was monitored, suggest that no significant turnover accompanies inactivation in the case of the BCI enzyme, i.e., $k_3 > k_4$, and thus, also taking into account earlier data (Pratt & Loosemore, 1978), one enzyme molecule becomes inactivated after covalent interaction with very close to a single inhibitor molecule. With the assumption of rapid binding and E–B′ as a steady-state intermediate, the following equation can be derived for the observed first-order rate constants, k_{obsd}, for inactivation of a constant concentration of BCI as a function of the β-BPA concentration, [B], and assuming [B] >> $[E]_0$, the total enzyme concentration:

$$k_{obsd} = \frac{[k_2k_3/(k_2 + k_3 + k_{-2})][B]}{[B] + K(k_{-2} + k_3)/(k_2 + k_3 + k_{-2})} \qquad (2)$$

Equation 2 describes a Michaelis–Menten type curve with

$$k_{obsd}^{max} = k_2k_3/(k_2 + k_3 + k_{-2})$$

where k_{obsd}^{max} is the first-order rate constant at saturating [B] and

$$K_m = K(k_{-2} + k_3)/(k_2 + k_3 + k_{-2})$$

where K_m is the apparent Michaelis dissociation constant.

Simplification of eq 2 would result from the assumptions $k_3 >> k_{-2}$ and $k_3 >> k_2$; the former is reasonable since recyclization to the strained β-lactam would likely be a slow step if it occurred at all, and the latter is reasonable since no induction period is observed in the appearance of the chromophore that accompanies the final product.

Thus

$$k_{obsd}^{max} = k_2$$

and

$$K_m = K$$

Since no saturation of inactivation rates was observed up to 2 mM β-BPA, $K \geq 2$ mM and $k_2 \geq 70$ s^{-1}. At low β-BPA concentrations ([B] << K) the observed second-order rate constant will be given by k_2/K (= 1.8×10^4 s^{-1} M^{-1}). That the dissociation constant of such an effective inhibitor is so large is perhaps surprising, but the weak binding is offset by the rapid subsequent reaction (k_2) and $k_3 > k_4$. The efficiency of the inhibitor is best appreciated through the high second-order rate constant k_2/K.

It is of interest to note that the K_m values of penicillins with small side chains are also in the millimolar range; e.g., the K_m of 6-aminopenicillanic acid with BCI is variously reported as 1.35 mM (Waley, 1974) and 3 mM (Batchelor et al., 1961). For this comparison to be valid, of course, it must be assumed that $K_m = K_s$ for 6-aminopenicillanic acid, and this would require the same assumptions as made above for β-BPA, i.e., that the rate-determining step in turnover is the formation of E–B′, acylation of the enzyme (i.e., that the same assumptions with respect to the formation and decay of the acyl-enzyme species are made in each case). With these assumptions, however, the apparent binding properties of β-BPA are in accord with its role as a poor substrate. Furthermore, it may be noted that k_{cat}/K_m for 6-aminopenicillanic acid, which should represent the second-order rate constant for the interaction of this substrate with the enzyme, has a value around 2×10^5 s^{-1} M^{-1} (calculated from data tabulated by Citri & Pollock, 1966), which is similar to, although somewhat larger than, the k_2/K value for β-BPA. In contrast, the value of this parameter for a good substrate like benzylpenicillin is around 3×10^7 s^{-1} M^{-1}. Consequently, not only the apparent binding properties of β-BPA but also the rate constants of inactivation, to the extent that they are available, are in agreement with what one would expect from the turnover of a poor substrate, as one would expect β-BPA to be, and this lends credence to the kinetic scheme developed above, whereby β-BPA behaves as a normal substrate up to and including the rate-determining step of the inactivation, which is suggested to be enzyme acylation.

The pH–rate profile (Figure 6) for inactivation of the enzyme, as measured by the appearance of the 326-nm chromophore, can also be considered in terms of eq 1 and the above discussion. In view of the latter the profile should in fact be that of k_{cat}/K_m for a poor substrate. The profile is obviously bell-shaped and can be fitted reasonably well to a classical bell curve (solid line of Figure 6), for which it is assumed that there are three forms of the enzyme between pH 4 and 9, but of these, it is only the intermediate species, present in greatest quantity at pH 6.9 in this case, which can interact with the substrate productively (Waley, 1953; Alberty & Massey, 1954). The fit to the classical bell does not appear perfect, however, since there is certainly the suggestion of a shallower decline at low pH and a steeper decline at high pH than allowed by single dissociations. If real, these deviations could indicate the presence of other ionizations occurring on the protein in the pH range considered that also affect activity, a not unlikely situation.

However, given the assumption of a classical bell curve, the data indicate apparent pK_a values of 5.25 and 8.6. These could represent pK_a values for the dissociation of acidic groups on the free enzyme that are required for catalysis, one in its basic form, the other as the free acid (Alberty & Massey, 1954; Peller & Alberty, 1959). In view of the dangers inherent in yielding to this interpretation, however, as emphasized recently, for example, by Knowles (1976), extensive discussion of the nature of these functional groups is not warranted in the absence of further evidence, but, irrespective of interpretation, it is useful to compare the pH–rate profile obtained here with those published earlier by Waley (1975) for the hydrolysis of good substrates (benzylpenicillin and ampicillin). Waley obtains in these cases k_{cat}/K_m profiles yielding pK_as of 4.85 and 8.6 for benzylpenicillin hydrolysis and 5.4 and 8.6 for ampicillin. The good agreement between these values and those from the β-BPA reaction is further evidence for the conclusion arrived at above, i.e., that the kinetics of the β-BPA inactivation are those of the interaction of BCI with a poor substrate.

The agreement between the present higher pK_a and those obtained by Waley is very good and reinforces his suggestion of an enzyme functional group with this pK_a; he suggests either tyrosine or lysine as the likely residue. Since we have shown that tyrosine, specifically tyrosine-105 [using the numbering system of Ambler (1979)], is not essential for β-lactamase activity in BCI (R. Myerowitz, B. L. Wolozin, C. A. Satler, and R. F. Pratt, unpublished results) and that there are indications, derived from chemical modification studies with phenylpropynal, of the requirement for a lysine residue (Schenkein & Pratt, 1980), it seems that at present a protonated lysine amine group is the most likely candidate for an essential acid of pK_a around 8.7. The N-terminal amine group would be another possibility.

Although Waley (1975) has also suggested the existence of an essential carboxyl group with a pK_a corresponding to the lower pK_a of the pH–rate profiles, the variability found in the latter value suggests a kinetic, rather than thermodynamic, source might be likely for this constant.

Note that, although the data have been discussed in terms of an acyl-enzyme intermediate, they do not in themselves require such an intermediate, so that a simpler kinetic scheme such as in eq 3 would be sufficient. Here the initial binding,

$$E + B \underset{K}{\rightleftharpoons} E{\cdot}B \xrightarrow{k_2} E\text{–}B'' \qquad (3)$$

however weak, is retained because of the stereospecificity of the inactivation with respect to substitution at C-6. However, the previous discussion is useful, since β-BPA is certainly a potential substrate and because of recent evidence for an acyl-enzyme intermediate in the case of the *E. coli* β-lactamase (Fisher et al., 1980).

Although, in terms of the enzyme/β-BPA complexes of eq 1 and the kinetics discussed above, the spectral changes and tritium exchange could occur at either stage, E–B′ or E–B″, it does not seem unlikely that all of these changes are associated with the formation of the inhibited enzyme complex E–B″. Certainly, the absorption spectral change must occur at the latter stage, since such changes are not observed with normal substrates. Furthermore, hydrogen exchange at C-6

is not seen to a significant extent during the turnover of normal substrates such as benzylpenicillin (R. Pratt, unpublished observations). Nor has fluorescence quenching been reported, although the absence of this observation might only reflect experimental constraints. The chemistry of the inactivation step, leading to the observations discussed here, is explored at length in the accompanying paper (Cohen & Pratt, 1980).

Conclusions

In summary, the kinetic results available are in accord with the scheme of eq 1 where, after initial reaction of β-BPA as a normal substrate (with acylation of the enzyme possibly rate determining), inactivation follows as a rapid step probably accompanied by the observed spectral changes and hydrogen loss from the 6 position. Since the inhibition would thus stem from diversion of a normal enzyme intermediate rather than simply from reaction of the original compound noncovalently bound at the active site, it seems likely that β-BPA is a suicide substrate of β-lactamases.

It is convenient at this point to summarize the case for β-BPA as a suicide substrate or Trojan horse substrate (Miesowitz & Bloch, 1979) of BCI in terms of the criteria proposed by Abeles & Maycock (1976). Firstly, the inactivation process, as measured by loss of activity, fluorescence quenching, and chromophore development, is a strictly first-order process, which argues against any migration of the inhibitor from the active site either in an intramolecular reaction or via a product species released into solution. Secondly, there is no evidence for release of an inactivating species into solution by the test of successive enzyme addition. Thirdly, the concentration dependence of the inactivation rates is as would be expected from a substrate with the structure of β-BPA; no saturation has been observed, but under the conditions of accessible β-BPA concentrations, this would not be expected. The complete ineffectiveness of α-BPA as a substrate or inhibitor also argues for an active-site role with β-BPA. Fourthly, the rate of inactivation at a given β-BPA concentration is decreased in the presence of a substrate (Pratt & Loosemore, 1978) or an inhibitor, and in the latter case the interaction is competitive. Fifthly, the inactivation is irreversible (Pratt & Loosemore, 1978) and the interaction is stoichiometric with a 1:1 enzyme to substrate ratio being required for inactivation and found to be present on the inactivated enzyme (Cohen & Pratt, 1980). Finally, and most definitively, we have identified both the functional group on the enzyme and the structural changes that the inhibitor has undergone (Cohen & Pratt, 1980). These results are in accord with the kinetic results discussed above and taken together point strongly toward β-BPA as a suicide substrate of BCI.

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