Kinetics of Inactivation of β -Lactamase I by 6 β -Bromopenicillanic Acid

Vroni KNOTT-HUNZIKER,* Barry S. ORLEK,† Peter G. SAMMES† and Stephen G. WALEY*

*Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.,
 and †Department of Organic Chemistry, University of Leeds, Leeds LS2 9JT, U.K.

(Received 11 October 1979)

The kinetics of the inactivation of β -lactamase I from *Bacillus cereus* 569 by preparations of 6 α -bromopenicillanic acid showed unexpected features. These can be quantitatively accounted for on the basis of the inactivator being the epimer, 6 β -bromopenicillanic acid. At pH 9.2, the rate-determining step in the inactivation is the formation of the inactivator. When pure 6 β -bromopenicillanic acid is used to inactivate β -lactamase I, simple second-order kinetics are observed. The inactivated enzyme has a new absorption peak at 326 nm. The rate constant for inactivation has the same value as the rate constant for appearance of absorption at 326 nm; the rate-determining step may thus be fission of the β -lactam ring of 6 β -bromopenicillanic acid. Inactivation is slower in the presence of substrate, and the observed kinetics can be quantitatively accounted for on a simple competitive model. The results strongly suggest that inactivation is a consequence of reaction at the active site.

β -Lactamases, the enzymes that catalyse the hydrolysis of the β -lactam ring in penicillins (e.g. structure I) and cephalosporins, are clinically important enzymes of whose mechanism we know all too little. β -Lactamase I from *Bacillus cereus* was inactivated by preparations of 6 α -bromopenicillanic acid (structure II), but there were, as described below, unexpected features in the kinetics. These apparent anomalies led to the suggestion that the active species was 6 β -bromopenicillanic acid (structure III), which has the same configuration at C-6 as natural penicillins (Knott-Hunziker *et al.*, 1979a). Pratt & Loosemore (1978) had found that the 6 α -bromo epimer (structure II) could be partially converted into the 6 β -bromo epimer (structure III), and that the inactivating power of the mixture depended on the proportion of 6 β -bromo epimer that it contained. We show, in the present paper, that the rate of epimerization governs the rate of inactivation of β -lactamase I by 6 α -bromopenicillanic acid at pH 9.2. The site covalently labelled is serine-44 (Knott-Hunziker *et al.*, 1979b) (on the

numbering system of Thatcher, 1975; serine-70 on the numbering system of Ambler, 1980). This is a conserved residue. Furthermore, there is kinetic evidence for an acyl-enzyme intermediate in the enzymic hydrolysis, probably involving this serine residue (Fisher *et al.*, 1980). Additional evidence that 6 β -bromopenicillanic acid reacts at the active site of β -lactamase I is provided by the experiments reported below on the kinetics of inactivation in the presence of substrate. The observed rates can be adequately explained by a simple scheme.

The inactivation of β -lactamase I by 6 β -bromopenicillanic acid is accompanied by the appearance of u.v. absorption at 326 nm, and Knott-Hunziker *et al.* (1979b) (see also Orlek *et al.*, 1979) suggested that a dihydrothiazine was formed; analogously, 6 α -chloropenicillanic acid reacts with sodium azide, or with sodium methoxide, to give a dihydrothiazine (McMillan & Stoodley, 1968). We have now compared the rates of the two processes, inactivation and formation of the chromophore. Both are second-order reactions, with the same rate

constant. This is a fact that must be taken into account in any detailed description of the mechanism of the inactivation.

Materials and Methods

Materials

6-Bromopenicillanic acids. The 6 α -epimer was prepared and converted into an equilibrium mixture as described previously (Knott-Hunziker *et al.*, 1979a). The reduction of 6,6-dibromopenicillanic acid with tri-*n*-butyltin hydride gave a mixture containing about 50% of the 6 β -bromo epimer. The kinetics described below were carried out with a purified specimen of the 6 β -bromopenicillanic acid kindly provided by Pfizer Central Research, Sandwich, Kent, U.K.

Other materials

β -Lactamase I was prepared as previously described (Davies *et al.*, 1974; Kiener, 1976).

Methods

Assays of β -lactamase I were carried out with benzylpenicillin as substrate, either in the pH-stat or spectrophotometrically (Waley, 1974), in 0.5M-NaCl/1mM-EDTA at 30°C. The ^1H n.m.r. spectra in $^2\text{H}_2\text{O}$ were measured as described by Kiener & Waley (1978).

Results and Discussion

Inactivation by preparations of 6 α -bromopenicillanic acid at pH 7

At pH 7 the main features of inactivation of β -lactamase I by preparations of 6 α -bromopenicillanic acid suggested that a minor component in the preparation of the 6 α -bromopenicillanic acid might be reacting with the enzyme; it would need less than 1% of stoichiometrically reacting minor component to account for the results shown.

Inactivation by 6 α -bromopenicillanic acid at pH 9.2

At pH 9.2, on the other hand, inactivation of β -lactamase I by 6 α -bromopenicillanic acid was progressive and did not come to a halt before all the enzyme was inactivated. Nevertheless the kinetics showed unexpected features. The initial rate of inactivation, although proportional to the concentration of reagent, was independent of the concentration of enzyme. This suggested that the rate-determining step was conversion of the 6 α -bromopenicillanate (A) into a species (B), which then inactivated the enzyme in a relatively rapid step. It may be shown that the kinetics of inactivation can be quantitatively accounted for by the formation of a reactive species B. The extent of conversion of A into B is small (of the order of 0.1%) in these

experiments, but the process is readily detected because the concentration of reagent is much larger than the concentration of enzyme.

Inactivation with 6 β -bromopenicillanic acid: rates of inactivation and of appearance of chromophore

Pratt & Loosemore (1978) suggested that the compound gradually formed at pH 9.2 was the epimeric 6 β -bromopenicillanic acid (structure III), because the inactivating power of the solution developed in parallel with the appearance of a compound in solution with spectroscopic (n.m.r.) properties consistent with those expected for 6 β -bromopenicillanic acid (Loosemore & Pratt, 1978). Results with material containing only 5% of the 6 β -bromopenicillanic acid misleadingly suggested that concurrent hydrolysis might be competing with inactivation (Knott-Hunziker *et al.*, 1979a), but with pure 6 β -bromopenicillanic acid there is no evidence for any hydrolysis. It is the kinetic results obtained with the pure material that are now described.

Comparison of rates of inactivation and of appearance of chromophore

The inactivation of β -lactamase I by 6 β -bromopenicillanic acid is accompanied by the appearance of u.v. absorbance at 326 nm (Knott-Hunziker *et al.*, 1979b). The kinetics of inactivation, and of the spectroscopic change, are now described, and compared.

Inactivation was second-order (Fig. 1) over the range of concentrations that could conveniently be studied. The intermediate formation of a non-covalent enzyme-inactivator complex might be expected, but it is not surprising that it is not detectable kinetically when low and comparable concentrations of both species are used, and higher concentrations could not be used without the rates becoming too fast. The appearance of the absorbance at 326 nm also obeyed second-order kinetics. Moreover, the increase in A_{326} and the inactivation proceeded at the same rate (Table 1). Knott-Hunziker *et al.* (1979b) suggested that the increase in A_{326} was due to formation of a 2,3-dihydro-1,4-thiazine; the present results show that, if the reaction of the hydroxy group of serine-44 of the enzyme with 6 β -bromopenicillanic acid takes place in two stages, then the first stage (presumably opening the β -lactam ring and forming the penicilloyl-enzyme) is the rate-determining one.

The rate constant is quite large, about $10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$. The ring-opening and rearrangement of 6 β -bromopenicillanic acid occurs readily in aqueous solution at pH 7, the reaction being complete in a few minutes on treatment with 0.1M-hydroxylamine (S. G. Waley, unpublished work). However, the reaction with the enzyme is much more rapid than this model reaction ($k \sim 1 \text{ min}^{-1}$ at 0.1M-

Fig. 1. Inactivation of β -lactamase I by 6 β -bromopenicillanate

β -Lactamase I ($0.2 \mu\text{M}$) and the sodium salt of 6 β -bromopenicillanate ($0.2 \mu\text{M}$) were kept together at 0°C in $50\text{mM-NaH}_2\text{PO}_4/50\text{mM-K}_2\text{HPO}_4$ containing 0.5M-NaCl and 1mM-EDTA ; the volume of the reaction mixture was 0.5ml , and $20\mu\text{l}$ portions were taken for assay. The concentration of inactivated enzyme at time t was calculated as $b(u_0 - u)/u_0$, where b is the initial concentration of β -lactamase I and u_0 and u are the enzymic activities initially and at time t respectively.

NH_2OH would correspond to $k \sim 10\text{M}^{-1} \cdot \text{min}^{-1}$ for a second-order rate constant), and so some features of enzymic catalysis probably play a part in the inactivation. This conclusion, however, does assume that inactivation is a consequence of reaction at the active site of the enzyme. This assumption can be tested by studying inactivation in the presence of substrate, and such experiments are now described.

Inactivation by 6 β -bromopenicillanate in the presence of benzylpenicillin

The rate (and extent) of hydrolysis of benzylpenicillin by β -lactamase I was measured in the presence and absence of 6 β -bromopenicillanate (Fig. 2). Comparison of the rates shows the effect of the inactivator. 'Protection' of the enzyme by the substrate can be detected and accounted for, even with a readily hydrolysed substrate in these 'combined incubations'. An alternative procedure (pre-incubation) is to measure inactivation directly, with and without substrate present. This procedure entails the use of high concentrations of substrate;

Table 1. Rate constants for inactivation and for appearance of chromophore

β -Lactamase I and the sodium salt of 6 β -bromopenicillanate were present in $50\text{mM-NaH}_2\text{PO}_4/50\text{mM-K}_2\text{HPO}_4$ containing 0.5M-NaCl and 1mM-EDTA (the pH 7 buffer) (total volume 0.5ml) at 0°C . Portions of volume $20\mu\text{l}$ or $50\mu\text{l}$ were taken for assay by the spectrophotometric method (Waley, 1974). The concentrations of β -lactamase I and of 6 β -bromopenicillanate varied from 0.2 to $0.4 \mu\text{M}$ and from 0.2 to $0.8 \mu\text{M}$ respectively. The appearance of chromophore was measured as follows. β -Lactamase I ($5\text{--}15\mu\text{l}$ of 10mg/ml solution) was added to 6 β -bromopenicillanate in 1ml of the pH 7 buffer at 0°C in the cuvette (1cm light-path) of a Cecil model 272 grating spectrophotometer (Cecil Instruments, Cambridge, U.K.) and the absorbance at 326nm recorded. The concentrations of β -lactamase I and of 6 β -bromopenicillanate varied from 2 to $4 \mu\text{M}$ and from 3 to $8 \mu\text{M}$ respectively. The values for the second-order rate constants k are given as the means $\pm$ S.E.M., with the numbers of experiments in parentheses.

Type of experiment	k ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)
Inactivation	0.77 ± 0.04 (10)
Appearance of chromophore	0.78 ± 0.07 (19)

Fig. 2. Hydrolysis of benzylpenicillin by β -lactamase I in the presence of 6 β -bromopenicillanate

The hydrolysis of 3mM -benzylpenicillin in 2ml of $0.5\text{M-NaCl}/1\text{mM-EDTA}$ in the pH-stat at pH 7 at 30°C by 1.5mM - β -lactamase I in the presence of $3 \mu\text{M}$ -6 β -bromopenicillanate (Δ) and in the absence of inactivator (O) is shown.

thus Pratt & Loosemore (1978) used 100mM -benzylpenicillin in an experiment of this kind; even so, there is considerable hydrolysis of the substrate

during the preincubation, and the 'combined incubation' procedure was preferred. The control hydrolysis in Fig. 2 is zero-order, the concentration of substrate (3 mM) being much greater than the K_m (0.077 mM; Waley, 1975). The velocity of the hydrolysis in the presence of 6 β -bromopenicillanate decreases. Although the velocity cannot be measured as accurately as the amount of product, it is simpler to compare the results with theoretical equations when the measured rates rather than the amounts of product are used. The rates of hydrolysis with (v_i) and without (v) inactivator were compared by plotting $\ln(v/v_i)$ against time (Fig. 3). The slopes of the straight lines obtained were directly proportional to the concentration of inactivator (Fig. 4) and inversely proportional to the concentration of substrate (Fig. 5). This behaviour can be accounted for, and the rate constant for inactivation inferred for comparison with the measured value, by the simple treatment now given. Aspects of the kinetics are given by Goldstein (1944) and Webb (1963), and have been described by Labia & Peduzzi (1978), and a similar situation has been discussed by Laidler & Bunting (1973). The scheme is:

where E, S, P and B stand for enzyme, substrate, product and inactivator (here 6 β -bromopenicillanic acid) respectively. The concentration of the inactivator may be regarded as constant, its concentration (1–4 μM) being much greater than that of the enzyme (about 1 nM).

Fig. 4. Variation of the slope (C) with concentration of inactivator

Experiments of the kind shown in Figs. 2 and 3 were performed at different concentrations of 6 β -bromopenicillanate, and the slope (C) in plots of $\ln(v/v_i)$ against time is plotted against concentration of inactivator. The concentrations of enzyme and substrate were 1.5 nM and 3 mM respectively.

Fig. 3. Progressive inactivation of β -lactamase I by 6 β -bromopenicillanate

The rates of the hydrolyses shown in Fig. 2 were measured as $(p_2 - p_1)/(t_2 - t_1)$, where p_1 and p_2 are the amounts of product (μmol of NaOH added) at times t_1 and t_2 . The interval $t_2 - t_1$ was 1 min and the rate was taken as applying at $\frac{1}{2}(t_1 + t_2)$. The rate of the control hydrolysis (v) was constant, but in the presence of inactivator the rate (v_i) decreased. The slope of the plot of $\ln(v/v_i)$ against time is referred to as C .

Fig. 5. Variation of the slope (C) with concentration of substrate

Experiments of the kind shown in Figs. 2 and 3 were performed at different concentrations of substrate (benzylpenicillin), and the slope C was plotted against the reciprocal of the substrate concentration. The concentration of enzyme was 0.75 nM or 1.5 nM, and the concentration of inactivator was 4 μM .

If the concentrations of the species are denoted by the lower-case symbols, the steady-state approximation gives, for the enzyme-substrate complex:

$$es = (e_0 - x)s/(s + K_m)$$

where $e + es + x = e_0$, the total concentration of enzyme, and $K_m = (k_{-3} + k_{+4})/k_{+3}$. In the present experiments, the initial concentration of substrate (s_0) was 1–3 mM, and, since $K_m = 0.077$ mM (Waley, 1975), the value of $K_m/(K_m + s)$, which is the fraction of active enzyme present as free enzyme (rather than as enzyme-substrate complex), changes little, and may be taken as approximately equal to K_m/s_0 . It may be shown that, since $e_0/(e_0 - x) = v/v_1$, where v and v_1 are the rates of hydrolysis without and with inactivator respectively:

$$\ln\left(\frac{v}{v_1}\right) = \left(\frac{k_{+2} K_m b}{s_0}\right) \cdot t \quad (1)$$

The linear relationship implied by this equation is obeyed (Fig. 3), with a slope (C) proportional to b (Fig. 4) and inversely proportional to s_0 (Fig. 5), and moreover the value of k_{+2} obtained from the slope in Fig. 5 is $2.2 \mu\text{M}^{-1} \cdot \text{min}^{-1}$, closely similar to the value obtained directly ($2.1 \mu\text{M}^{-1} \cdot \text{min}^{-1}$, Table 3). This agreement shows that binding of inactivator is competitive with substrate binding: one interpretation is that reaction occurs at the active site of the enzyme. In fact, the structural resemblance between substrate and inactivator makes this the most likely interpretation.

The situation at the end of the reaction is that all the enzyme has been inactivated but not all of the substrate has been hydrolysed. It may be shown that the final concentration of substrate (s_∞) is:

$$s_\infty = s_0 \exp(-k_{+4} e_0/k_{+2} K_m b) \quad (2)$$

The extent of formation of product, $(s_0 - s_\infty)/s_0$, thus calculated is compared with the observed values in Table 2; the agreement is satisfactory.

Variation of rate of inactivation with temperature and with pH

Inactivation, which followed second-order kinetics under all the conditions examined, gave the rate constants shown in Table 3. Comparison of the rates of inactivation at 30°C with those at 0°C gave an approximate value of 23 kJ/mol for the energy of activation; the same (relatively low) value has been reported for the enzymic hydrolysis of benzylpenicillin (Abraham & Newton, 1956). A non-covalent complex between 6 β -bromopenicillanate and enzyme formed exothermically would contribute to a low value for the energy of activation.

The rate of inactivation is appreciably higher at pH 7 than it is at pH 5 or at pH 9. Similarly, the values of k_{cat} for the hydrolysis of benzylpenicillin

Table 2. Extent of reaction when benzylpenicillin is hydrolysed by β -lactamase I in the presence of 6 β -bromopenicillanate

The reaction was carried out at pH 7 at 30°C in 0.5 M-NaCl/1 mM-EDTA in the pH-stat. The concentrations of enzyme and substrate were 1.5 nM and 3 mM respectively. The extent of reaction was calculated from eqn. (3) in the text, with values of $k_{+4} = 1.44 \times 10^3 \text{ min}^{-1}$, $K_m = 0.077$ mM (Waley, 1975) and $k_{+2} = 2.12 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ (from Table 3).

Concn. of 6 β -bromopenicillanate (μM)	Extent of final conversion of substrate into product (%)	
	Observed	Calculated
2	55	48
3	43	36
4	23	28

Table 3. pH-dependence of inactivation of β -lactamase I by 6 β -bromopenicillanate

Inactivations were carried out at 30°C; the concentrations of enzyme was 30 nM and the concentration of inactivator varied from 50 to 300 nM. The buffers were: 50 mM-sodium acetate/50 mM-acetic acid, pH 5; 50 mM- NaH_2PO_4 /50 mM- K_2HPO_4 , pH 7; 50 mM- KHCO_3 /50 mM- NaCO_3 , pH 9; in each case containing 0.5 M-NaCl and 1 mM-EDTA. Portions were taken for assay as described in the legend to Table 1. The values for the second-order rate constant k are given as the means $\pm$ s.d., with the numbers of experiments in parentheses.

pH	k ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)
5	0.76 ± 0.12 (4)
7	2.12 ± 0.54 (9)
9	0.35 ± 0.03 (3)

show a maximum (at pH 6.7) (Waley, 1975). Nothing is known of the groups (or factors) responsible for the pH optimum of the enzymic reaction.

The inactivation of β -lactamase I by the epimeric mixture containing 6 β -bromopenicillanate was slowed by the presence of 100 mM-benzylpenicillin (Pratt & Loosemore, 1978). This experiment led Pratt & Loosemore (1978) to suggest that the dissociation constant of the enzyme-6 β -bromopenicillanate complex was of the order of 1 nM. However, this cannot be so, since the inactivation obeys second-order kinetics, and a second-order rate constant of $2 \mu\text{M} \cdot \text{min}^{-1}$ combined with a dissociation constant of 1 nM leads to a 'saturation' rate constant of only 0.002 min^{-1} , corresponding to a half-life of about 350 min, which is much larger than is observed.

Few other studies of the kinetics of inactivation of β -lactamases have been reported. The inactivation of the RTEM β -lactamase from *Escherichia coli* by clavulanic acid both without (Fisher *et al.*, 1978) and with (Labia & Peduzzi, 1978) added substrate has been investigated, but the existence of a variety of forms of inactivated enzyme (Charnas *et al.*, 1978) complicates the interpretation of the kinetic observations. In these experiments the clavulanic acid is extensively hydrolysed, but in contrast there is little or no hydrolysis when it inactivates the β -lactamases from *Staphylococcus aureus* (Cartwright & Coulson, 1979; Reading & Hepburn, 1979), and some hydrolysis when it inactivates β -lactamase I (Durkin & Viswanatha, 1978; S. G. Waley, unpublished work). It seems that more work is needed before simple generalizations emerge. But meanwhile the use of β -lactams as inactivators is at last throwing light on the active sites of β -lactamases.

The support of the Science Research Council and the Medical Research Council is gratefully acknowledged. We thank Glaxo Research Laboratories, Beecham Research Laboratories and Pfizer Central Research for gifts, Professor E. P. Abraham for helpful discussions and Professor J. R. Knowles and Dr. R. P. Ambler for sending us manuscripts before publication.

References

- Abraham, E. P. & Newton, G. G. F. (1956) *Biochem. J.* **63**, 628–634
- Ambler, R. P. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, in the press
- Cartwright, S. J. & Coulson, A. F. W. (1979) *Nature (London)* **278**, 360–361
- Charnas, R. L., Fisher, J. & Knowles, J. R. (1978) *Biochemistry* **17**, 2185–2189
- Davies, R. B., Abraham, E. P. & Melling, J. (1974) *Biochem. J.* **143**, 115–127
- Durkin, J. P. & Viswanatha, T. (1978) *J. Antibiot.* **31**, 1162–1169
- Fisher, J., Charnas, R. L. & Knowles, J. R. (1978) *Biochemistry* **17**, 2180–2184
- Fisher, J., Belasco, J. G., Charnas, R. L., Khosla, S. & Knowles, J. R. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, in the press
- Goldstein, A. (1944) *J. Gen. Physiol.* **27**, 529–580
- Kiener, P. A. (1976) D.Phil. Thesis, University of Oxford
- Kiener, P. A. & Waley, S. G. (1978) *Biochem. J.* **169**, 197–204
- Knott-Hunziker, V., Orlek, B. S., Sammes, P. G. & Waley, S. G. (1979a) *Biochem. J.* **177**, 365–367
- Knott-Hunziker, V., Waley, S. G., Orlek, B. S. & Sammes, P. G. (1979b) *FEBS Lett.* **99**, 59–61
- Labia, R. & Peduzzi, J. (1978) *Biochim. Biophys. Acta* **526**, 572–579
- Laidler, K. J. & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, p. 177, Clarendon Press, Oxford
- Loosemore, M. J. & Pratt, R. F. (1978) *J. Org. Chem.* **43**, 3611–3613
- McMillan, I. & Stoodley, R. J. (1968) *J. Chem. Soc. C* 2533–2537
- Orlek, B. S., Sammes, P. G., Knott-Hunziker, V. & Waley, S. G. (1979) *J. Chem. Soc. Chem. Commun.* 962–963
- Pratt, R. F. & Loosemore, M. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4145–4149
- Reading, C. & Hepburn, P. (1979) *Biochem. J.* **179**, 67–76
- Thatcher, D. R. (1975) *Biochem. J.* **147**, 313–326
- Waley, S. G. (1974) *Biochem. J.* **139**, 789–790
- Waley, S. G. (1975) *Biochem. J.* **149**, 547–551
- Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors*, vol. 1, p. 553, Academic Press, New York