Irreversible inactivation of β -lactamase I from *Bacillus cereus* by chlorinated 6-spiroepoxypenicillins

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On incubation of the chlorinated 6-spiroepoxypenicillin anilides (I) and (II)

with β -lactamase I from *Bacillus cereus*, three distinct processes are observed. The inhibitors act as (a) substrates, the turnover of which respectively results in a single product, namely 6-substituted 2(*H*)-3,4-dihydro-1,4-thiazine, (b) a transiently inhibited enzyme complex, and finally (c) an irreversibly inactivated enzyme complex. Although differing only in their stereochemistry at one centre, the anilide (I) is a more potent irreversible inactivator of β -lactamase I than is compound (II). Analysis of irreversibly inactivated β -lactamase I by isoelectric focusing and inspection of peptide fragmentation maps indicated that irreversible inactivation appears to be accompanied by covalent modification. These studies reveal that the chlorinated 6-spiroepoxypenicillin anilide (I) is a mechanism-based β -lactamase inhibitor.

INTRODUCTION

β -Lactamase-mediated resistance to therapeutically important penicillins and cephalosporins severely limits the use of this class of antibiotics. β -Lactamases inactivate these antimicrobial agents by hydrolysing the β -lactam ring, which is prerequisite for their antibacterial activity. To overcome these problems, attempts have been made to develop either β -lactamase-stable antibiotics or β -lactamase inhibitors. The second approach, which neutralizes the hydrolytic activity of the β -lactamase, permits the use of otherwise redundant antibiotics against resistant bacteria. A rational approach to the design of such compounds is limited by the lack of sufficient detailed structural and mechanistic knowledge of β -lactamases. The study of a number of mechanism-based β -lactamase inhibitors, in particular the clinically important clavulanic acid (Charnas & Knowles, 1981), penicillanic acid sulphone (Brenner & Knowles, 1984a), 6-acetylmethylene-penicillanic acid (Arisawa & Then, 1983), 6-(methoxymethylene)-penicillanic acid (Brenner & Knowles, 1984b) and 6- β -bromo-penicillanic acid (Orlek *et al.*, 1979, 1980), has nevertheless contributed to this important area. Typical class A β -lactamases (Ambler, 1980), e.g. the R-TEM series from *Escherichia coli* and β -lactamase I from *Bacillus cereus*, recognize the above inhibitors as substrates, but deviation from the 'normal' catalytic pathway leads to the formation of an inactive acylated enzyme complex. Most of these, all of which contain a heteroatom at position 1 (Knowles, 1985), show not only irreversible enzyme inactivation but also alternative re-arrangement pathways leading to transient reversible inhibition. Experimental evidence supports the view that all these pathways centre around a transient acyl-enzyme intermediate formed by the nucleophilic attack of an active-site serine hydroxy group on the β -lactam carbonyl group.

It is generally agreed that β -lactams exert their antibacterial activity by acylating the active-site serine residue of bacterial transpeptidases, thus preventing peptidoglycan cross-wall formation. There is evidence that these target enzymes, which are part of a larger group known as penicillin-binding proteins, are related to the β -lactamases (Waxman & Strominger, 1980; Samraoui *et al.*, 1986). Indeed, some groups of β -lactam antibiotics possess both antibacterial and β -lactamase-inhibitory activity.

We have previously reported the synthesis of two novel chlorinated 6-spiroepoxypenicillin anilides (I) and (II) that show dual antimicrobial and β -lactamase inhibitory action (Bycroft *et al.*, 1988a). These anilides differ only with respect to their stereochemistry at the C-3 position of the side chain, but show marked differences in their biological activities (Gledhill *et al.*, 1987; Gledhill, 1988). Compound (I) possesses notable Gram-positive antibacterial activity and is a potent class A β -lactamase inhibitor. Conversely compound (II) is antibacterially inactive and is a relatively poor β -lactamase inhibitor. Thus these compounds offer the opportunity for detailed molecular studies into the active-site relationship between penicillin-binding proteins and β -lactamases. In the present paper we describe the

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kinetics of inhibition of β -lactamase I from *Bacillus cereus* by compound (I), which indicates that this highly modified penicillin is a mechanism-based inhibitor.

MATERIALS AND METHODS

Materials

Benzylpenicillin and nitrocefin were obtained from Sigma Chemical Co. and Glaxo Research respectively. The chlorinated 6-spiroepoxybenzylpenicillin anilides (I) and (II) were synthesized as described previously (Bycroft *et al.*, 1988a).

β -Lactamase I from *Bacillus cereus* 569H was isolated from a crude mixture of β -lactamase I and β -lactamase II obtained from the Centre for Applied Microbiology and Research (Porton Down, Wilts., U.K.). β -Lactamase I was purified by using CM-Trisacryl cation-exchange chromatography (Pharmacia-LKB, Milton Keynes, Bucks., U.K.) essentially as described by Davies *et al.* (1974). Protein concentrations were determined by the method of Lowry *et al.* (1951), with BSA (fraction V; Sigma Chemical Co.) as a standard, and by activity towards the substrates nitrocefin and benzylpenicillin. Kinetic analysis of pure β -lactamase I with nitrocefin as substrate gave a $V_{\max}$ of $4.5 \times 10^{-5} \text{ mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and a K_m of $98 \mu\text{M}$ (k_{cat} $1.5 \times 10^3 \text{ min}^{-1}$). Using benzylpenicillin as substrate gave a $V_{\max}$ of $3.9 \times 10^{-3} \text{ mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and a K_m of $78 \mu\text{M}$ (k_{cat} $1.2 \times 10^5 \text{ min}^{-1}$). These values are in close agreement with those reported by Christensen *et al.* (1990).

Determination of enzyme activity and inhibition

Enzyme activity was measured by spectrophotometric assay in a Perkin-Elmer 554 UV-VIS spectrophotometer fitted with a thermostat-controlled heated cell holder, by adding enzyme samples either to benzylpenicillin (Waley, 1974) and monitoring the decrease in absorbance at 232 nm, or to nitrocefin (O'Callaghan *et al.*, 1972) and measuring the increase in absorbance at 500 nm. All assay reactions were performed in 0.5 M-NaCl/50 mM-Tris buffer, pH 7.0, at 30 °C.

The appearance of a new chromophore absorbing at 350 nm on hydrolysis of both compounds (I) and (II) was utilized to measure K_m and $V_{\max}$ for these substrates by using the initial-rate method.

Inhibition studies were performed in either 50 mM- or 100 mM-Mops buffer, pH 7.0, containing 0.5 M-NaCl at 30 °C.

Isoelectric focusing

Isoelectric focusing was carried out on preformed LKB Ampholine polyacrylamide plates with a pH range of 3.5–9.5 at 10 °C by using an LKB 2117 Multiphor I electrophoresis unit at a constant power of 10 W for 2.5 h. Isoelectric points were determined by comparison with the focused positions of reference proteins (Electran 4.7–10.6 isoelectric-point marker kit; BDH Chemicals). Isoelectric focusing was used to monitor the change in isoelectric point of β -lactamase I due to inhibition by compound (I). To retain adequate enzyme concentrations for direct gel loading and to expose the β -lactamase to sufficient inhibitor, β -lactamase I was withdrawn from a dialysis bag suspended in buffered inhibitor solution.

Peptide fragmentation patterns of inhibited β -lactamase I

β -Lactamase I (0.7 mg) was incubated with 17 mg of compound (I) in 5 ml of 100 mM-Mops buffer, pH 7.0, containing 0.5 M-NaCl and maintained at 30 °C. After 100 min, 12% of the original activity remained. The reaction was dialysed against

2 × 2 litres of 1 mM-Mops buffer, pH 7.0, containing 1 mM-NaCl for 48 h at 4 °C. Exhaustive dialysis was completed against 2 litres of 1 mM-Mops buffer, pH 7.0, for a further 24 h at 4 °C. The inactivated enzyme was freeze-dried to yield a pale-yellow powder.

Inactivated enzyme in 1% (w/v) NH_4HCO_3 (100 μl) was treated with 0.2 mg of trypsin (type XIII, 1-chloro-4-phenyl-3-tosylaminobutan-2-one-treated; Sigma Chemical Co.), dissolved in 1 mM-HCl (20 μl), for 2.5 h (37 °C). The mixture was diluted to 1 ml with water and the u.v.-absorption spectrum analysed. The digest was freeze-dried in 100 μl portions. The above procedure was repeated without inhibitor and the digest was used as a control.

Peptide fragments were separated on a C_{18} reverse-phase silica column (Hichrom S50DS2 Spherisorb, 25 cm × 0.49 cm) by using a gradient elution system provided by two LKB Bromma 2150 pumps controlled by an LKB Bromma 2152 h.p.l.c. unit. The peak elution profile was monitored at 230 and 320 nm almost simultaneously by two variable-wavelength monitors linked in series. Digested samples were dissolved in 60 μl of elution buffer A (50 mM-ammonium bicarbonate buffer, pH 7.83) and injected on to the h.p.l.c. column by using a 50 μl loading loop. Gradient elution was performed by controlled changes in the ratio of buffers A and B [20% (v/v) 50 mM-ammonium bicarbonate buffer, pH 7.83, in acetonitrile]. Peptide fragments were separated by a linear elution gradient from 1% to 60% (v/v) buffer B (30 min) and the column was washed with a gradient from 60% to 100% (v/v) buffer B (5 min). A return to 1% (v/v) buffer B (1 min) prepared the system for the next injection. Eluted fragments showing strong absorbance at 320 nm were collected and their full u.v. spectra were analysed.

Elution profiles produced from digests of inhibited β -lactamase were compared with uninhibited controls and in turn with blank injections in order to eliminate changes in gradient baseline.

RESULTS

Compounds (I) and (II) as substrates

Reaction of compound (I) with β -lactamase I was monitored by u.v.-absorption spectroscopy, which revealed the production of a new absorption maximum at 350 nm (ϵ 6800 $\text{M}^{-1} \cdot \text{cm}^{-1}$; Fig.

Fig. 1. Changes in the absorption spectra of compound (I) during hydrolysis by β -lactamase I

Compound (I) (0.13 mM) was incubated at 30 °C with β -lactamase I (0.37 μM) in 50 mM-Tris/HCl buffer, pH 7.0, containing 0.5 M-NaCl. The 1 ml solution was scanned against a reference cell [at 5 min intervals, from 1 (0 min) to 7 (30 min)] containing β -lactamase I (0.37 μM) in the same buffer.

Table 1. Rate constants for the hydrolysis of compounds (I) and (II) by β -lactamase I

Compound	K_m (μM)	V_{max} ($\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	k_{cat} (min^{-1})	Turnover/ inactivation
(I)	735	1.13×10^{-6}	32.8	2083:1
(II)	962	7.63×10^{-6}	221.0	10800:1

Fig. 2. Time courses for the irreversible inactivation of β -lactamase I by compounds (I) and (II)

β -Lactamase I ($4.6 \mu\text{M}$) was allowed to react with compound (I) ($\circ$) and compound (II) ($\square$) at a concentration of 9.0 mM in 50 mM -Mops buffer, pH 7.0, containing 0.5 M -NaCl at 30°C . At various time intervals, $20 \mu\text{l}$ reaction samples were removed and mixed with 1 ml of benzylpenicillin (1.34 mM). Then $100 \mu\text{l}$ of this solution was added to 1 ml of 1.34 mM -benzylpenicillin solution. After a 10 min incubation period, a sample from this dilution was added to 1 ml of benzylpenicillin (1.34 mM) and the reaction was monitored spectrophotometrically as described in the Materials and methods section.

1). Similar results were obtained on incubation of compound (II) with β -lactamase I ($\epsilon 6900 \text{ M}^{-1} \cdot \text{cm}^{-1}$). When the same reactions were monitored by h.p.l.c. [C_{18} reverse-phase column with isocratic elution with 0.1 M -phosphate buffer, pH 3.96, in 30% (v/v) acetonitrile], the turnover of both compound (I) (retention time 9.4 min) and compound (II) (retention time 14.0 min) resulted in the concomitant production of essentially one compound with a retention time of 6.4 min . The appearance of this single turnover product was verified by using silica t.l.c. [R_f 0.3 in chloroform/methanol/acetic acid ($4:1:1$, by vol.)]. This turnover product has subsequently been characterized and found to be a 6-substituted 1,4-dihydrothiazine-3-carboxylate α -ketoamide (Bycroft *et al.*, 1988b).

K_m and V_{max} (Table 1) were subsequently determined by using an initial-rate method to minimize possible effects from progressive irreversible inhibition.

Progressive irreversible inactivation

Progressive irreversible inactivation of β -lactamase I by compounds (I) and (II) was measured after decay of the transiently stable complex by dilution of the reaction mixtures into benzylpenicillin. Both isomers gave biphasic reaction courses (Fig. 2) at

Fig. 3. Logarithmic plot of the time course for the irreversible inactivation of β -lactamase I by compound (I)

Data are derived from Fig. 2.

Fig. 4. Effect of inhibitor/enzyme molar ratio on the extent of irreversible inactivation

Experiments were performed at 30°C in 50 mM -Mops buffer, pH 7.0, containing 0.5 M -NaCl. (a) Concentrations ranging from 0 to 3.95 mM of compound (I) were incubated with β -lactamase I ($1.2 \mu\text{M}$, $\circ$; $2.5 \mu\text{M}$, $\square$; $2.8 \mu\text{M}$, $\triangle$) at 30°C for 3 h. Then $10 \mu\text{l}$ samples of the reaction mixtures were withdrawn and assayed for residual enzyme activity by incubation with 0.9 ml of nitrocefin ($77 \mu\text{M}$) as described in the Materials and methods section. (b) Concentrations ranging from 0 to 5.5 mM of compound (II) were incubated with β -lactamase I ($0.6 \mu\text{M}$, $\bullet$; $2.5 \mu\text{M}$, $\blacksquare$) at 30°C for 3 h and the residual enzyme activity was measured as described in the Materials and methods section.

a concentration of 9.0 mM . A rapid fall to 70% residual enzyme activity was seen in the first 3 min, followed by a second, more gradual, phase lasting 200 min. Complete hydrolysis of compounds (I) and (II) by β -lactamase I resulted in residual enzyme activities of 10% for the former and 55% for the latter. Re-analysis of data for inhibition by compound (I) gave the first-

Fig. 5. Decay of transiently inhibited enzyme after incubation with compound (I) monitored by dilution into nitrocefin substrate

β -Lactamase I ($1.86 \mu\text{M}$) was incubated for 22 min without (curve A) or with (curve B) 4.58 mM -compound (I) in 100 mM -Mops buffer, pH 7.0, containing 0.5 M -NaCl at 30°C . Samples ($20 \mu\text{l}$) were then withdrawn and diluted into 1 ml of nitrocefin ($85.3 \mu\text{M}$) in 50 mM -Mops buffer, pH 7.0, containing 0.5 M -NaCl. The change in absorbance at 500 nm was monitored spectrophotometrically as a measure of enzyme recovery.

Fig. 6. Isoelectric-focusing gel showing the shift in isoelectric points of β -lactamase I isoenzymes following irreversible inactivation by compound (I)

A 0.5 ml portion of β -lactamase I ($33 \mu\text{M}$) was dialysed against 15 ml of a 2.27 mM solution of compound (I) at 30°C in 50 mM -Mops buffer, pH 7.0, containing 0.5 M -NaCl. At appropriate time intervals $50 \mu\text{l}$ samples of the enzyme were withdrawn, $20 \mu\text{l}$ of which was assayed for residual enzyme activity with nitrocefin as substrate. The remaining $30 \mu\text{l}$ was focused on isoelectric-focusing gels (pH range 3.5–9.5) and after electrophoresis stained with Coomassie Brilliant Blue. Lane A, native β -lactamase I isoenzymes; lanes B–E, inhibited β -lactamase I isoenzymes with percentage inhibition of 6% (B), 40% (C), 58% (D) and 65% (E).

order rate constant of $4.3 \times 10^{-4} \text{ s}^{-1}$ for the second phase of the inactivation process (Fig. 3).

Number of hydrolytic events required for enzyme inactivation

Incubation of β -lactamase I with a range of molar excesses of either compound (I) or compound (II) revealed that a linear relationship exists between residual enzyme activity and initial molar ratio (Fig. 4). Extrapolation of this relationship to zero residual activity demonstrated that compound (I) is 5 times more efficient as an irreversible inhibitor of β -lactamase I than is compound (II) (Table 1). Similar experiments with greater enzyme concentrations, and therefore inhibitor concentrations to retain initial molar ratios, had no effect on the final residual

Fig. 7. Peptide fragmentation maps obtained after tryptic digestion of native and inhibited β -lactamase I

Both native β -lactamase (a) and β -lactamase inhibited by compound (I) (b and c) samples were digested with trypsin and the resulting peptide fragments were analysed by h.p.l.c. (a) Peptide elution profile of native β -lactamase I monitored at 230 nm ; (b) elution profile of β -lactamase I inhibited by compound (I) and monitored at 230 nm ; (c) elution profile of β -lactamase I inhibited by compound (I) and monitored at 320 nm .

enzyme activity. This indicates that the extent of irreversible inactivation of β -lactamase I by compounds (I) and (II) was due to the number of turnover events and not to the initial inhibitor concentration.

Incubation periods of 3 h were sufficient to allow the reactions to reach completion and to eliminate the transiently stable complex. Prolonged incubation (10 h) of the assay reactions did not result in recovery of enzyme activity, indicating the irreversible nature of the inactivation.

Decay of transiently stable enzyme-inhibitor complex

β -Lactamase I was preincubated with compound (I), and samples were withdrawn and diluted into nitrocefin solution. An example of a progress curve of nitrocefin hydrolysis for a partially inhibited enzyme mixture (preincubation for 22 min) compared with preincubated non-inhibitor-exposed control enzyme is shown in Fig. 5. A number of features were apparent from this type of curve. Unlike the control reaction, the initial rate of nitrocefin hydrolysis was almost zero, indicating that at first there was little or no free enzyme to react with the nitrocefin. This hydrolysis then accelerated, indicating recovery of enzyme activity. This acceleration continued for up to 3 min, resulting in a constant rate.

The data from this type of curve were processed in accordance with the graphical method of Glick *et al.* (1978), giving rate constants for the decay of transient inhibition of $9.8 \times 10^{-3} \pm 2.5 \times 10^{-3} \text{ s}^{-1}$ (mean $\pm$ S.D.). This was shown to be independent of inhibitor exposure time (within 5–30 min pre-exposure at 4.58 mM).

Characteristics of irreversibly inactivated β -lactamase I

Three β -lactamase I isoenzymes with pI values of 8.7, 8.6 and 8.4 were observed by analytical isoelectric focusing (Fig. 6). After incubation with an excess of compound (I), isoenzymes focusing at new positions corresponding to pI values of 8.1 and 7.9 were observed (Fig. 6). To investigate this modification further, a tryptic digest was prepared, the u.v.-absorption spectrum of which showed a new chromophore absorbing at 350 nm. Reverse-phase h.p.l.c. of this digest monitored at 230 nm (Fig. 7) revealed a modified peptide profile compared with that of the native enzyme digest. In addition, simultaneous monitoring of the peptide elution profile at 320 nm showed that, unlike the native protein, a number of peptide fragments possessed chromophores absorbing at this wavelength (Fig. 7).

DISCUSSION

Kinetic characteristics

The hydrolysis of compounds (I) and (II) by β -lactamase I was demonstrated by observing changes in the u.v.-absorption spectra and by monitoring the reactions by reverse-phase h.p.l.c. Both anilides have the same product, with a strong maximum at 350 nm, indicating an extended chromophore. Subsequent purification and spectral analysis of this product, which are reported elsewhere (Bycroft *et al.*, 1988b; Gledhill, 1988), gave data consistent with a dihydrothiazine derivative. Further support for the six-membered ring system was provided by modification of the product to form the methyl ester (Bycroft *et al.*, 1988b; Gledhill, 1988), which enabled the full ^1H -n.m.r. spectra to be analysed in ^2H chloroform. Thus both compounds (I) and (II) act as substrates for β -lactamase I with a reaction mechanism that must involve β -lactam hydrolysis, re-arrangement of both ring systems and decarboxylation.

K_m and $V_{\max}$ for these reactions were determined by using an initial-rate method to minimize effects from progressive irreversible inhibition. Comparison of k_{cat}/K_m for compound (I) ($7.48 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) and compound (II) ($3.82 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) reveals that both anilides are poor β -lactamase I substrates compared with benzylpenicillin ($2.62 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; Christensen *et al.*, 1990). It is also apparent that the 3S side-chain configuration rather than the 3R configuration of compounds (II) and (I) respectively favoured hydrolysis by β -lactamase I.

On monitoring the hydrolysis of nitrocefin in the presence of each anilide, a complex reaction process was observed that prevented reliable determination of competitive inhibition con-

stants. Comparison of these reactions with uninhibited control reactions implicated progressive irreversible inactivation and/or transient inhibition.

To study the contribution made by irreversible inactivation, the average number of turnover events required for a single inactivation was obtained by incubating β -lactamase I with a range of molar excesses of either inhibitor. Compound (I) was shown to be approx. 5 times more efficient as an irreversible inactivator of β -lactamase I than compound (II). In addition, compound (I) showed a partition ratio between inactivation and turnover of 1/2083, which compares favourably with penicillanic acid sulphone at 1/20000 and quinacillin sulphone at 1/1300 (Mezes *et al.*, 1982). The 3R analogue, compound (I), which showed a lower turnover rate, proved to be a better irreversible inactivator. This is probably because the mechanistic pathway leading to hydrolysis competes with that leading to irreversible inactivation, a common feature of mechanism-based inhibitors, such as the penam sulphone analogues (Fisher *et al.*, 1981). It was also apparent from these studies that the extent of irreversible inactivation of β -lactamase I by compounds (I) and (II) was due to the initial enzyme/inhibitor ratio and not to inhibitor concentration (within the range 0–5.5 mM). Enzyme activity could not be restored by prolonged incubation, indicating the irreversible nature of the inactivation pathway. This finding was later verified by exhaustive dialysis to eliminate the possibility of a tightly bound non-covalent inhibitor.

Preincubation of either compound (I) or compound (II) in buffer alone before addition of enzyme did not increase the inhibitory properties of these compounds. This indicated that the active constituent of the anilide preparations was not formed *in situ* by the action of buffer. This ruled out a buffer activation mechanism similar to that observed for 6 α -bromopenicillanic acid, where epimerization to the active 6 β -epimer is required (Knott-Hunziker *et al.*, 1980) for inhibitory activity.

The kinetic characteristics of the irreversible inactivation process were observed by using a dilution method, allowing time for decay of any transiently inhibited complexes. A comparison of the progress curves of irreversible inactivation obtained with compounds (I) and (II), each at a molar excess of approx. 2000, revealed a number of features common to other mechanism-based inhibitors. These included an initial rapid loss of enzyme activity followed by a more gradual phase. A logarithmic plot of fraction of residual enzyme activity against time for inhibition by compound (I) gave a first-order rate constant of $4.3 \times 10^{-4} \text{ s}^{-1}$ for the second phase of the inactivation process. The linearity of this plot (before depletion of inhibitor) indicated that the inactivation process was saturated as expected at an inhibitor concentration of $12.8 \times K_m$.

The observation of a two-phase irreversible inactivation process was reminiscent of the behaviour of other β -lactamase inhibitors, particularly clavulanic acid (Charnas *et al.*, 1978; Charnas & Knowles, 1981) and penicillanic acid sulphone (Fisher *et al.*, 1981) with R-TEM β -lactamase. In line with the kinetic observations made for these inhibitors, the work concentrated on the search for a transiently inhibited complex that was responsible for the retardation of irreversible inactivation.

β -Lactamase I was preincubated with a 2500 molar excess of compound (I), and at different time intervals a sample of the reaction was mixed with a nitrocefin solution that was used as a reporter substrate. Acceleration of nitrocefin hydrolysis revealed that a proportion of the inhibited β -lactamase was only temporarily inhibited. As expected, recovery of activity did not proceed to completion, owing to the presence of irreversibly inactivated enzyme. A series of such experiments revealed that formation of the transient species was rapid: within 1 min much of the enzyme was in the transiently inhibited form. Secondly,

Scheme 1. Postulated reaction scheme for the interaction of compound (I) with β -lactamase I

decay of the transient species followed a first-order reaction process with a rate constant of $9.8 \times 10^{-3} \text{ s}^{-1}$, which was independent of the duration of exposure to the inhibitor. The rate of enzyme recovery was 50-fold slower than K_{cat} , again pointing to a branched pathway where release of the dihydrothiazine product is not responsible for the recovery from the transiently inhibited state.

Nature of the irreversible inactivated enzyme-inhibitor complex

The stability of the inactivated β -lactamase I-compound (I) complex was demonstrated by extensive dialysis. However, as inactivation of the enzyme was also accompanied by enzyme precipitation, it was necessary to show that extensive conformational changes were not totally responsible for inactivation. Irreversible inactivation of β -lactamase I was therefore studied by isoelectric focusing. This revealed a shift to lower isoelectric points for all three of the isoenzymes present in the β -lactamase I preparation. This shift is consistent with covalent linkage of an inhibitor-derived moiety resulting in a net increase in acidity of the β -lactamase. Similar isoelectric-point changes have been demonstrated in the inhibition of R-TEM β -lactamase by penicillanic acid sulphone (Brenner & Knowles, 1984a) and 6-acetyl-methylene penicillanic acid (Arisawa & Then, 1983).

In an attempt to demonstrate the covalent nature of the modification of β -lactamase I by compound (I), the irreversibly inactivated complex was digested by trypsin and the resulting peptide fragments were analysed by h.p.l.c. Analysis of enzyme inhibited by compound (I) by this procedure revealed complex changes in the peptide profile, with many fragments apparently showing inhibitor-derived chromophores that absorbed at 320 nm. Fractions containing these chromophores were analysed

for their full u.v.-absorbance spectra, which revealed a complex assortment of chromogenic species. The one-hit labelling of the active-site serine-44 observed for the inhibition of β -lactamase I by 6- β -bromopenicillanic acid (Knott-Hunziker *et al.*, 1979) did not apply to compound (I). However, fragments with absorption maxima in the region of 314 nm were obtained, which are characteristic of dihydrothiazine derivatives (Orlek *et al.*, 1979, 1980).

The apparent complexity of the labelling by compound (I) may have arisen for a number of reasons, including the occurrence of several different tryptic cleavages or because of cross-linking of normally separate peptide fragments. On the other hand, the multiplicity of chromophores may reflect different covalently associated products or attachment at different sites within the active site and/or at other non-catalytic sites. Obviously interpretation of such complex data is difficult without more detailed knowledge of the chemical nature of the labelled peptides. Further work is required to characterize the nature of these modified peptides.

Conformation effects

There are many reports in the literature concerning the conformational flexibility of class A β -lactamases. For example, penicillin analogues such as methicillin, cloxacillin and oxacillin, which are poor substrates, sensitize β -lactamase I to irreversible inactivation by heat, iodine, urea, *p*-chloromercuribenzoate, photo-oxidation and proteolysis (Citri *et al.*, 1964; Citri & Zyk, 1965). These so-called A-type substrates are hydrolysed in a biphasic manner, which is consistent with a substrate-induced deactivation process involving reversible conformational changes (Kiener & Waley, 1977). The carbapenem antibiotic imipenem

has been shown to induce reversible conformational changes in β -lactamase I that correspond to transient inhibition (Monks & Waley, 1988). It would therefore be inappropriate to consider compound (I) as a classical mechanism-based inhibitor solely on the findings of the present study. Kinetic behaviour involving the formation and decay of transiently inhibited enzyme as well as biphasic progressive irreversible inactivation can also occur through conformational changes. In the present study, transient inhibition could be therefore due to a less active enzyme conformation, induced by turnover of compound (I). The rate of relaxation of this conformation to the active native state may be lower than the rate of decay of the inhibitor-enzyme complex. Such behaviour has been implied from the study of the conformation response induced by β -lactamase A-type substrates (Zyk & Citri, 1967).

There are indications that compound (I) is capable of inducing major changes in the conformation of β -lactamase I. Firstly, concentrations of compound (I) above 0.05 M cause very rapid precipitation of β -lactamase I without significant turnover of the inhibitor. Secondly, irreversibly inactivated enzyme tends to be precipitated, particularly during dialysis. Precipitation of β -lactamase I when exposed to high inhibitor concentrations may reflect secondary binding of the inhibitor to non-specific sites exposed by extensive conformational changes (Citri *et al.*, 1984).

The importance of conformational change in both the active site and the bulk tertiary structure of β -lactamases following interaction with substrates or inhibitors is often neglected. Future investigations similar to those by Dmitrienko *et al.* (1985), which concentrate on the conformational changes induced by inhibitors, may reveal the more subtle aspects of the normal catalytic behaviour of β -lactamases.

Hypothetical reaction pathway for the interaction of compounds (I) and (II) with β -lactamase I

A hypothetical pathway for the interaction of both compounds (I) and (II) with β -lactamase I that involves a complex active-site re-arrangement can be postulated (Scheme 1). Nucleophilic attack of the β -lactam carbonyl group by the active-site serine residue of the β -lactamase (E-OH in Scheme 1) gives a tetrahedral intermediate, which in turn collapses (k_2), with loss of both ring systems, to an acyl-enzyme complex. Nucleophilic attack of the epoxide by the resulting thiolate and the displacement of chlorine (k_3) results in the formation of a 6,6-disubstituted dihydrothiazine derivative. Deacylation (k_4) and loss of CO₂ liberates active enzyme and the observed dihydrothiazine hydrolysis product.

As both isomers are fully substituted at the 6-position, neither transient inhibition nor irreversible inactivation can occur by the usual imine-enamine tautomerism (Knowles, 1985) postulated for penicillanic acid sulphone and clavulanic acid inhibition of TEM β -lactamase. Reaction of the side-chain keto function generated by opening of the epoxide with an active-site nucleophile could give rise to a Schiff base and may account for irreversible inactivation. The proximity and orientation of reactive functionality such as this must be influenced by the locked side-chain geometry initially present in the two inhibitors, and is probably the reason for the striking difference in the respective abilities of compounds (I) and (II) to inhibit β -lactamases.

Further work is required to confirm the reaction course given above and to discover the nature of the irreversibly inactivated species. Similarly, at present it is difficult to postulate a suitable reaction mechanism for transient inhibition, which may ultimately prove to be conformational in nature.

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