Kinetic and physical studies of β -lactamase inhibition by a novel penem, BRL 42715

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The interactions of *Staphylococcus aureus*, *Bacillus cereus* I, TEM, *Klebsiella pneumoniae* K1 and *Enterobacter cloacae* P99 β -lactamases with the novel penem inhibitor BRL 42715 were investigated kinetically and, in some cases, by electrospray mass spectrometry (e.s.m.s.). All the β -lactamases were rapidly inactivated by BRL 42715, with second-order rate constants ranging from 0.17 to 6.4 $\mu\text{M}^{-1}\cdot\text{s}^{-1}$. The initial stoichiometry of β -lactamase inhibition was essentially 1:1, with the exception of the K1 enzyme. In this instance about 20 molecules of BRL 42715 were hydrolysed before the enzyme was completely inhibited. Inhibited β -lactamases did not readily regain activity in the absence of BRL 42715, the half-lives for regeneration of

free enzyme ranging from 5 min for the K1 β -lactamase to over 2 days for the staphylococcal enzyme. Recovery of activity was incomplete for TEM-1, K1 and P99 β -lactamases, suggesting partitioning of the inhibited enzymes to give a permanently (or at least very stable) inactivated species. Examination of the interactions of the penem with TEM, *B. cereus* I and P99 β -lactamases by e.s.m.s. also showed rapid and stoichiometric binding of the inhibitor. In all cases a mass increase of 264 Da over the native enzyme was observed, corresponding to the molecular mass of BRL 42715, showing that no fragmentation of the penem occurred on reaction with the β -lactamases.

INTRODUCTION

The discovery and development of compounds such as clavulanic acid (Hunter *et al.* 1980; Reading and Cole, 1977) and sulbactam (English *et al.*, 1978) have established enzyme inhibition as a valid means of overcoming β -lactamase-mediated resistance. A further sulphone, tazobactam, has also been examined for its ability to synergize β -lactamase-labile antibiotics (Aronoff *et al.*, 1984) and is now also available clinically. More recently, a novel class of inhibitors, the 6-(substituted methylene) penems, have been synthesized in our laboratories (Bennett *et al.*, 1991). One of these, BRL 42715 [(5*R*)-(Z)-6-(1-methyl-1,2,3-triazol-4-yl-methylene) penem-3-carboxylic acid (Figure 1) has been shown to be both a very potent inhibitor of a wide range of β -lactamases and an effective synergist of β -lactamase-labile antibiotics (Coleman *et al.*, 1989). The development of BRL 42715 as a therapeutic agent has now ceased, because it lacks other required technological features. However, such inhibitors are useful probes to investigate mechanistic aspects of β -lactamase catalysis.

Studies with inhibitors such as clavulanic acid, sulbactam and 6 β -bromopenicillanic acid have shown them to be k_{cat} - or mechanism-based inhibitors, often exhibiting complex kinetics [see reviews by Knowles (1983), Cartwright and Waley (1983) and Reading and Cole (1986)]. Despite the complexities observed with β -lactamase inhibitors, certain common features have emerged. Effective inhibition appears to require extensive structural reorganization of the inhibitor molecule following the formation of an initial acyl-enzyme complex, with the exception of the carbapenems. The chemical events are mirrored kinetically by the tendency of β -lactamase inhibition to follow branched reaction pathways. Recently, we have demonstrated that the K1 β -lactamase from *Klebsiella pneumoniae* generates a seven-membered cyclic product (dihydrothiazepine) from BRL 42715 (Broom *et al.*, 1992). In the present paper, inhibition kinetics for

BRL 42715 and this enzyme are presented, together with data for *Staphylococcus aureus*, *Bacillus cereus* I, TEM-1 β -lactamases (all class A enzymes; Ambler, 1980) and *Enterobacter cloacae* P99, a class C β -lactamase (Jaurin and Grundström, 1981). A full understanding of the processes involved in β -lactamase catalysis and its inhibition requires the combination of classical biochemical information with that obtained from site-directed mutagenesis and biophysical studies. While in the latter case the ultimate goal may seem to be resolution of enzyme crystal structures, other techniques can also provide valuable information. Electrospray mass spectrometry (e.s.m.s.) has successfully been used to observe acyl-enzyme intermediates in β -lactamase catalysis (Aplin *et al.*, 1990). This technique has the advantage that it can provide very accurate mass measurements of β -lactamase inhibitor complexes, and the additional benefit that it can be carried out under conditions and time scales similar to those employed in kinetic experiments. Results obtained using this method with BRL 42715 and TEM, *B. cereus* I and *Ent. cloacae* P99 β -lactamases are also presented.

EXPERIMENTAL

Antibiotics and reagents

Amoxycillin, benzylpenicillin, nitrocefin and BRL 42715 were all prepared in our laboratories; cephaloridine was purchased from Sigma. Bio-Gel P2 and Bio-Lyte ampholine solutions were obtained from Bio-Rad. Buffer reagents were of AnalaR grade, and distilled water was used to prepare all buffers and reagents.

Methods

Bacterial strains and β -lactamase preparation

Escherichia coli JT4 (TEM-1), *K. pneumoniae* 1082E (K1), *Ent. cloacae* P99 and *S. aureus* N.C.T.C. 11561 are strains used in our

Abbreviation used: e.s.m.s., electrospray mass spectrometry.

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Figure 1 Structure of BRL 42715

laboratories for antibiotic-susceptibility testing. Bacteria were grown in shake flasks, and β -lactamases were released from the cells by ultrasonication as described previously (Reading and Farmer, 1981). TEM-1 β -lactamase from *E. coli* JT4 and the K1 enzyme from *K. pneumoniae* 1082E were further purified in a preparative isoelectric-focusing cell (Bio-Rad Rotofor). A 1 ml portion of cell-free β -lactamase was diluted to 40 ml in 1% (w/v) pH 3–10 Bio-Lyte ampholine and loaded into the cell. The power supply was set at 2000 V and 200 mA, with 12 W constant power. A circulating water system maintained the apparatus at 4 °C. After 4.5 h, the run was terminated and the fractions collected. The pH of each fraction was measured prior to assaying for β -lactamase activity with benzylpenicillin (see below). In the fraction used for the inhibition studies, β -lactamase accounted for 25–40% of the total protein.

B. cereus β -lactamase I was a gift kindly donated by (the late) Professor S. G. Waley (then at the University of Oxford). TEM-2 and P99 used for e.s.m.s. studies were purchased from Porton Products. Solutions of these two enzymes were dialysed overnight at 4 °C with 5 mM Tris/HCl buffer, pH 7. Each enzyme was then diluted in 5 mM Tris to give between 55 and 17 pmol·ml⁻¹.

β -Lactamase assays

β -Lactamase activities were determined by using direct spectrophotometric assays (O'Callaghan et al., 1972; Waley, 1974; Samuni, 1975). The assay substrates and the wavelengths used to measure their hydrolysis were: benzylpenicillin (240 nm), amoxycillin (250 nm), cephaloridine (299 nm) and nitrocefin (482 nm). Nitrocefin gave an increase of absorbance on hydrolysis, whereas the other compounds showed reduced absorbance. All assays were carried out in 0.05 M sodium phosphate, pH 7.3, at 37 °C, except where mentioned otherwise. Changes in absorbance were monitored using either a Perkin-Elmer λ 7 UV/Vis double-beam spectrophotometer or a Philips PU 8700 single-beam instrument. In both cases reactions were maintained at the desired temperature using thermostatically controlled water-jacketted cuvette holders.

Stoichiometry and duration of β -lactamase inhibition

Concentrations of β -lactamase (generally from 0.02 to 1.3 μ M) were calculated by using published data (see the Results section),

and the amount of BRL 42715 varied to give from 0.2 to 40 mol. equiv. Samples were periodically removed and assayed for remaining β -lactamase activity using 1000 μ M benzylpenicillin as substrate. BSA was added to give 5 mg·ml⁻¹ to stabilize enzyme activity. Nevertheless, incubation of the staphylococcal β -lactamase at 37 °C resulted in a rapid loss of activity, and experiments with this enzyme were repeated at 30 °C. The stoichiometry of inhibition of the β -lactamases by BRL 42715 was calculated as described in the Results and discussion section.

Rate of β -lactamase inactivation

Inactivation rates were too high to measure accurately by sequential incubation of β -lactamase with BRL 42715 and substrate. The rates of inactivation were therefore determined in the presence of 1000 μ M amoxycillin (TEM-1), 2000 μ M benzylpenicillin (K1 and *B. cereus* I), 100 μ M nitrocefin (staphylococcal) and 2000 μ M cephaloridine (P99) (all at pH 7.3 and 37 °C). Typically hydrolysis of substrate was monitored for 5–10 min, with rates being recorded at timed intervals. Comparison of the reaction rates in the presence of inhibitor with those for control reactions enabled the degree of enzyme inhibition at each time point to be calculated. First-order rate constants for inactivation were then determined from plots of ln (percentage of enzyme activity remaining) against time. These values were then plotted against inhibitor concentration to obtain the second-order rate constants for the inactivation of β -lactamase by BRL 42715 after correcting for the influence of the assay substrate.

Stability of β -lactamase inhibition

The stability of inhibition achieved by BRL 42715 was measured in three ways. First, the data from the stoichiometry experiments with limiting concentrations of BRL 42715 were used to calculate the rates of decay of enzyme inhibition by measuring the re-establishment of β -lactamase activities. Secondly, enzyme was allowed to react with sufficient BRL 42715 to give 100% inhibition, then diluted into excess substrate and the breakdown of β -lactamase-inhibitor complex calculated from the increase in the rate of substrate hydrolysis with time. Thirdly, reactions of *S. aureus* and *Ent. cloacae* β -lactamase were carried out with excess penem, the reaction mixture was then gel-filtered using a Bio-Gel P2 column to remove the inhibitor. The β -lactamase-containing fractions were pooled, incubated and periodically assayed for β -lactamase activity as previously described. Prolonged incubation of uninhibited staphylococcal β -lactamase resulted in some loss of activity even at 30 °C, although the enzyme was stabilized by the presence of substrate. Consequently, staphylococcal enzyme samples were diluted into 1200 μ M amoxycillin, and increases in substrate hydrolysis with time used to assess the rate of re-establishment of β -lactamase activity.

E.s.m.s.

BRL 42715 was preincubated at 37 °C for $\leq$ 3 min with between 17 and 55 pmol/ml of β -lactamase in approximately equimolar proportions. E.s. mass spectra were obtained as described previously (Payne et al., 1994).

RESULTS AND DISCUSSION

Inhibition of staphylococcal β -lactamase (class A)

Incubation of staphylococcal β -lactamase with limiting concentrations of BRL 42715 at 30 °C resulted in rapid, partial inhibition of enzyme activity (Figure 2). The degree of inhibition

Figure 2 Inhibition of *S. aureus* β -lactamase by limiting concentrations of BRL 42715

S. aureus β -lactamase [$0.4 \mu\text{M}$, determined from the catalytic-centre activity ('turnover number') for benzylpenicillin] was incubated with BRL 42715. The molar ratios of inhibitor to enzyme were: 0.16:1.0 (●), 0.31:1.0 (○), 0.62:1.0 (△). Over a period of 4 h samples ($10 \mu\text{l}$) were removed and added to 2.5 ml of $1000 \mu\text{M}$ benzylpenicillin and assayed for β -lactamase activity.

remained constant for the duration of the experiment ($> 4 \text{ h}$), indicating that the enzyme-inhibitor complex did not break down to release active β -lactamase. The stoichiometry of inhibition by BRL 42715 was calculated with concentrations of staphylococcal β -lactamase determined from published k_{cat} values for the PC1 enzyme. Using a k_{cat} for benzylpenicillin of 185 s^{-1} (Hardy and Kirsch, 1989), the stoichiometry of inhibition was calculated to be 1.3:1, whereas when a value of 128 s^{-1} was used (Rizwi et al., 1989), the stoichiometry was found to be 0.9:1. Although these k_{cat} determinations were carried out at 25°C , this is unlikely to alter the conclusion that the penem inhibits *S. aureus* β -lactamase with a 1.0:1.0 stoichiometry. Removal of excess inhibitor by gel filtration followed by prolonged incubation of the β -lactamase in the presence of amoxycillin resulted in a very slow recovery of activity. The half-life for re-activation was estimated to be approx. 50 h (Table 1), suggesting that the enzyme-inhibitor complex was very stable.

Figure 3 Rates of inactivation of *S. aureus* and TEM-1 β -lactamases as a function of BRL 42715 concentration

(a) Rates of β -lactamase inactivation were used to construct a double-reciprocal plot as described by Kitz and Wilson (1962). The slope of the line enables the second-order rate constant, k'_{inhib} , to be determined (allowing for the presence of the substrate). (b) TEM-1 β -lactamase was incubated with 0.0125 – $0.2 \mu\text{M}$ BRL 42715 in the presence of $1000 \mu\text{M}$ amoxycillin. The rate of enzyme inactivation was determined at each inhibitor concentration, then these rates used for the double-reciprocal plot shown.

Inactivation of staphylococcal β -lactamase by BRL 42715 followed first-order kinetics. Figure 3(a) shows inactivation rates plotted as a function of BRL 42715 concentration using the method of Kitz and Wilson (1962). The line intersects the axes at their origin, indicating that saturation kinetics were not obeyed. It was not, therefore, possible to find either the inhibitor constant (K_i) or the maximum rate of inactivation (k_2 in Scheme 1 below). The reciprocal of the slope of the line did, however, provide a value of $0.17 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ for the second-order rate constant for inactivation of *S. aureus* β -lactamase (see Table 1). The 1:1 stoichiometry of the inactivation of *S. aureus* N.C.T.C. 11561 β -lactamase by BRL 42715, together with the detection of only one inhibited species show that inhibition of this enzyme can be described by a linear kinetic scheme. Although it was not possible to detect a Michaelis complex experimentally, both current and previous studies (Coleman et al., 1989) have shown that the presence of substrate decreases the rate of inactivation. This observation shows that the inhibitor and substrate compete for

Table 1 Summary of the kinetic data

β -Lactamase	Initial stoichiometry of inhibition	Second-order rate constant ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	Stability of the enzyme-inhibitor complex (h)	$t_{1/2}$ for inactivation at $6 \times 10^{-2} \mu\text{g} \cdot \text{ml}^{-1}$ BRL 42715 (s)
<i>S. aureus</i> N.C.T.C. 11561	1.1:1.0	0.17	53	20.4
TEM-1	1.0:1.0	6.4	1.2	17.4
<i>Ent. cloacae</i>	1.1:1.0	2.5	16	1.39
K1	23:1.0	2.5	0.089	39.9
<i>B. cereus</i> 1	1.6:1.0	0.11	1.3	31.5

Scheme 1 Inhibition of staphylococcal β -lactamase by BRL 42715

EC is the Michaelis complex; EC* is the acyl-enzyme; $K_1 = k_{-1}/k_1$; k_2 is the rate of formation of EC* and k_3 is the rate of decay of EC*.

Figure 4 Mass determinations by e.s.m.s. for *B. cereus* β -lactamase

(a) *B. cereus* I β -lactamase and (b) *B. cereus* I β -lactamase + 1:1 BRL 42715 after 3 min incubation.

the same binding site. As interactions of β -lactamases with mechanism-based inhibitors are generally represented as proceeding via a Michaelis complex (Cartwright and Waley, 1983; Knowles, 1983), it is reasonable to propose that such a complex is involved in the inhibition of staphylococcal β -lactamase by BRL 42715. However, competition between inhibitor and substrate does not provide unequivocal proof of a Michaelis complex in this case, and it is possible that the acyl-enzyme (EC*) is formed directly between free penem and β -lactamase. Inhibition probably proceeds via such a complex, and Scheme 1 is proposed as representing the inhibition of staphylococcal β -lactamase by BRL 42715. The terminology is based on that of Frère et al. (1982). For this β -lactamase, the k_3 reaction is very slow.

Inhibition of *B. cereus* β -lactamase I (class A)

The second-order rate constant for the inactivation of this enzyme by BRL 42715 was found to be $0.11 \mu\text{M}^{-1}\text{s}^{-1}$, and

Figure 5 Inhibition of TEM-1 β -lactamase by limiting concentrations of BRL 42715

TEM-1 β -lactamase ($2 \times 10^{-2} \mu\text{M}$) was incubated with the concentrations of BRL 42715 shown. Periodically, $25 \mu\text{l}$ samples were removed and added to 2.5 ml of $1000 \mu\text{M}$ benzylpenicillin to assay for β -lactamase activity. The molar ratios of inhibitor to enzyme were: 0.4:1.0 (●), 0.8:1.0 (○), 1.6:1.0 (▲), and 3.1:1.0 (△).

saturation kinetics were not observed. The stoichiometry of inhibition was 1.6:1.0. In this respect, inhibition of *B. cereus* β -lactamase I resembles that of the staphylococcal enzyme, but the decay of EC* with a half-life of 1.3 h was much more rapid (Table 1). The inhibition of the *B. cereus* enzyme by BRL 42715 can also be described by Scheme 1. Mathematically transformed data of the e.s.m.s. results for *B. cereus* β -lactamase I showed a major species (Figure 4a) with a mass of $28943.08 \pm 3.94 \text{ Da}$, with a minor species of molecular mass $28815.99 \pm 1.66 \text{ Da}$. The former species is in good agreement with the value of 28813.67 Da , the molecular mass calculated from the amino acid sequence (Madgwick and Waley, 1987). The latter protein corresponds to enzyme which had lost the N-terminal lysine residue ('ragged end'). When the β -lactamase was incubated for 3 min with an equimolar concentration of BRL 42715 a mass of shift of $\sim 266 \text{ Da}$ was observed for both the major and minor peaks. No free enzyme was apparent (Figure 4b). The increase in mass is close to the theoretical increase of 264 expected for the binding of the penem to the β -lactamase. The results indicate that *B. cereus* β -lactamase I rapidly formed a 1:1 covalent adduct with BRL 42715. It is also apparent that no fragmentation of the inhibitor occurred.

Inhibition of TEM β -lactamase (class A)

The inhibition of TEM-1 β -lactamase by low concentrations of BRL 42715 is shown in Figure 5. The enzyme was rapidly inhibited, but, like the *B. cereus* β -lactamase, a return in activity was apparent. However, unlike the situation with the *B. cereus*

enzyme, the recovery of enzyme activity was incomplete. Calculation of the stoichiometry for the initial phase of inhibition using published k_{cat} data for TEM-1 β -lactamase with benzylpenicillin (Arisawa and Then, 1983), gave a value of less than 1 (0.4). As it is difficult to visualize how this could arise, it was assumed that the initial stoichiometry was 1:1. On this basis it was calculated that approx. 20 molecules of BRL 42715 were needed to achieve complete inactivation of TEM β -lactamase, following decay of the transiently inhibited species. TEM-2 differs from TEM-1 by only one amino acid (Gln-37 $\rightarrow$ Lys), and kinetic experiments indicated that TEM-2 behaved similarly to TEM-1 with regard to the stoichiometry and stability of inhibition (T. H. Farmer, J. W. J. Page, D. J. Payne and D. J. C. Knowles, unpublished work). Statistical analysis of the charged peaks from e.s.m.s. of TEM-2 β -lactamase gave a mass of 28906.10 ± 1.55 Da. This is within 1 Da of the mass given by the deduced amino acid sequence (28905.24 Da) (Barthelemy et al., 1985). Analysis of the peaks following incubation with an equimolar concentration of BRL 42715 for 2 min gave a calculated molecular mass of 29169.70 ± 1.53 Da, which is almost 264 Da higher than that of the free enzyme. These data are again consistent with the rapid formation of a covalent complex with a 1:1 stoichiometry, and support the kinetic results obtained with TEM-1 β -lactamase. As with the *B. cereus* I enzyme, there was no evidence of fragmentation of the inhibitor on binding to the β -lactamase, as has been observed with other inhibitors (Knowles, 1983; Cartwright and Waley, 1983).

Using the procedure of Kitz and Wilson (1962) to plot rates of β -lactamase inactivation against BRL 42715 concentration, it is evident that saturation kinetics were obeyed for TEM-1 (Figure 3b). Values of $6.4 \times 10^{-3} \mu\text{M}$ were obtained for K_i and $4.1 \times 10^{-2} \text{ s}^{-1}$ for the maximal rate of inhibition. Since the line intersects close to the origin, these values can only be regarded as approximate. The second-order rate constant could be reliably measured from the slope of the line, and was found to be $6.4 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (Table 1). Similar values were obtained regardless of whether cell-free extract or purified β -lactamase was used.

Recovery of enzyme activity is incomplete when concentrations of BRL 42715 are limiting, showing that, unlike the situation pertaining to the *B. cereus* enzyme, irreversibly inactivated TEM β -lactamase must be generated on reaction with the penem. As 20 molecules of BRL 42715 are hydrolysed before all the enzyme is sequestered in this form, this species cannot be the only one arising from the initial acyl-enzyme (EC^*). It follows, therefore, that a branched kinetic scheme (Charnas and Knowles, 1981; Cartwright and Waley, 1983; Charnas and Then, 1988) is required to describe the inhibition of TEM-1 β -lactamase by BRL 42715. Scheme 2 is put forward as the minimal mechanism based on our data, where EC_i^* represents the permanently inactivated form.

Inhibition of K1 β -lactamase (class A)

Superficially, the results obtained for K1 β -lactamase resembled those acquired with the TEM enzyme: there was a rapid loss of β -lactamase activity, a re-establishment of enzyme activity at limiting inhibitor concentrations, and the formation of a permanently inactivated species. However, the recovery of β -lactamase activity was much faster than observed for the other β -lactamases, with a $t_{1/2}$ of 5.3 min (Table 1). Furthermore, using the published k_{cat} for K1 β -lactamase (Emanuel et al., 1986) to calculate enzyme concentration, showed that a 23-fold excess of BRL 42715 was required to inhibit the β -lactamase in the initial (i.e. the first few minutes) phase of the reaction. These results are consistent with our earlier demonstration of turnover of the penem by the K1 enzyme (Broom et al., 1992). However, with

Scheme 2 Inhibition of TEM-1 β -lactamase by BRL 42715

Scheme 3 Inhibition kinetics of K1 β -lactamase by BRL 42715

excess inhibitor, turnover of BRL 42715 ceases and the β -lactamase becomes permanently inactivated. The steady-state rate for this inactivation process was found to be $3.4 \times 10^{-4} \text{ s}^{-1}$, while a 67-fold molar excess of the penem was required to achieve permanent inactivation. The need for excess BRL 42715 initially to inhibit the enzyme reflects a burst of inhibitor hydrolysis which cannot be accommodated by Scheme 2. An additional branch is thus required for the pathway. The kinetics of K1 β -lactamase by BRL 42715 can be described by either Scheme 3(a) or 3(b), these being kinetically indistinguishable. In these Schemes, EC_i represents a transiently inhibited species.

This mechanism is the same as that proposed for the inhibition of TEM β -lactamase by clavulanic acid (Fisher et al., 1978; Reading and Farmer, 1981). Interestingly, the stoichiometry for the initial and final inactivation and the rates of decay of the transient complexes are very similar for TEM/clavulanic acid and K1/BRL 42715. However, the second-order rate constant for the inactivation of K1 β -lactamase by BRL 42715 is about 60-fold higher than that for clavulanic acid with the TEM enzyme. Plots of inactivation rates against inhibitor concentration indicated that saturation kinetics were followed by BRL 42715 and K1 β -lactamase; values of $7.30 \times 10^{-3} \mu\text{M}$ and $1.8 \times 10^{-2} \text{ s}^{-1}$ were found for K_i and the maximum inhibition rate respectively.

Interestingly the protein content in the reactions influenced the partitioning of K1 β -lactamase between EC_i and EC_i^* . This

behaviour is reminiscent of that observed with the effects of ionic strength on the inhibition of the same β -lactamase and β -halogenopenicillanates (De Meester et al., 1989). In the presence of BSA the amount of enzyme sequestered as EC_1^* was about 40% of that obtained in its absence. Experiments with sonicated cells showed that the β -lactamase behaved in the same way as pure enzyme in the presence of BSA. These observations may reflect subtle conformational changes which are influenced by protein concentration.

Inhibition of *Ent. cloacae* P99 β -lactamase (class C)

The class C β -lactamase from *Ent. cloacae* P99 was rapidly inactivated by BRL 42715 with an initial stoichiometry of 1.1:1.0 and a second-order rate constant of $2.5 \mu\text{M}^{-1}\cdot\text{s}^{-1}$. Saturation kinetics were not observed. Experiments undertaken with limiting penem concentrations, or where excess inhibitor was removed by gel filtration, showed that there was slow recovery of enzyme activity. This process had a half-life of 16 h, but was also incomplete showing that, as with TEM β -lactamase, irreversibly inactivated enzyme was also generated. The stoichiometry for irreversible inactivation was 2:1. The inhibition of *Ent. cloacae* β -lactamase by BRL 42715 can readily be described by Scheme 2. The mass of *Ent. cloacae* P99 β -lactamase as determined by e.s.m.s. was 39196.56 ± 4.26 Da, which is only 9.8 Da lower than the value calculated from the deduced amino acid sequence (Galleni et al., 1988) and 6.8 Da lower than recently published molecular masses found by e.s.m.s. (Aplin et al., 1993). Following 3 min incubation with an equivalent concentration of BRL 42715, a major peak of molecular mass 39460.18 Da was obtained, with free enzyme being present as a minor component. The mass shift of almost 264 Da corresponds to the covalent binding of BRL 42715 in stoichiometric proportions. Again, as with the other β -lactamases examined, there was no indication of fragmentation of the penem.

CONCLUSIONS

The results from the kinetic experiments show that BRL 42715 is a very effective inhibitor of all the β -lactamases studied, readily explaining its excellent activity as a synergist for β -lactamase-labile antibiotics (Coleman et al., 1989). The critical parameter relating the time needed to inhibit an enzyme to inhibitor concentration is the second-order rate constant. Data for the penem (see Table 1) fall in the region of 0.1 – $6.4 \mu\text{M}^{-1}\cdot\text{s}^{-1}$, showing it is generally superior to other β -lactamase inhibitors (Cartwright and Waley, 1983), including tazobactam, for which inhibition kinetics have recently been described (Bush et al., 1993). An exception is the olivanic acid MM 4550, which has a second-order rate constant of $100 \mu\text{M}^{-1}\cdot\text{s}^{-1}$ (Easton and Knowles, 1982). Two other important requirements for efficient enzyme inhibition are low inhibitor turnover coupled with stability of inhibition. Except for the K1 β -lactamase, initial stoichiometries for β -lactamase inhibition by BRL 42715 are 1:1 and enzyme-inhibitor complexes are relatively stable, exceptionally so for staphylococcal and P99 β -lactamases. The results from the e.s.m.s. studies confirm the rapid stoichiometric inhibition of β -lactamases by the penem. Interestingly, these experiments did not suggest any fragmentation following initial binding to the β -lactamases, but do not preclude ring opening and rearrangement to form the dihydrothiazepine, as has been

shown with BRL 42715 and K1 β -lactamase using other techniques (Broom et al., 1992)

Like BRL 42715 β -halogenated penicillanic acids also undergo cyclic rearrangements on reaction with β -lactamases (Orlek et al., 1979), and have recently been examined by e.s.m.s. (Aplin et al., 1993). To what extent formation of a cyclic entity from BRL 42715 is a prerequisite for effective β -lactamase inhibition is presently unknown, and further studies will be necessary to more fully understand the factors responsible for its activity.

We thank (the late) Professor S. G. Waley and Dr. R. Aplin of the University of Oxford for advice on the e.s.m.s. experiments and operation of the mass spectrometer. We also appreciate the advice and assistance of Dr. P. Skett and C. Reading of SmithKline Beecham.

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