Biochem. J. (1988) 253, 323–328 (Printed in Great Britain)

Imipenem as substrate and inhibitor of β-lactamases

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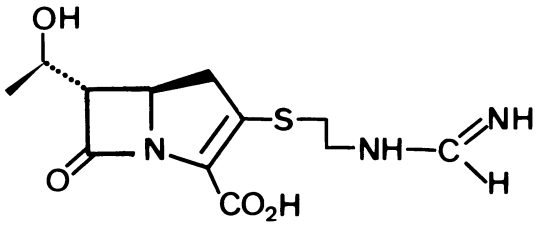
The interaction between imipenem, a carbapenem antibiotic, and two representative β-lactamases has been studied. The first enzyme was β-lactamase I, a class-A β-lactamase from *Bacillus cereus*; imipenem behaved as a slow substrate ($k_{cat.}$ 6.7 min^{-1}, K_m 0.4 mM at 30 °C and at pH 7) that reacted by a branched pathway. There was transient formation of an altered species formed in a reversible reaction; this species was probably an acyl-enzyme in a slightly altered, but considerably more labile, conformation. The kinetics of the reaction were investigated by measuring both the concentration of the substrate and the activity of the enzyme, which fell and then rose again more slowly. The second enzyme was the chromosomal class-C β-lactamase from *Pseudomonas aeruginosa*; imipenem was a substrate with a low $k_{cat.}$ (0.8 min^{-1}) and a low K_m (0.7 μM). Possible implications for the clinical use of imipenem are considered.

INTRODUCTION

β-Lactams occupy pride of place among antibiotics, and imipenem, a carbapenem, is perhaps the most potent β-lactam antibiotic. The resistance of pathogens to β-lactam antibiotics, an important clinical problem, is often mediated by β-lactamases. Imipenem is generally regarded as 'β-lactamase-stable' (Kropp *et al.*, 1985; Hashizume *et al.*, 1984). The stability of imipenem to β-lactamases, however, has usually been tested with low concentrations of enzyme and high (saturating) concentrations of substrate; such tests may mislead.



Imipenem

The classification by mechanism assigns a β-lactamase to one of two classes: those which function by acylation and deacylation of a particular serine residue (serine enzymes) and those that require zinc ions for activity (Coulson, 1985; Frere & Joris, 1985). Crystallographic studies on both serine and zinc β-lactamases are well advanced (Kelly *et al.*, 1986; Samraoui *et al.*, 1986; Herzberg & Moult, 1987; Dideberg *et al.*, 1987; Sutton *et al.*, 1987) and have established the relationship between serine β-lactamases and transpeptidases, the targets of β-lactam action. Further classification by amino acid sequence splits the serine β-lactamases into two classes, A and C (Ambler, 1980; Jaurin & Grundström, 1981). Zinc β-lactamases are known to hydrolyse imipenem quite well (Saino *et al.*, 1982; Bicknell *et al.*, 1985); just how well serine β-lactamases do so is the subject of this paper. The main features of the reaction between imipenem and representatives of the two classes of serine β-lactamase are described in the present paper.

MATERIALS AND METHODS

β-Lactamase I was prepared as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980), except that a Millipore Pellicon cassette system was used to concentrate and desalt the extract after adsorption on, and elution from, Celite. The β-lactamase from *Pseudomonas aeruginosa* was prepared by affinity chromatography (Cartwright & Waley, 1984). Nitrocefin was from Becton–Dickinson, Oxford, U.K., and imipenem was a gift from Merck Sharp & Dohme Research Laboratories, Rahway, NJ, U.S.A. Benzylpenicillin and cephalosporin C were gifts from Glaxo Laboratories (Greenford, Middx., U.K.).

Assays for β-lactamase activity were carried out in a Cary 219, or Cecil 272 spectrophotometer, or in a Radiometer pH-stat; the change in absorption coefficient on hydrolysis was 9.393 $mM^{-1} \cdot cm^{-1}$ for imipenem at 300 nm, 7.5 $mM^{-1} \cdot cm^{-1}$ for cephalosporin C at 261 nm, and 16.65 $mM^{-1} \cdot cm^{-1}$ for nitrocefin at 500 nm.

Initial rates were determined from progress curves by the method of Duggleby (1984), unless the extent of reaction was so small (e.g. less than 3%) that the trace was linear. The hyperbolic form of the Michaelis–Menten equation was then used to obtain $V_{max.}$ and K_m by non-linear regression (Duggleby, 1984), which was also used to obtain parameters from the biphasic progress curves described below.

THEORY

Re-activation during assay

When a sample of partially inactivated enzyme is diluted into an assay mixture (substrate and buffer), the progress curve may show an increase in rate if the enzyme is recovering activity (Fig. 1). This is the converse of the more usual situation in which activity is lost during an assay (e.g. Fig. 2). The rate constant, k, for re-activation may be most simply obtained from progress curves as follows. The rate of consumption of substrate is:

$$[-ds/dt = [V - B \cdot \exp(-k \cdot t)][s/(s + K_m)]$$

where V is the maximum velocity, s is the concentration of substrate at time t, and $B = V$ − activity at zero time. When this is integrated, and expressed in terms of the measured absorbance, A, we have:

$$\left|\frac{A_0 - A}{\Delta\epsilon}\right|\left(1 + \frac{K_m}{S_0}\right) = Vt - [B(1\text{-}e^{-kt})/k]$$

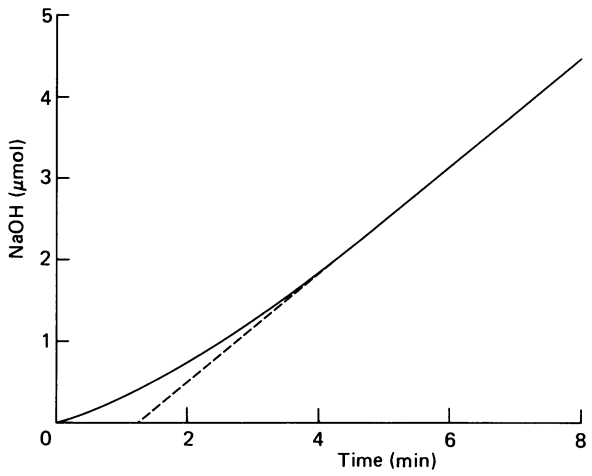


Fig. 1. Regain of activity of *Pseudomonas* β-lactamase during assay

The β-lactamase (2.185 μM) and imipenem (50 μM) in 20 mM-triethanolamine/HCl buffer, pH 8, were incubated together for 1 min at 30 °C; a portion of the solution was then assayed in the pH-stat at pH 8 with 5 mM-cephalosporin C as substrate. The vertical distance between the broken line and the curve is used in a semi-logarithmic plot as described in the Theory section.

where A_0 is the absorbance at zero time and $\Delta\epsilon$ is the absorption coefficient. The final rate is obtained when the right-hand side equals $V \cdot t - B/k$ and is linear when there is little change in the concentration of substrate during the course of the experiment (about 2% in the 'lag' experiment with imipenem and the *Pseudomonas* β-lactamase). Thus the difference (D) in alkali uptake in the pH-stat (or absorbance in a spectrophotometer) at a given time between the final line (extrapolated back) and the curve is an exponential function of the rate constant, and:

$$\ln(D) = \text{constant} - (k \cdot t)$$

whence k is easily found graphically. This procedure has some advantages over the rate method (Fink *et al.*, 1987) and the method restricted to saturating concentrations of substrate (Kemal & Knowles, 1981).

Activity loss and regain

The kinetics of transient inactivation, applied to experiments such as those illustrated in Fig. 3, may be developed as follows. Numerical solution of the differential equations corresponding to the scheme:

$$\begin{array}{c} \overset{K_s}{\rightleftharpoons} \quad \overset{k_2}{\rightarrow} \quad \overset{k_3}{\rightarrow} \\ E + S \rightleftharpoons X \rightarrow Y \rightarrow E + P \\ \quad {\scriptstyle k_5}\uparrow\downarrow{\scriptstyle k_4} \\ \quad\quad Z \end{array}$$

showed that (with the rapid equilibrium assumption applied to substrate-binding) [X]/[Y] was approximately constant throughout the reaction, and so there was a constant flux (v) through the 'non-branching' part of the scheme, and hence we have (cf. Kiener *et al.*, 1980):

$$\frac{e_0}{v} = \left(\frac{K_s}{k_2} \cdot \frac{1}{s}\right) + \frac{1}{k_2} + \frac{1}{k_3} + \frac{z}{v}$$

where e_0 is the total concentration of enzyme, and s and z are the concentrations of substrate and of inactive enzyme respectively. The fractional activity is defined as $A = 1 - (z/e_0)$, and hence:

$$e_0 \cdot A \cdot s/v = (K_s/k_2) + \left(\frac{1}{k_2} + \frac{1}{k_3}\right)s$$

The left-hand side of this equation was plotted against s to obtain K_s/k_2 from the intercept and $1/k_2 + 1/k_3$ from the slope. These constraints were used in fitting the 'loss and regain' curves (e.g. Fig. 3 below) as follows. The program utilized the trapezoidal method for solving the differential equations (see, e.g., Fox & Mayers, 1987) and the pattern-search algorithm for optimization (Hooke & Jeeves, 1961; Bunday, 1984); the sum of the absolute differences between the observed and calculated activities was minimized, with three parameters, K_s, k_4 and k_5 as variables. These three parameters and the two constraints referred to above gave values for K_s, k_2, k_3, k_4 and k_5 and the fit was acceptable (Fig. 3).

RESULTS

Imipenem as a substrate for β-lactamase I

The initial rates of hydrolysis of imipenem by β-lactamase I at 30 °C and pH 7 gave the following values: $k_{cat.}$, 6.7 (±0.7)/min; K_m, 0.4 (±0.1) mM. Thus $k_{cat.}/K_m$ was 17 $mM^{-1} \cdot min^{-1}$, a value about 10^{-5} that for benzylpenicillin (Waley, 1975). At higher concentrations of substrate the progress curves were perceptibly biphasic, a feature which was much more marked at 23 °C (Fig. 2). The curves at 23 °C were fitted to eqn. (1) (Frieden, 1970):

$$p = v_f t + (v_0 - v_f)[1 - \exp(-kt)] + d \qquad (1)$$

where p is the concentration of product formed at time t, v_0 and v_f are the original and final velocities, k is the rate constant governing the transition and d is a correction to allow for uncertainty about the origin. The values of v_0 from 12 experiments over the concentration range 0.1–1.5 mM-imipenem varied hyperbolically with the concentration and gave a $k_{cat.}$ value of 3.2 min^{-1} and a K_m value of 0.4 mM, consistent with the values given for initial rates at 30 °C. The final steady-state rate was not reached until there had been appreciable depletion of substrate. The value of k at infinite substrate concentration, obtained by extrapolation, was 0.67 min^{-1}; this is less than $k_{cat.}$, suggesting a branched pathway. This suggestion was confirmed by the observation that the burst (Fig. 2) was greater than the concentration of enzyme. Thus a minimal mechanism is:

$$\begin{array}{c} E + S \rightleftharpoons ES \rightarrow \text{E-acyl} \rightarrow E + P \\ \quad\quad\quad\quad\quad\quad \uparrow\downarrow \\ \quad\quad\quad\quad\quad\quad E_i \end{array}$$

This mechanism, first put forward for the RTEM β-lactamase from *Escherichia coli* and clavulanic acid (Fisher *et al.*, 1978) was suggested for β-lactamase I and methicillin (Kiener *et al.*, 1980) and has been corroborated recently (Fink *et al.*, 1987).

When the hydrolysis of imipenem was measured in the pH-stat (as opposed to spectrophotometrically) at 30 °C, the rate soon declined, even though the initial concentration, 10 mM, was high, and the reaction came to an end after about 7% of the substrate had been consumed (Fig. 4). On addition of β-lactamase II, which hydrolyses

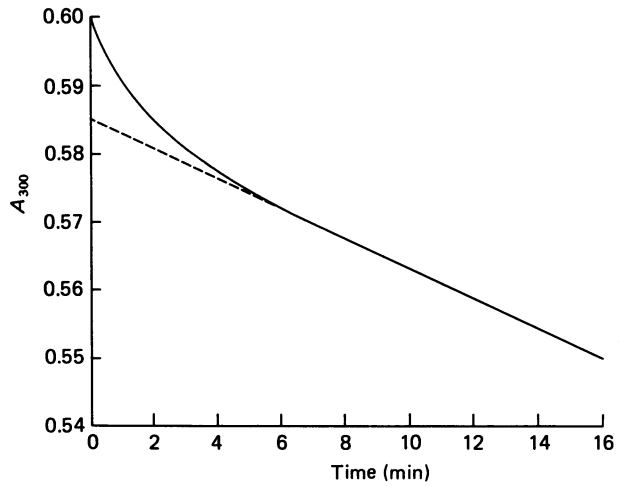


Fig. 2. Progress curve for the hydrolysis of imipenem by β-lactamase I at 23 °C

The change in A_{300} during the action of β-lactamase I (5.95 μM) on imipenem (600 μM) in 0.1 M-Mops/0.1 M-NaCl/0.05 mM-EDTA, pH 7; the path length was 1 mm.

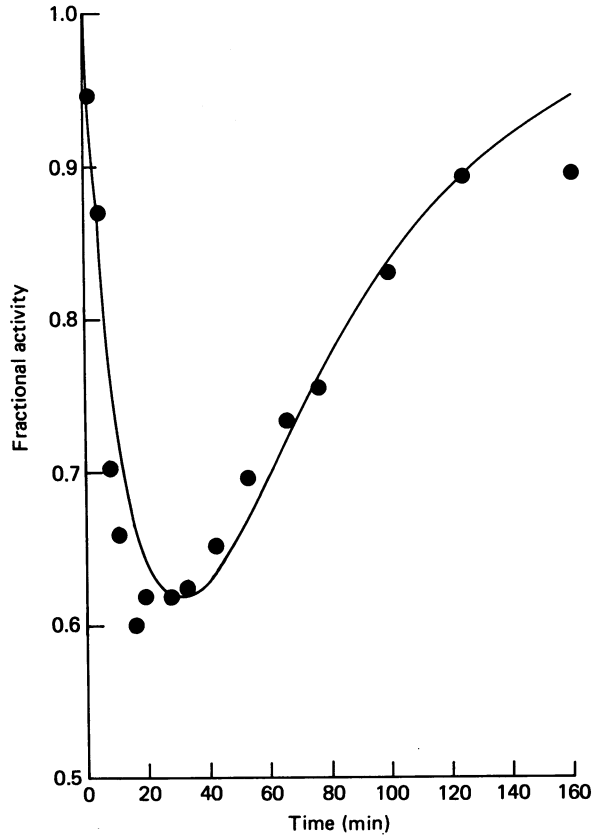


Fig. 3. Loss and regain of activity of β-lactamase I during hydrolysis of imipenem at 30 °C

Imipenem (1 mM) was incubated with β-lactamase I (10 μM) in 0.1 M-potassium phosphate buffer/0.05 mM-EDTA; the course of hydrolysis was monitored by measuring A_{300}, and residual activity was measured by taking portions and assaying with benzylpenicillin as substrate in the pH-stat.

imipenem well, there was rapid production of protons to the extent expected for the consumption of the rest of the substrate; this shows that the reason for the cessation of the reaction with β-lactamase I was the inactivation of the enzyme.

State of the enzyme after hydrolysing imipenem

When a second portion of imipenem was added to a reaction mixture in which β-lactamase I had hydrolysed some imipenem, the rate, measured spectrophotometrically, was only about 25% of the first rate; subsequent additions of imipenem, however, did not depress the rate nearly so much (Table 1, expt. 1). The product formed was not an inhibitor (Table 1, expt. 2). This effect was only revealed when imipenem was the 'second substrate'. If nitrocefin was used, the kinetic parameters of the β-lactamase I were unchanged after hydrolysis of imipenem. Thus the enzyme appears to be in a reversibly altered conformation after hydrolysing imipenem; a good substrate [nitrocefin or benzylpenicillin (results not shown)] brings about a return to the usual conformation. Indeed, the same applied when a 'non-distorting' poor substrate, cephalosporin C, was used (Table 1, expt. 3). Apparently the minimal mechanism given above may need to be expanded to take into account the possibility that the enzyme, after hydrolysing imipenem, emerges in a reversibly altered conformation.

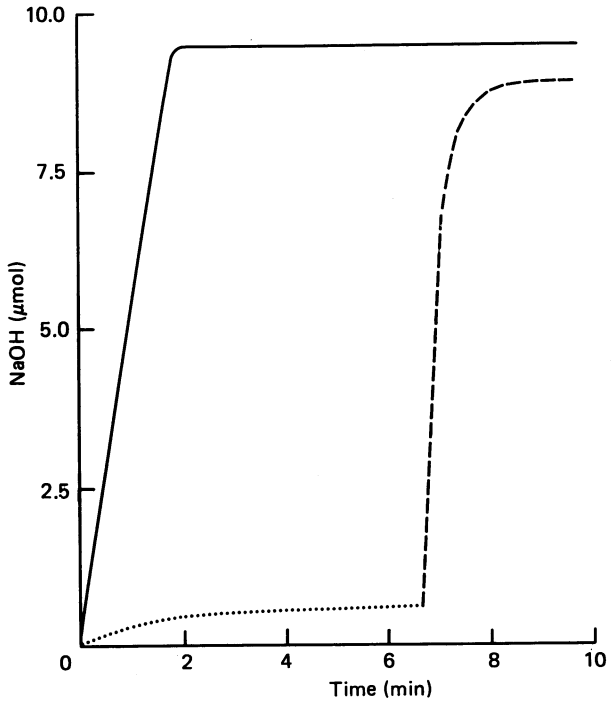


Fig. 4. Inactivation of β-lactamase I during hydrolysis of imipenem in the pH-stat

Imipenem (9 mM) in 0.1 M-NaCl/0.05 mM-EDTA, was treated with β-lactamase I (0.1 mg/ml) at 30 °C in the pH-stat at pH 7 (·····). After about 7 min, β-lactamase II (4 μg/ml) was added; rapid hydrolysis of the remaining imipenem ensued (----). The hydrolysis of benzylpenicillin (10 mM) in a separate experiment is also shown (——).

Table 1. Kinetic characterization of β-lactamase I after hydrolysis of imipenem

Expt. 1: the rate of hydrolysis of imipenem (1 mM) by β-lactamase I (1 mg/ml) in 100 mM-potassium phosphate buffer (pH 7)/0.05 mM-EDTA at 30 °C was measured by the change in A_{300} (first rate). At the end of the reaction, another portion of imipenem was added and the second rate measured, and similarly for the third and fourth rates. The rates were corrected for the effect of the 5% increase in volume on the concentration of enzyme, but not for the relatively small effect due to the lower concentration of substrate (initially at 2.5 times the K_m). Expt. 2: the effect of the presence of product was tested for by hydrolysing 1 mM-imipenem with β-lactamase II (0.887 μg/ml) in 100 mM-potassium phosphate, pH 7, the solution made 5.2 mM in EDTA to inactivate β-lactamase II, and then made 1 mM in imipenem and 0.809 mg/ml in β-lactamase I and, as in Expt. 1, a first rate and second rate were measured. Expt. 3: after hydrolysis of 1 mM-imipenem by β-lactamase I (1 mg/ml), either cephalosporin C (1.14 mM) or imipenem (1 mM) was added, and the rates measured, at 261 nm in the first case and at 300 nm in the second case (path length 0.1 cm). Percentage changes are shown in parentheses.

Expt.	Rate	Rate after 1 min (μM/min)
1	First	170.3 (100)
	Second	44.6 (26)
	Third	29.5 (17)
	Fourth	26.6 (16)
2	First	145.6 (100)
	Second	35.7 (25)
3	First	40 (100)
	Second, with cephalosporin C	33.9 (85)
	First	115 (100)
	Second, with imipenem	35.1 (31)

Table 2. Kinetic parameters for the hydrolysis of imipenem by the *Pseudomonas* β-lactamase

Conditions were as follows. Method 1: hydrolysis of 0.15 mM- or 1.5 mM-imipenem by 5 μM-β-lactamase in 0.1 M-NaCl/0.1 M-Mops, pH 7, at 37 °C. Method 2: 0.1 mM-imipenem and 238 nM-β-lactamase were kept at 37 °C for 3 min or 6 min and then portions taken for measurement of regain of activity in the hydrolysis of 1.5 mM-cephalosporin C observed at 261 nm (path length 0.1 cm). Method 3: hydrolysis of 1.88 mM-cephalosporin C by 12.5 mM-β-lactamase inhibited by 20–70 μM-imipenem; other conditions were as for methods 1 and 2; initial rates were obtained by fitting to eqn. (1) in the text.

Method	$k_{cat.}$ (min^{-1})	K_m (μM)
1. Initial rates*	0.83±0.2	
2. Regain of activity	1.3±0.3	
3. Inhibition		0.7

* $k_{cat.}$ values at 30 °C and 23 °C were 0.52 min^{-1} and 0.32 min^{-1} respectively.

Imipenem as inhibitor of β-lactamase I

The behaviour of imipenem as a transient inhibitor was examined as follows. Imipenem (1 mM) and β-lactamase I (0.003–0.017 mM) were incubated together at 30 °C and samples periodically withdrawn for assay (Fig. 3); the course of the hydrolysis of the imipenem was simultaneously measured. The characteristic loss and regain of activity may be noted. The points were fitted to curves calculated as described in the Theory section; the values of K_s/k_2 and $1/k_2+1/k_3$ were 100–120 μM·min and 0.04–0.06 min respectively, and with the optimized values of K_s, k_4 and k_5, gave values for K_s, k_2, k_3, k_4 and k_5 of 9–30 mM, 90–300 min^{-1}, 16–24 min^{-1}, 0.1–0.2 min^{-1} and 0.01–0.02 min^{-1} respectively. These results, from four experiments, showed that the initial binding was relatively weak and that the rate constant for acylation was greater than that for deacylation, neither being particularly low. Moreover, the enzyme was mainly present as the inactive form, E_i, and as free enzyme, with relatively little acyl-enzyme and even less enzyme–substrate complex.

The enzyme was freed from imipenem and its hydrolysis product by exclusion chromatography at 4 °C and compared with a solution of untreated enzyme. The solutions were adjusted so as to have the same A_{280}; their spectra then coincided in the 275–285 nm region, but the activity of the treated sample was only 55% of the untreated one. After 2.2 h at 30 °C the activity had risen to 90% of the control. Thus after hydrolysis of imipenem the β-lactamase I was partially, and reversibly, inactivated.

Imipenem as a substrate for a class-C β-lactamase

The chromosomally encoded β-lactamase from *Pseudomonas aeruginosa* (Sabath *et al.*, 1965; Flett *et al.*, 1976; Berks *et al.*, 1982) is a class-C β-lactamase (Jaurin & Grundström, 1981; Knott-Hunziker *et al.*, 1982*a*); the enzymes belonging to this class resemble each other closely and are responsible for resistance to many β-lactam antibiotics (Lindberg & Normark, 1986; Sanders, 1987). Imipenem, however, had been regarded as stable to class-C β-lactamases, but this is only true up to a point. Thus the $k_{cat.}$ was nearly 1/min, and the K_m was approx. 1 μM (Table 2), so that $k_{cat.}/K_m$ is about 0.25% that of a good substrate. The value for $k_{cat.}$ was low enough to test for a lag as follows. Imipenem and the *Pseudomonas* β-lactamase were incubated at 37 °C and then a portion taken and the rate constant for regain of activity during an assay (with cephalosporin as substrate) was determined, as described in the Theory section (Fig. 1). The value for $k_{cat.}$ thus found (Table 2) did not differ significantly from that measured directly; this suggests that deacylation is the rate-determining step in the hydrolysis of imipenem, as it is for other substrates with this enzyme (Knott-Hunziker *et al.*, 1982*b*).

The K_m was too low to be measured directly and so was obtained from the use of imipenem as an inhibitor of the hydrolysis of cephalosporin C (Table 2). The progress curves could not be used to determine K_i by the 'direct-comparison' method (Waley, 1982) because there was progressive inactivation; instead, progress curves were fitted to eqn. (1) to obtain initial rates. Although imipenem is actually a competing substrate, its relative specificity constant (Waley, 1983) is less than 0.01 and so it may be treated as a competitive inhibitor and the found K_i is identical with the K_m. Although kinetic parameters could be obtained from progress curves for

the hydrolysis of imipenem by the *Pseudomonas* β-lactamase, the instability of the enzyme at 37 °C makes this procedure unreliable.

DISCUSSION

Structural changes in β-lactamase I brought about by imipenem

The changes in β-lactamase I during hydrolysis of imipenem were inferred from the altered properties of the enzyme. There were two ways in which this was shown. One was from the repeated hydrolyses of imipenem by one enzyme solution. The fact that it was only the first (and not subsequent) courses of hydrolysis that had a marked effect shows that there is a branched pathway, with the branch leading, reversibly, to an altered form of the (acyl-)enzyme. The other altered property is the loss in activity of the enzyme during hydrolysis of imipenem in the pH-stat (Fig. 4). This is attributed to the vigorous stirring in the pH-stat; there was no comparable loss in activity when the reaction with imipenem was monitored in the spectrophotometer, nor when the hydrolysis of benzylpenicillin was monitored in the pH-stat (Fig. 4). The heightened sensitivity of the enzyme brought about by a slow substrate is thus an example of the phenomenon that we called 'substrate-induced de-activation' (Kiener & Waley, 1977) which has been extensively studied by Citri and his co-workers (Citri *et al.*, 1976; Klemes & Citri, 1979). That the acyl-enzyme is the intermediate that alters in conformation seems likely, from work with β-lactamase I and methicillin or cloxacillin (Kiener *et al.*, 1980; Fink *et al.*, 1987). This behaviour thus resembles that shown by olivanic acids with the β-lactamase from *Escherichia coli*, where the branch leads to a relatively stable acyl-enzyme in which the double bond in the five-membered ring has migrated (Charnas & Knowles, 1981; Easton & Knowles, 1982). Olivanic acids are carbapenems, but, unlike imipenem, they have a double bond in the side chain carrying the sulphur atom. Imipenem is known to hydrolyse to a Δ^1 product in which the double bond has migrated and which only shows end absorption (R. W. Ratcliffe, K. J. Wildonger, L. Di Michele, A. W. Douglas, R. Hajdu, R. T. Goegelman, J. P. Springer & J. Hirshfield, unpublished work).

Hysteretic behaviour is now known for a number of enzymes, but the structural basis is often obscure (Frieden, 1970; Ricard & Cornish-Bowden, 1987). Dynamic behaviour, as shown by hydrogen exchange, for example, may be closely associated with catalytic competence. The change in motility of β-lactamase I in the presence of substrates such as methicillin or cloxacillin was revealed by hydrogen exchange; the changes were greater than would be expected had they been confined to the immediate environment of the active site (Kiener & Waley, 1977). It should be stressed that these are changes in *motility*; it is not unreasonable that small changes in structure can lead to large changes in motility, and there is thus no discrepancy between the results of hydrogen exchange and those from circular dichroism, which failed to reveal large changes in the overall conformation of the altered acyl-enzyme (Persaud *et al.*, 1986; Fink *et al.*, 1987). The detailed structural basis for the change will only be understood when there has been crystallographic study of an altered acyl-enzyme.

At the steady state, the branched-pathway model gives kinetics in which the effect of branching depends on the magnitude of k_3 relative to k_2 $[1+(k_4/k_5)]$ (Kiener *et al.*, 1980; Frere, 1981); the larger the rate constant for acylation the greater the extent to which the acyl-enzyme accumulates and the more pronounced the effect of branching.

Implications of the sensitivity of imipenem to the *Pseudomonas* β-lactamase

The kinetic parameters given in Table 2 imply that, given a high enough periplasmic concentration, a class-C β-lactamase could diminish the effectiveness of imipenem as an antibiotic if the permeability of the bacterial outer membrane were also low. The interplay between β-lactamase activity and permeability is best described in terms of the dimensionless permeability number (Waley, 1987). Imipenem has such a high rate of diffusion through the OmpF channel of *E. coli* (Yoshimura & Nikaido, 1985) that even for *Pseudomonas aeruginosa*, where the permeability is about 100-fold lower (Yoshimura & Nikaido, 1982), the permeability number would be 0.8 if the periplasmic concentration of β-lactamase were 10 μM. A value of the order of 1 for the permeability number indicates that the periplasmic concentration of the antibiotic approaches the external concentration (Waley, 1987). If this were 100 μM, the parameters in Table 2 show that the half-life for hydrolysis by the β-lactamase would be about 6 min. This is sufficiently long to suggest that imipenem might have preferentially reacted with the target transpeptidase. That permeability can play a major part is clear from the fact that strains of *Pseudomonas aeruginosa* that are resistant to imipenem show diminished expression of an outer-membrane protein, presumably important in transport of the antibiotic (Quinn *et al.*, 1986; Buscher *et al.*, 1987).

The general consensus that imipenem is relatively resistant to β-lactamases (see, e.g., Cullmann, 1986; Labia *et al.*, 1986; Williams *et al.*, 1986) appears to be well founded, although exceptions continue to be encountered (Shannon *et al.*, 1986). The work described above puts these ideas on a more quantitative basis.

We thank the Medical Research Council for support, and Merck Sharp & Dohme Research Laboratories and the Glaxo Group for gifts. This is a contribution from the Oxford Enzyme Group.

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Received 4 February 1988; accepted 31 March 1988