Biochem. J. (1990) 272, 613–619 (Printed in Great Britain)

Site-directed mutagenesis of β-lactamase I

Single and double mutants of Glu-166 and Lys-73

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Two single mutants and the corresponding double mutant of β-lactamase I from *Bacillus cereus* 569/H were constructed and their kinetics investigated. The mutants have Lys-73 replaced by arginine (K73R), or Glu-166 replaced by aspartic acid (E166D), or both (K73R+E166D). All four rate constants in the acyl-enzyme mechanism were determined for the E166D mutant by the methods described by Christensen, Martin & Waley [(1990) Biochem. J. **266**, 853–861]. Both the rate constants for acylation and deacylation for the hydrolysis of benzylpenicillin were decreased about 2000-fold in this mutant. In the K73R mutant, and in the double mutant, the rate constants for acylation were decreased about 100-fold and 10000-fold respectively. All three mutants also had lowered values for the rate constants for the formation and dissociation of the non-covalent enzyme–substrate complex. The specificities of the mutants did not differ greatly from those of wild-type β-lactamase, but the hydrolysis of cephalosporin C by the K73R mutant gave 'burst' kinetics.

INTRODUCTION

β-Lactamases are clinically important and mechanistically interesting hydrolytic enzymes. The first milestone in the task of unravelling the mechanism of their action was the conclusion that β-lactamases were either zinc enzymes or serine enzymes (Coulson, 1985; Waley, 1988), together with the establishment of the acyl-enzyme mechanism for the latter (Fisher *et al.*, 1980; Knott-Hunziker *et al.*, 1982). Serine β-lactamases were divided into two classes, class A (Ambler, 1980) and class C (Jaurin & Grundström, 1981), on the basis of their amino acid sequences. The central role of a serine residue [Ser-70 in the numbering system of Ambler (1980)] has been established by now for several β-lactamases, and the same applies to the related D-alanyl-D-alanine peptidases (Frère & Joris, 1985; Kelly *et al.*, 1989). Site-directed mutagenesis has been used to show that, for activity to be retained, cysteine, but not threonine, can replace Ser-70 (Dalbadie-McFarland *et al.*, 1982; Sigal *et al.*, 1984). Structural studies have shown that the active sites of serine β-lactamases contain a lysine residue (Lys-73) and an interacting residue of either glutamic acid (Glu-166) (class A) (Herzberg & Moult, 1987; Moews *et al.*, 1990; Baguley, 1990) or tyrosine (Tyr-150) (class C) (Oefner *et al.*, 1990). The critical role of Ser-70 immediately raises the question: what group activates this serine residue? Candidates that have been put forward are Lys-73 and either Glu-166 (class A) or Tyr-150 (class C); their precise role has evoked considerable discussion.

Site-directed mutagenesis has provided further evidence for the importance of Lys-73 and Glu-166 (Madgwick & Waley, 1987), which is considerably extended in the present paper. We have developed methods for expression of the mutants in *Bacillus subtilis* (R. M. Gibson & J. Errington, unpublished work). Two advantages accrue: the β-lactamase can be isolated more readily and in higher yield from the extracellular medium, and the *N*-terminal extension found when expression is in *Escherichia coli* is absent (Mézes *et al.*, 1985).

Understanding the effects of a mutation in an enzyme demands thorough characterization of the kinetic consequences. Fortunately, all the rate constants in the acyl-enzyme mechanism (Scheme 1) have been determined for several class A β-lactamases (Martin & Waley, 1988; Christensen *et al.*, 1990). The present paper reports the determination of the rate constants for the mutants Lys-73→Arg (K73R), Glu-166→Asp (E166D) and the double mutant (K73R, E166D). Here we use the widely adopted nomenclature in which, with the single-letter code for amino acids, K73R is a mutant enzyme having Lys-73 replaced by arginine.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E\text{-acyl} \xrightarrow{k_{+3}} E + P$$

Scheme 1.

MATERIALS AND METHODS

Materials

Restriction enzymes and [α-[^{35}S]thio]dATP were obtained from Amersham International, Amersham, Bucks., U.K. DNA polymerase (Klenow fragment) was obtained from Anglian Biotechnology, Colchester, Essex, U.K. DNA ligase was prepared by N. Gascoyne in this department. Celite was obtained from Fluka Chemicals, Glossop, Derbyshire, U.K.

Bacterial strains and plasmids

Escherichia coli strain RZ1032 {HFr KL16 PO/45 [*lysA* (16–62)], *dut1*, *ung1*, *thi1*, *relA1*, *Zbd-279*::*Tn10*, *supE44*} was obtained from P. Handford, and XL1-Blue {*endA1*, *hsdR17* (*rk*−, *mk*+), *supE44*, *thi*−, λ−, *recA1*, *gyrA96*, *relA1*, (*lac*), [F′, *proAB*, *lacI*^{q}, *lacZ*Δ *M15*, *Tn10Tc*]} was from Stratagene, Cambridge, U.K. Bacteria were grown in 2×TY broth [16 g of tryptone T (Oxoid, Basingstoke, Hants., U.K.), 10 g of yeast extract and 5 g of NaCl per litre]. M13mp8 was obtained from K. Gould, pUC18 and pSGMU2, a pUC13 derivative, were from

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J. Errington and pPJM9 was from P. J. Madgwick. The mutagenic oligonucleotides were synthesized by K. Gould.

Construction of the mutants of β-lactamase I

The *penPC* gene, encoding β-lactamase I, was excised from pPJM9 by digestion with *Xba*I and *Bam*HI and cloned into pUC18, from which it was subsequently cloned into M13mp8 as an *Sal*I–*Bam*HI fragment. The gene was sequenced by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). The mutants were constructed by the uracil template protocol (Kunkel, 1985; Kunkel *et al.*, 1987), by the use of oligonucleotides 20 bases long, and the introductions of the base changes were confirmed by sequencing. The mutant genes were cloned into pSGMU2 and expressed in *B. subtilis* by the use of a bacteriophage vector (R. M. Gibson & J. Errington, unpublished work).

Purification of mutants of β-lactamase I

The mutant enzymes were isolated from the culture medium of *B. subtilis* (R. M. Gibson & J. Errington, unpublished work) by the procedures used for wild-type β-lactamase I (Davies *et al.*, 1974), except that purification was followed by Western blots (Burnette, 1981) rather than by enzymic activity.

Estimation of steady-state kinetic parameters

The parameters were determined by analysis of progress curves, or from initial rates. The half-time method (Wharton & Szawelski, 1982) was mainly used for progress curves, but the results for the hydrolysis of benzylpenicillin in solutions containing glycerol were also analysed by the method of Duggleby & Nash (1989); the program ENZYMMRT (kindly supplied by Dr. J. Nash) was used, modified so that measurements were made from an arbitrary origin. The fit to the data for individual progress curves was much improved, but the scatter in the values of $k_{cat.}/K_m$ was, disappointingly, only marginally reduced. For the slow hydrolysis of 6-aminopenicillanic acid by the double mutant, K_m was found by inhibition of the hydrolysis of ampicillin; similarly, for the hydrolysis of cephalosporin C by the mutant E166D and by the double mutant the inhibition of hydrolysis of *N*-(2-furyl)-acryloylpenicillin was used. The found K_i gives the K_m accurately when the $k_{cat.}/K_m$ of the inhibiting substrate is less than 5% of that of the monitoring substrate (Waley, 1983).

Initial rates, obtained from the first 5% of reaction, were fitted to the Michaelis–Menten equation by non-linear regression with the DNRP53 program of Duggleby (1984).

RESULTS

Sequencing the wild-type β-lactamase I gene and isolation of mutant enzymes

Sequencing revealed two discrepancies with the published sequence of the wild-type β-lactamase I gene (Madgwick & Waley, 1987): one was an insertion of a G residue before the start of the coding sequence and the second was a change of C to T at position 404, which did not alter the amino acid sequence since both CAC and CAT encode histidine. The efficiencies of the mutagenesis were 22% (double mutant), 44% (E166Q), 64% (K73R) and 75% (E166D).

The amounts of purified mutants obtained, homogeneous by PAGE, were: K73R, 3 mg; E166D, 30 mg; K73R+E166D (double mutant), 4 mg; E166Q, 1 mg. There are several possible reasons for the differences in the amounts obtained, including differences in stabilities of the mutants, but we have no experimental evidence to decide between the alternatives.

The failure of E166Q β-lactamase I to protect *E. coli* against ampicillin led to the conclusion that replacement of the carboxylate (CO_2^-) group by the amide ($CONH_2$) group led to loss in activity (Madgwick & Waley, 1987). The samples of the E166Q mutant enzyme that we have so far obtained were not pure, and showed some activity. We believe that this activity was due to wild-type enzyme, arising by hydrolysis in the culture medium or during purification.

Active-site titration

It is helpful to be able to measure the concentration of active enzyme by active-site titration, especially for comparison of mutant and wild-type values of $k_{cat.}$ and $k_{cat.}/K_m$. We used radioactive 6β-iodopenicillanic acid for this purpose with E166D β-lactamase I; the sample of purified enzyme turned out to be fully active. The other mutants, however, did not furnish a separate peak of labelled protein on exclusion chromatography, for reasons that are not altogether clear, but may well be connected with the much smaller amounts of these mutants that were obtained.

Determination of k_{+1} and k_{-1}/k_{+2}

The effect of increasing the viscosity of the medium by adding glycerol was investigated in four instances: with either ampicillin or benzylpenicillin for E166D, and with benzylpenicillin for K73R and the double mutant. The variation of $k_{cat.}/K_m$ with viscosity has been used to obtain values of k_{+1} and k_{-1}/k_{+2} for β-lactamase I (Hardy & Kirsch, 1984; Christensen *et al.*, 1990) by the use of the following equation:

$$\frac{K_m}{k_{cat.}} = \frac{1}{k_{+1}}\eta_{rel.} + \frac{k_{-1}}{k_{+1}k_{+2}} \quad (1)$$

A necessary check that the effects of glycerol are restricted to its effect on diffusion is a lack of effect on $k_{cat.}$. The results for the K73R mutant in Fig. 1 clearly show that $K_m/k_{cat.}$ increases with the relative viscosity, whereas the values of $k_{cat.}$ show scatter, but no trend. In Fig. 2 the relative values of $K_m/k_{cat.}$ for the mutant

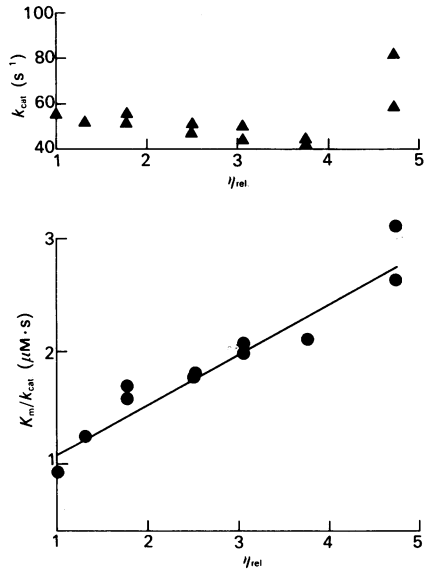


Fig. 1. Dependence of $k_{cat.}$ and $K_m/k_{cat.}$ on relative viscosity for the hydrolysis of benzylpenicillin by mutant K73R

Progress curves at pH 7 at 20 °C for the reaction in solutions containing 0–45% (v/v) glycerol were measured at 232 nm and analysed by the half-time method. The lower plot shows $K_m/k_{cat.}$ and the upper plot shows $k_{cat.}$.

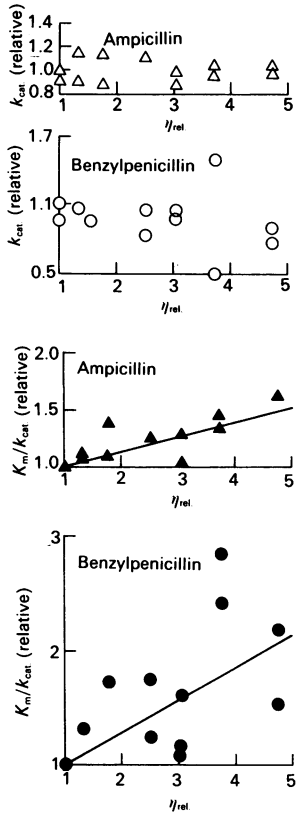


Fig. 2. Dependence of $k_{cat.}$ and $K_m/k_{cat.}$ on relative viscosity for the hydrolysis of ampicillin and benzylpenicillin by mutant E166D

Progress curves were measured as for Fig. 1. Relative values of $K_m/k_{cat.}$ and $k_{cat.}$ (i.e. values relative to those in solution lacking added glycerol) for benzylpenicillin (● and ○) and ampicillin (△ and ▲) are shown.

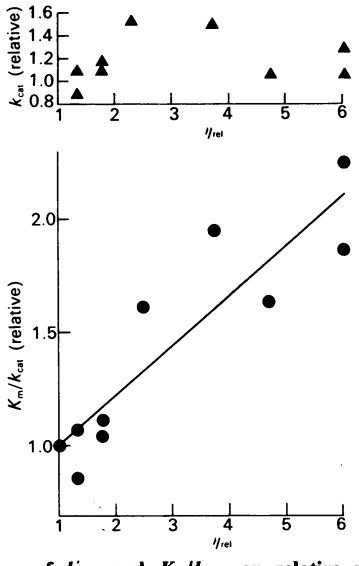


Fig. 3. Dependence of $k_{cat.}$ and $K_m/k_{cat.}$ on relative viscosity for the hydrolysis of benzylpenicillin by the double mutant K73R + E166D

Progress curves were measured as for Fig. 1. Relative values of $K_m/k_{cat.}$ and $k_{cat.}$ are shown.

E166D are plotted against the relative viscosity with either benzylpenicillin or ampicillin as substrate, and again there is an increase, not seen for the relative values of $k_{cat.}$ (top of Fig. 2). The same features were also seen with the double mutant (Fig. 3). Use of eqn. (1) gave the values of k_{+1} and k_{-1}/k_{+2} (Table 1).

Table 1. Rate constants for the hydrolysis of benzylpenicillin

Rate constants were determined in 0.05 M-phosphate buffer, pH 7, containing 0.5 M-NaCl and 0.1 mM-EDTA at 20 °C. For K73R and the double mutant, the value of k_{+2} was approximate, and that of k_{+3} was only a lower limit. The values in parentheses are standard errors of the mean derived from at least three experiments for the values immediately above, or for k_{+1} and k_{-1} the values derived from the standard errors of the intercepts and slopes of the lines fitted to eqn. (1) in the text.

Rate constant	Wild-type	K73R mutant	E166D mutant	Double mutant
k_{+1} ($\mu M^{-1} \cdot s^{-1}$)	41	2.1	0.65	0.7
	(3)	(0.2)	(0.3)	(0.1)
k_{-1} (s^{-1})	2320	77	6.7	1.4
	(700)	(23)	(5)	(0.6)
k_{+2} (s^{-1})	4100	55	2.1	0.47
	(200)	(1)	(0.1)	(0.02)
k_{+3} (s^{-1})	3600	$\geqslant 220$	2.3	$\geqslant 1.9$
	(200)		(0.2)	
$k_{cat.}$ (s^{-1})	2200	55	0.67	0.47
	(60)	(1)	(0.07)	(0.02)
$k_{cat.}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)	34	0.55	0.077	0.047
	(4)	(0.07)	(0.035)	(0.02)
K_m (μM)	65	101	8.6	10
	(5)	(10)	(3)	(3)
K_s (μM)	57	35	12	2.1
	(21)	(14)	(14)	(1)

Determination of the rate constants for acylation and deacylation

The acid-quench method (Martin & Waley, 1988; Christensen *et al.*, 1990) was used to find k_{+2} and k_{+3} for the hydrolysis of benzylpenicillin by E166D; their values were comparable (Table 1), as had been found for the wild-type enzyme. Unfortunately, no acyl-enzyme could be detected when either K73R or the double mutant was used. This suggests that for these mutants $k_{+2} \ll k_{+3}$. When $k_{+2} \ll k_{+3}$ the second term on the right-hand side of eqn. (2) becomes negligible and the value of $k_{cat.}$ gives an approximate value for k_{+2}; this is the value given in Table 1:

$$1/k_{cat.} = 1/k_{+2} + 1/k_{+3} \quad (2)$$

Only an approximate lower limit can be placed on k_{+3}; we would expect to be able to detect acyl-enzyme if k_{+3} were less than about 4 times k_{+2}, and so this value is given, as a lower limit, in Table 1.

Specificity of mutants of β-lactamase I

The effects of charged groups in the side chains of penicillins on their activities as substrates for the mutants were explored by the use of ampicillin (in which an amino group replaces a hydrogen atom in the methylene group of benzylpenicillin) and carbenicillin (in which a carboxylate group is similarly situated). The effects of removal of the side chain were probed by the use of 6-aminopenicillanic acid as substrate, and cephalosporins were also used. With one exception (described in the next paragraph), deviations from Michaelis–Menten kinetics were not detected, and $k_{cat.}$ and K_m were obtained from initial rates (usually) or progress curves. There were no dramatic changes in specificity, but 6-aminopenicillanic acid was hydrolysed particularly poorly by the double mutant (Table 2). When the values of $k_{cat.}/K_m$ are compared, 6-aminopenicillanic acid has the lowest value of the penicillins examined, and less than that of nitrocefin. Nitrocefin itself has a lower value than that of carbenicillin, the slowest hydrolysed of the penicillins with a side chain.

Table 2. Kinetic parameters for hydrolysis of β-lactams by mutant enzymes

The steady-state kinetic parameters were determined from initial rates (usually) or from progress curves. The reactions were carried out in 0.1 M-sodium phosphate buffer, pH 7, containing 0.5 M-NaCl and 0.01 mM-EDTA at 20 °C. The substrates were ampicillin, carbenicillin, *N*-(2-furyl)acryloylpenicillin, cephalosporin C and nitrocefin. The parameters shown for the K73R mutant and cephalosporin C are for the final rate, after the burst.

	K_m (μM)			
Substrate	Wild-type	K73R mutant	E166D mutant	Double mutant
Ampicillin	250	82±13	460±37	180±49
Carbenicillin	150	46±6	280±42	110±20
N-(2-Furyl)acryloylpenicillin	35	6.8±0.9	86±20	4±1
6-Aminopenicillanic acid	1600	120±20	810±70	160±20
Cephalosporin C	220	270±100	380±70	180±90
Nitrocefin	55	78±4	52±4	1.5±0.3
	$k_{cat.}$ (s^{-1})			
Substrate	Wild-type	K73R mutant	E166D mutant	Double mutant
Ampicillin	5000	1.3±0.08	140±3	1.4±0.2
Carbenicillin	440	0.46±0.03	24±1	0.37±0.03
N-(2-Furyl)acryloylpenicillin	1700	0.2±0.01	30±4	0.24±0.02
6-Aminopenicillanic acid	260	0.049±0.003	1.8±0.1	0.001±0.0001
Cephalosporin C	0.2	0.001±0.0004	0.011±0.003	0.0004±0.0002
Nitrocefin	34	0.21±0.01	0.81±0.02	0.0007±0.0002
	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)			
Substrate	Wild-type	K73R mutant	E166D mutant	Double mutant
Ampicillin	20000	16±3	308±31	8±3
Carbenicillin	2800	10±2	86±18	3.3±0.7
N-(2-Furyl)acryloylpenicillin	49000	30±5	343±140	55±20
6-Aminopenicillanic acid	170	0.4±0.1	2.2±0.3	0.006±0.002
Cephalosporin C	0.9	0.005±0.004	0.028±0.003	0.002±0.002
Nitrocefin	618	2.7±0.2	16±2	0.4±0.2

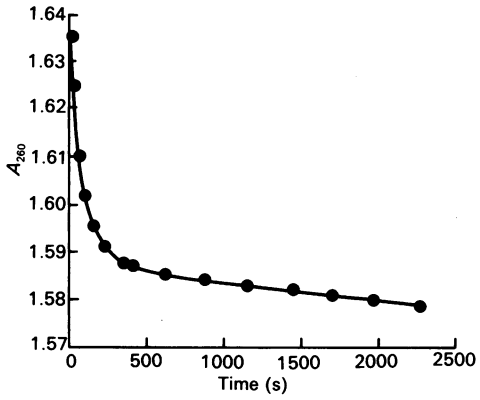
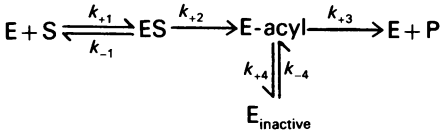


Fig. 4. Progress curve for the hydrolysis of cephalosporin C by mutant K73R

The hydrolysis of 250 μM-cephalosporin C by 400 nM mutant β-lactamase at pH 7 at 20 °C was measured spectrophotometrically; the change in A_{260} shown in the plot is equivalent to consumption of approx. 4% of the substrate.

Hydrolysis of cephalosporins by K73R β-lactamase I

The hydrolysis of cephalosporin C or cephalothin by the K73R mutant showed a burst, i.e. a rapid initial rate changing to a much lower rate (Fig. 4). This feature was not observed with wild-type enzyme (Abraham & Newton, 1956; Crompton *et al.*, 1962; Hardy & Kirsch, 1984; Bicknell & Waley, 1985), nor with the other mutants studied here. Both rates were proportional to the concentration of enzyme (results not shown). Moreover the size of the burst was proportional to the concentration of enzyme, but was much larger. This is characteristic of a branched pathway, often encountered with β-lactamases (Fisher *et al.*, 1978; Kiener *et al.*, 1980; Persaud *et al.*, 1986; Fink *et al.*, 1987) (Scheme 2). The progress curves were fitted to the equation:



Scheme 2.

$$p = v_t t + (v_0 - v_t)(1 - e^{-kt})/k \quad (3)$$

where v_0 and v_t are the original and final rates, and k is the first-order rate constant for the burst. Estimates of k_{-4} and k_{+3}/k_{+4} of $3.5 \times 10^{-4} \pm 1 \times 10^{-4}$ s^{-1} and 61±30 respectively were obtained by the procedure of M. T. Martin & S. G. Waley (unpublished work). The values of the original and final rates were linear in the concentration of cephalosporin C and gave values for $k_{cat.}/K_m$ of 2.7±0.3 $mM^{-1}\cdot s^{-1}$ and 0.028±0.003 $mM^{-1}\cdot s^{-1}$ respectively. It is noteworthy that the former value is about 3 times the value (0.91 $mM^{-1}\cdot s^{-1}$) for the wild-type enzyme.

DISCUSSION

Effect of mutations on enzyme–substrate combination

The relatively low value for k_{+1} (of the order of 10^5 $M^{-1} \cdot s^{-1}$) for the hydrolysis of benzylpenicillin by the mutant β-lactamases was unexpected. Admittedly, the reaction is only 23% diffusion-controlled, compared with 76% for the wild-type (Christensen *et al.*, 1990), but the finding focuses attention on the condition for detecting any appreciable effect of increase in viscosity. It can be seen from eqn. (1) that this condition is that the two terms on the right-hand side be comparable in magnitude. Now the term in k_{+1} is common, and so we must compare $\eta_{rel.}$ with k_{-1}/k_{+2}; the former can be about 6, and so if the latter is 30 or above then the first term is no more than 20% of the second, and in practice any effects of increase in viscosity will not be discernible. Conversely, when the slope in a plot of $K_m/k_{cat.}$ against the relative viscosity is greater than zero then it is likely that k_{-1} and k_{+2} are comparable. The values for k_{+1} for the mutants were 20–60-fold less than those for the wild-type; this cannot be accounted for by a two-step mechanism for binding. As pointed out by Bazelyansky *et al.* (1986), if there is more than one enzyme–substrate complex before the first irreversible step, the usual value for k_{+1} will be obtained from the dependence of $k_{cat.}/K_m$ on viscosity. In the formation of the enzyme–substrate complex, the proportion of fruitful encounters is apparently lower for the mutants. Moreover, the values for k_{-1} were lowered by approx. 500-fold compared with wild-type. These findings suggest that the mutants have an altered conformation in which access to, and egress from, the active site is impeded. The need for structural studies of the mutants is emphasized.

'Efficiency' of mutants

The results in Table 1 reveal the unexpected finding that all three mutants have comparable k_{-1} and k_{+2} values. This feature, which was observed for wild-type β-lactamase I and for two other β-lactamases, was regarded as a sign of a 'fully efficient' enzyme (Christensen *et al.*, 1990; but see Brocklehurst & Topham, 1990; Waley, 1990). The mutants that we are discussing here are clearly not fully efficient: this is obvious both from the diminished values of the kinetic parameters and from their diminished power to protect *E. coli* from ampicillin (R. M. Gibson & S. G. Waley, unpublished work). Thus it appears that the commonality of the rate constants may be a necessary but not sufficient condition for a fully efficient enzyme.

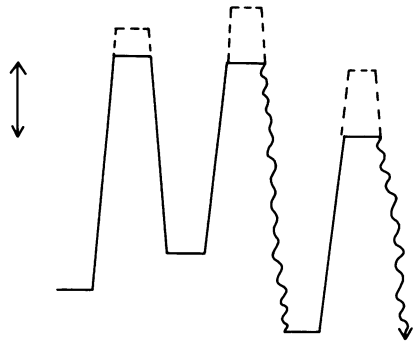


Fig. 5. Kinetic barrier diagram for the hydrolysis of benzylpenicillin by wild-type β-lactamase I and mutant E166D

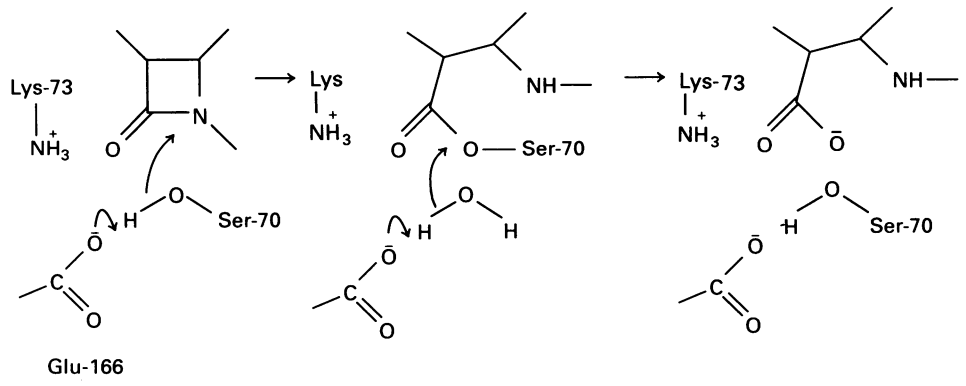
Unbroken lines denote wild-type β-lactamase I and broken lines the mutant E166D in this kinetic barrier diagram (Burbaum *et al.*, 1989) for 1 μM substrate. The wavy lines indicate uncertainty about the level of the second minimum, the acyl-enzyme, and the fact that the reaction is in practice irreversible. The arrow denotes 20 kJ/mol.

Kinetic barriers

The results for the E166D mutant, for which all four rate constants were determined, are shown in the kinetic barrier diagram (Burbaum *et al.*, 1989) (Fig. 5). The substrate concentration was arbitrarily fixed at 1 μM here, and this enables the first barrier height to be drawn; in a kinetic barrier diagram bimolecular steps are treated by replacing the second-order rate constant, here k_{+1}, by its product with the concentration, here k_{+1} [substrate]. Thus the barrier heights are represented by numbers with the same dimensions throughout. The heights of all three barriers were raised in the E166D mutant (Fig. 5); on the other hand, the level of the first stable species, the non-covalent enzyme–substrate complex was somewhat lowered. This implies that the substrate bound more tightly to the mutant than to wild-type enzyme. The kinetic barrier diagram clearly brings out the similar effects of the Glu-166→Asp mutation on the acylation and deacylation rate constants.

Rate enhancement by wild-type and mutant

The twin props to our understanding of β-lactamase action are knowledge of the structure of the enzyme and knowledge of the mechanism of the non-enzymic reaction. The structures of the



Scheme 3. Schematic picture of β-lactam hydrolysis by class A β-lactamases

active sites of β-lactamases (Herzberg & Moult, 1987; Dideberg *et al.*, 1987; Oefner *et al.*, 1990; Moews *et al.*, 1990; Baguley, 1990) have been compared with those of the well-studied serine proteinases. On this basis, Glu-166 or Tyr-150 could function like His-57 in the serine proteinases, and Lys-73 could, like Asp-102, function as an 'electrostatic anchor'. The backbone NH groups of Ser-70 and Ala-237 hydrogen-bond to the β-lactam carbonyl group and function as the oxy-anion hole. Our current working hypothesis for catalysis by class A serine β-lactamases is shown in Scheme 3.

The non-enzymic hydrolysis of benzylpenicillin is catalysed by both acids and bases (Page, 1987). In the enzymic reaction the reaction proceeds by way of the acyl-enzyme, in which the penicilloyl moiety forms an ester bond with Ser-70, and there are two steps to consider. The first step, acylation, is an alcoholysis of benzylpenicillin; in the reactions of alkoxide ions with benzylpenicillin there is formation, and rate-limiting breakdown, of a tetrahedral intermediate, and there is general acid catalysis by the solvent (Page, 1987). As mentioned above, a favoured candidate for the group in class A β-lactamases that (partially) converts the hydroxy group of serine-70 into an alkoxide ion is Glu-166. In deacylation, it can play a similar role by deprotonation of the incoming molecule of water (Scheme 3). The rates of hydrolysis of α-penicilloyl esters are similar to those of simple alkyl esters (Page, 1987). This contrasts with the first stage, where β-lactams are more reactive than simple (acyclic) amides. If, as is generally believed, serine β-lactamases originated from transpeptidases (Pollock, 1971; Kelly *et al.*, 1986; Samraoui *et al.*, 1986), the novel feature was the catalysis of deacylation. Thus in the reaction of β-lactams with transpeptidases $k_{+2} \gg k_{+3}$; in wild-type β-lactamases $k_{+2} \simeq k_{+3}$, and in the K73R and double mutants $k_{+2} \ll k_{+3}$. Hence it could be said that we have here yet a further step along the course of evolution.

Substrate-induced inactivation of K73R by cephalosporin C

One of the generalizations that Citri and co-workers put forward on the basis of their extensive investigations of substrate-induced inactivation (Citri *et al.*, 1976; Klemes & Citri, 1979) was that cephalosporins did not elicit inactivation of class A β-lactamases. Now we see that this no longer applies for β-lactamase I when Lys-73 is replaced by arginine. Even more striking is the finding that the activity of the wild-type enzyme is here surpassed, albeit briefly: for some 20 turnovers $k_{cat.}/K_m$ is larger for the mutant than for the wild-type enzyme.

Site-directed mutagenesis of other β-lactamases

β-Lactamases have been used for random mutagenesis (Hall & Knowles, 1976) and were the first enzymes to be subjected to oligonucleotide-directed mutagenesis (Dalbadie-McFarland *et al.*, 1982). Alterations in the specificity have been noted. In the RTEM β-lactamase, A237N has markedly altered specificity, with the activity towards cephalosporins enhanced 4-fold and towards penicillins decreased 10-fold (Healey *et al.*, 1989). On the other hand, in the β-lactamase from *Streptomyces albus* G, the mutant N132S has selectively decreased activity towards cephalosporins (Jacob *et al.*, 1990). The counterpart of Lys-73 in the class C β-lactamase from *Citrobacter freundii* GN346 has been replaced by arginine to give an enzyme that had about 2% of the activity (as measured by $k_{cat.}/K_m$) of the wild-type with either cephalothin or benzylpenicillin as substrate (Tsukamoto *et al.*, 1990). Since in our case mutant K73R of β-lactamase I had $k_{cat.}/K_m$ for benzylpenicillin 1.6% of wild-type this feature is similar. However, the replacement in the class C enzyme gave a 20-fold increase in K_m and 3-fold decrease in $k_{cat.}$, contrasted with about 1.5-fold increase in K_m and 30-fold decrease in $k_{cat.}$ in our case. One would not perhaps expect a very close similarity in the effects of such a mutation because the environments of the lysine residue differ in class A and class C β-lactamases.

Replacement of glutamic acid by aspartic acid in some other enzymes

The change of glutamic acid to aspartic acid might well be considered a conservative change. Two contrasting examples of the consequences in other enzymes may be cited. The key active-site residue in triose-phosphate isomerase is Glu-165 (Waley *et al.*, 1970; de la Mare *et al.*, 1972; Corran & Waley, 1973); the effects of this change (E165D in the isomerase) have been analysed in detail: the upshot is that the change essentially affected the transition states for the two enolization steps; the rate constant for proton transfer was down about 1000-fold in the mutant, but there was little or no effect on association of the substrates (Raines *et al.*, 1986; Blacklow *et al.*, 1988). There are different theoretical explanations for the structural and energetic consequences (Alber *et al.*, 1987; Daggett *et al.*, 1989). On the other hand, the same change in staphylococcal nuclease had structural consequences not confined to the active site (Hibler *et al.*, 1987). As always, mechanistic inferences from mutagenesis must remain tentative until confirmed from structural evidence.

We thank Glaxo Group, Beecham Research Laboratories and Leo Pharmaceutical Products for gifts, Joan Monks and Sue Thornewell for the preparation of β-lactamase I, Dr. J.-M. Frère for the labelled 6β-iodopenicillanic acid and the Medical Research Council for financial support.

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Received 24 April 1990/30 May 1990; accepted 18 June 1990