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Site-directed mutagenesis and substrate-induced inactivation of β-lactamase I

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The substrate-induced inactivation of β-lactamase I from *Bacillus cereus* 569/H has been studied. Both the wild-type enzyme and mutants have been used. The kinetics follow a branched pathway of the type recently analysed [Waley (1991) Biochem. J. **279**, 87–94]. The substrate cloxacillin (a penicillin) formed an acyl-enzyme (characterized by m.s.), and it was probably the instability of this intermediate that brought about inactivation. A disulphide bond was introduced into β-lactamase I (the wild-type enzyme lacks this bond) by site-directed mutagenesis: Ala-77 and Ala-123 were replaced by cysteine. Spontaneous oxidation yielded the disulphide. The activity of this newly cross-linked enzyme was a little diminished, but the stability towards inactivation by cloxacillin was not increased. A second mutant of β-lactamase I was studied: this mutant lacked the first 17 residues, i.e. the first α-helix. The mutant had reduced activity towards ordinary (non-inactivating) substrates and no hydrolysis of cloxacillin could be detected. These mutant enzymes were expressed in *Bacillus subtilis*, and were purified from the extracellular medium.

INTRODUCTION

β-Lactamases, enzymes that play a major part in the resistance of bacteria to β-lactam antibiotics, are mechanistically diverse (Waley, 1988), yet they all share the unusual property of being inactivated by certain substrates. This behaviour, noted for β-lactamase I from *Bacillus cereus* (Crompton *et al.*, 1962), was subsequently studied with several other β-lactamases (Citri *et al.*, 1976; Fisher *et al.*, 1978; Samuni & Citri, 1979; Kiener *et al.*, 1980; Frère, 1981; Persaud *et al.*, 1986). There is general agreement that an intermediate in the hydrolysis of certain substrates is particularly unstable. There is also general agreement that there are two intermediates in catalysis by serine β-lactamases, the non-covalent enzyme–substrate complex and the covalent acyl-enzyme (Waley, 1992). The inactivated enzyme from cloxacillin and β-lactamase I contains a penicilloyl moiety, as judged by the penamaldyl reaction (Kiener *et al.*, 1980), and clear-cut physical evidence for this structure is described below. Thus the inactive form may be described as an altered (and less stable) acyl-enzyme.

Since β-lactamase I lacks disulphide bonds, which may confer stability by cross-linking polypeptide chains, we have introduced a disulphide bond by site-directed mutagenesis to see whether this stabilizes the acyl-enzyme. Two cysteine residues were introduced, replacing alanine residues in the wild-type enzyme at positions 77 and 123 [the ABL numbering of Ambler *et al.* (1991) is used throughout]. The double mutant, A77C + A123C, in fact formed a disulphide bond (this mutant is referred to as the S–S mutant). The homologous RTEM1 β-lactamase from *Escherichia coli* contains a disulphide bond in this position, and several other class A β-lactamases contain cysteine (or half-cystine) residues in positions 77 and 123 or only in position 123 (Ambler *et al.*, 1991; Jelsch *et al.*, 1992).

The S–S mutant was designed to enhance stability; by contrast, for the other mutant enzyme described below, it was more a question of whether the protein would have adequate stability and any activity: this mutant enzyme (called the deletion mutant protein) lacks the first 17 residues of the polypeptide chain. Unfortunately, only small amounts of this mutant protein were obtained (yield about 0.25% of that of wild-type enzyme), but it has been possible to characterize its activity towards ordinary (non-inactivating) substrates; no hydrolysis of cloxacillin could be detected.

The favoured kinetic mechanism for the hydrolysis of cloxacillin by β-lactamase I is a branched pathway (Kiener *et al.*, 1980; Fink *et al.*, 1987):

$$\text{Substrate} + \text{enzyme} \leftrightarrow \text{ES complex} \rightarrow \text{acyl-enzyme} \rightarrow \text{product} + \text{enzyme}$$
$$\uparrow\downarrow$$
$$\text{dead enzyme}$$

Recent work on the kinetics of this branched-pathway mechanism (Waley, 1991) has shown how certain rate constants can be evaluated. This makes it possible, in principle, to describe quantitatively the effects of mutations on substrate-induced inactivation.

MATERIALS AND METHODS

Materials

Restriction endonucleases and (α-[^{35}S]thio)]dATP were obtained from Amersham International, Amersham, Bucks., U.K. DNA polymerase I (Klenow fragment) was from Anglian Biotechnology, Colchester, Essex, U.K. DNA ligase was from Northumbria Biologicals Ltd., Cramlington, Northumbria, U.K. Celite was from Fluka Chemicals, Glossop, Derbys., U.K.

Abbreviation used: TY broth, 16 g of Tryptone T/l, 10 g of yeast extract/l and 5 g of NaCl/l.

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The RTEM β-lactamase was a kind gift from Professor J. Richards.

Bacterial strains and plasmids

E. coli strain RZ1032 {HFr KL16 PO/45 [*lysA* (16–21)], *dut1*, *thi1*, *relA1*, *Zbd-279::Tn10*^{Tc}, *supE44*} was obtained from P. A. Handford, and XL1-Blue {*endA1*, *hsdR17* (r_k^-, m_k^+), *supE44*, *thi*$^-$, λ^-, *recA1*, *gyrA96*, *relA1*, *ΔlacZ* [F′, *proAB*, *lacI*^{q}, *lacZΔM15*, *Tn10*^{Tc}]} 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/l]. The *penPC* gene, encoding β-lactamase I, was cloned into M13mp8 as a *Sal*I–*Bam*HI fragment by R. M. Gibson, and pSG703, a pUC12 derivative, was obtained from J. Errington. The mutagenic oligonucleotides were synthesized by K. Gould.

Construction of the mutant forms of β-lactamase I

The mutants were constructed by the uracil-template protocol (Kunkel, 1985; Kunkel *et al.*, 1987), by the use of oligonucleotides between 20 and 24 bases in length. The introduction of the base changes in the double mutant gene and the removal of 51 nucleotides in the deletion mutant gene were confirmed by sequencing with the dideoxynucleotide-chain-termination method of Sanger *et al.* (1977). Mutants were obtained with efficiencies of 33% (S–S mutant) or 50% (deletion mutant). The mutant genes were cloned into, and expressed in, *B. subtilis* by the use of a bacteriophage vector (S. J. Thornewell, A. K. East & J. Errington, unpublished work).

Purification of mutant forms of β-lactamase I

The mutant enzymes were isolated from the culture medium of *B. subtilis* by the procedures used for wild-type β-lactamase I (Davies *et al.*, 1974). Purification was followed either by enzymic action on nitrocefin, or by Western blots (Burnette, 1981).

Estimations of kinetic parameters

Proton evolution during the hydrolysis of cloxacillin was measured in the pH-stat at pH 7 at 20 °C in 0.1 M- or 0.5 M-NaCl, or in a Varian Cary model 219 or model 3 spectrophotometer at 400 nm using *p*-nitrophenol (0.1 mM) as indicator, in 1 mM-Mops buffer, pH 7, containing 0.1 M-NaCl and 0.01 mM-EDTA. The extinction coefficient (1040 $M^{-1} \cdot cm^{-1}$) was measured by the addition of accurately known small amounts of HCl, and confirmed by the complete hydrolysis of known amounts of cloxacillin by β-lactamase II. The indicator method was convenient but could only be used to measure concentrations of protons up to about 2 mM; at higher concentrations the change in A_{400} was reduced, and the pH-stat method had to be used. The *p*-nitrophenol method was also used for measurements in the stopped-flow (HiTech SF-42, Hi-Tech Scientific, Salisbury, Wilts., U.K.) instrument. Spectrophotometric measurements on the hydrolysis of other substrates were carried out as described previously (Christensen *et al.*, 1990).

Steady-state kinetic parameters were estimated from measurements of initial rates (from the first 5% of the reaction) by fitting the Michaelis–Menten equation with the non-linear regression program of Duggleby (1984), or from progress curves. The half-time method (Wharton & Szawelski, 1982) was used for progress curves obtained with non-inactivating substrates (e.g. benzylpenicillin or nitrocefin).

Electrospray m.s.

Electrospray mass spectra were measured on a VG BIO Q triple-quadrupole atmospheric-pressure mass spectrometer equipped with an electrospray interface (VG Biotech, Altrincham, Cheshire, U.K.). Samples (10 μl) were injected into the electrospray source via a loop injector (Rheodyne 5717) as a solution, typically 20 pmol/μl, in water/methanol (1:1) containing 1% (v/v) acetic acid at a flow rate of 2 μl/min (Applied Biosystems model 140A dual-syringe pump). The reaction mixture contained 50 μl of β-lactamase I (1 mg/ml) and 5 μl of cloxacillin (0.44 mg/ml) in Milli-Q water at pH 7. Samples were taken after 3 min at 20 °C, mixed with an equal vol. of methanol containing 2% (v/v) acetic acid (resulting pH 3.5) and immediately analysed. The mass spectrometer was scanned over the mass range 600–1400 Da. The instrument was calibrated with myoglobin (20 pmol/μl, molecular mass 16951.5 Da).

RESULTS

Hydrolysis of non-inactivating substrates by the mutant β-lactamases

The S–S mutant enzyme had similar kinetic parameters to the wild-type enzyme when benzylpenicillin was the substrate (Table 1). The K_m for nitrocefin was increased, as was the K_m for cephalosporin C; the latter value was obtained by the use of cephalosporin C as an inhibitor and nitrocefin as substrate. This procedure is valid because the $k_{cat.}/K_m$ of cephalosporin C is only about 1% of the $k_{cat.}/K_m$ of nitrocefin (Waley, 1983). Since the deletion-mutant enzyme was not obtained pure, the values for $k_{cat.}$ and $k_{cat.}/K_m$ are only lower limits, but it is clear that this mutant has at least 1% of the activity of the wild-type enzyme.

The quotient ($k_{cat.}/K_m$ for cephalosporin C)/($k_{cat.}/k_m$ for benzylpenicillin) was 2.7×10^{-5} and 3.6×10^{-5} for the wild-type enzyme and the S–S mutant enzyme respectively. Both these values are appreciably lower than that for the RTEM1 β-lactamase, 8.6×10^{-4} (Healey *et al.*, 1989), which has a disulphide bond in the same position as the S–S mutant protein. Thus the introduction of a disulphide bond into the *B. cereus* β-lactamase I has not markedly changed its rather extreme preference for a penicillin rather than a cephalosporin. The same probably applies to the deletion-mutant enzyme.

Table 1. Kinetic parameters for hydrolysis of β-lactams by mutant enzymes

The reactions were carried out in 50 mM-sodium phosphate buffer, pH 7, containing 0.1 M-NaCl and 0.01 mM-EDTA at 20 °C. The parameters were obtained from measurements of initial rates by non-linear regression. The values for wild-type β-lactamase I are from Gibson *et al.* (1990).

	K_m (μM)			$k_{cat.}$ (s^{-1})			$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)		
Substrate	Wild-type enzyme	S–S-mutant enzyme	Deletion mutant enzyme	Wild-type enzyme	S–S mutant enzyme	Deletion mutant enzyme	Wild-type enzyme	S–S mutant enzyme	Deletion mutant enzyme
Benzylpenicillin	65	73±50	146±11	2200	1600±200	28±5	33800	22000±13000	190±24
Nitrocefin	55	370±40	380±70	34	33±2	0.55±0.06	620	90±5	1.5±0.08
Cephalosporin C	220	1400±800	1100±400	0.2	1.1±0.3	0.02±0.006	0.9	0.8±0.7	0.02±0.01

Table 2. Kinetic parameters for the hydrolysis of cloxacillin

The hydrolysis of 0.5–5 mM-cloxacillin by 6 nM-β-lactamase I was measured at pH 7 and 20 °C, in the presence of 0.1 mM-*p*-nitrophenol (for the spectrophotometric assay) and 1 mM-Mops, or in 0.5 M-NaCl in the pH-stat; 0.02 mg of lysozyme/ml was also present.

Enzyme	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Wild type	270±30	2.3±0.6	120±20
S–S mutant	100±20	7.0±2	14±1

Hydrolysis of cloxacillin by wild-type β-lactamase I

There are two possible outcomes in the interaction of cloxacillin and β-lactamase I: either complete hydrolysis of the cloxacillin, with little or no inactivation of the enzyme, or little hydrolysis of the cloxacillin with more or less complete inactivation of the enzyme. The pH, and temperature, greatly affect the outcome (Kiener *et al.*, 1980; Fink *et al.*, 1987), as does the ratio of [cloxacillin]/[enzyme]. It is convenient to describe experiments with low and high concentrations of cloxacillin separately.

Hydrolysis of low concentrations of cloxacillin

At cloxacillin concentrations of 0.5–5 mM there was no activation of 3 nM or 6 nM wild-type β-lactamase I. The steady-state kinetic parameters given in Table 2 show that the $k_{cat.}$ and $k_{cat.}/K_m$ of cloxacillin are about 12% and 0.4% of the corresponding values for benzylpenicillin; moreover cloxacillin is a much better substrate than cephalosporin C (Table 1). Nevertheless, there were features (see below) in the behaviour of even low concentrations of cloxacillin not displayed in the hydrolysis of non-inactivating substrates.

Hydrolysis of low concentrations of cloxacillin in the presence of glycerol

Unexpectedly, the hydrolysis of cloxacillin proceeded more rapidly in solutions containing high concentrations of glycerol. Previously, viscosogens had been shown to decrease the rate of hydrolysis, as measured by $k_{cat.}/K_m$, of good substrates of β-lactamase I (Hardy & Kirsch, 1984; Christensen *et al.*, 1990); poor substrates were unaffected. The dependence of $k_{cat.}/K_m$ on the viscosity of the medium enables one to determine k_{+1} and k_{-1}/k_{+2}. The values of $k_{cat.}/K_m$ for mutants of β-lactamase I were also decreased by viscosogens (Gibson *et al.*, 1990); this illustrated the usefulness of the procedure even when the values of $k_{cat.}/K_m$ were not high. With good substrates, combination of enzyme and substrate is, at least partially, rate determining, and this combination is slowed by an increase in the viscosity of the medium. The value of $k_{cat.}/K_m$ for cloxacillin is higher than most of the values for mutants of β-lactamase I given by Gibson *et al.* (1990) and so a decrease in the presence of viscosogens was expected; however, measurements of the hydrolysis of 0.5 mM-cloxacillin by stopped-flow spectrophotometry showed an increase of rate with respect to the concentration of glycerol (Fig. 1). The results, perhaps fortuitously, are fitted by a linear increase of the first-order rate constant with respect to the concentration of glycerol. The intercept, $(2.58 \pm 0.12) \times 10^{-4}$ $M^{-1} \cdot s^{-1}$ (when the rate constant is divided by the concentration of enzyme) is appreciably greater than the value of 1.2×10^{-4} $M^{-1} \cdot s^{-1}$ for $k_{cat.}/K_m$ given in Table 2. Since the concentration of cloxacillin is only about 20% of K_m, the first-order rate constant would not be expected to be very different from $k_{cat.}/K_m$. Part, at least, of the difference could be due to the fact that the stopped-flow results, especially those at higher concentrations of glycerol, were not well-fitted by a single exponential function. Since the effects of glycerol in the hydrolysis of non-inactivating substrates were as expected, the present increase in rate might be attributed to a step in the branched pathway described below. However, there are no other signs of this pathway being operative at such low concentrations of cloxacillin. It is not clear what the explanation of the effect of glycerol is, but there have been other reports of unexpected increases in $k_{cat.}/K_m$ brought about by glycerol (Grissom & Cleland, 1988).

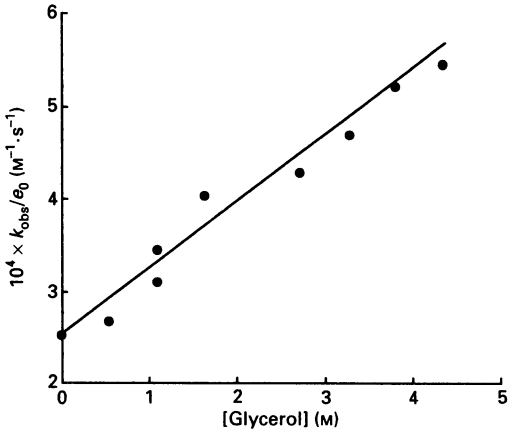


Fig. 1. Variation of rate constant with concentration of glycerol

The hydrolysis of 0.5 mM-cloxacillin by 1.39 μM-β-lactamase I in the presence of 100 μM-*p*-nitrophenol, 0.02 ml of lyxozyme/ml, and 0–40% (w/v) glycerol was measured in the stopped-flow spectrophotometer; six to eight progress curves were obtained at each concentration of glycerol, signal-averaged, and fitted to a single-exponential equation.

Structure of the intermediate

Previous work has been interpreted on the basis of a branched-pathway kinetic mechanism in which an acyl-enzyme is an intermediate; inactivation is caused by some alteration of this intermediate (Kiener *et al.*, 1980; Fink *et al.*, 1987). The formation of the acyl-enzyme has now been placed on a firmer footing by electrospray m.s. This method had been used to observe an acyl-enzyme intermediate in reactions catalysed by a β-lactamase (Aplin *et al.*, 1990). In this study β-lactamase I was treated with 100 mM-cloxacillin for 3 min and the resulting spectrum compared with that of the untreated enzyme. The treated enzyme showed shifted peaks, and the observed mass shift was 434 Da, compared with the mass shift calculated for acyl-enzyme of 435 Da (R. T. Aplin & S. J. Thornewell, unpublished work).

Stability of the intermediate

The decreased stability of β-lactamase I when acting on cloxacillin is shown by the differential effects of guanidinium chloride (Fig. 2). The rate declined with the concentration of denaturant when cloxacillin was substrate, but not when benzylpenicillin was substrate. The curve drawn in Fig. 2 is theoretical, based on the assumption that denaturant bound preferentially to n identical non-interacting sites on the denatured form with association constant k. This leads to

$$F = 1 - \frac{K_0(1+kC)^n}{1+K_0(1+kC)^n} \quad (1)$$

where F is the fraction of enzymic activity ($F = 1$ when the concentration C of denaturant = 0), and K_0 is the equilibrium

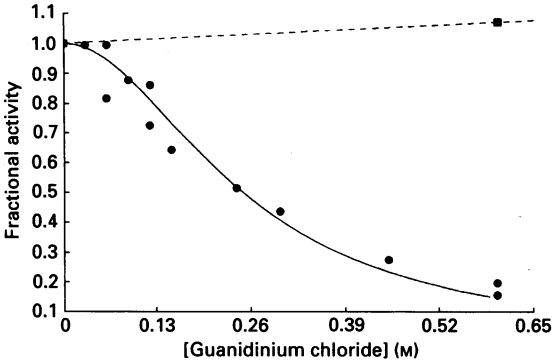


Fig. 2. Dependence of rates of hydrolysis of benzylpenicillin and cloxacillin on concentration of guanidinium chloride

β-Lactamase [20 μl of either 0.44 mg/ml for cloxacillin (●) or 0.0044 mg/ml for benzylpenicillin (■)] was added to 1 ml of substrate in 0.5 M-NaCl, 1 mM-EDTA, containing various concentrations of guanidinium chloride, and the rate of hydrolysis measured in the pH-stat at pH 7. The rate is given relative to the rate in the absence of guanidinium chloride.

constant for denaturation when $C = 0$ (Tanford, 1970). Attempts to fit the results in Fig. 2 to eqn. (1) [with the program of Duggleby (1984)] showed that K_0 and k could not be determined separately, but only their product; and that n was approx. 1.7. On the model suggested, n should be integral and the curve was drawn in Fig. 2 with $n = 2$ and $K_0 k = 16 \pm 2\ \text{M}^{-1}$; the fit is satisfactory. Larger values of n are commonly reported for more extensive unfolding of proteins at higher concentration of guanidinium chloride; indeed, results for β-lactamase I in the absence of substrate, followed by changes in fluorescence, were fitted with $n = 13$ (J. Monks & S. G. Waley, unpublished work). The lower value of n for the experiments in the presence of cloxacillin seems consistent with a local change in tertiary structure that leads to loss of activity.

Estimation of rate constants from progress curves for the hydrolysis of high concentrations of cloxacillin

Progress curves for the hydrolysis of 1–50 mM-cloxacillin by 20 nM-β-lactamase I clearly show the transition from virtually complete reaction at relatively low concentrations of substrate to inactivation at high concentrations of substrate (Fig. 3). The interpretation of the kinetics according to Scheme 1 requires that

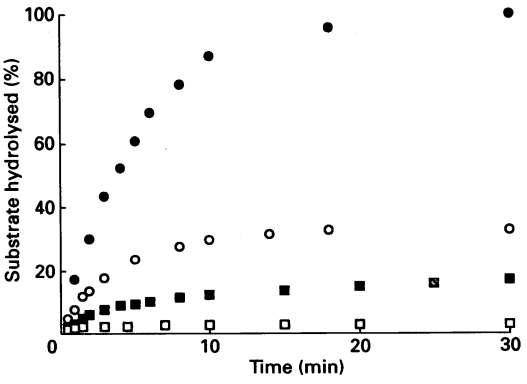
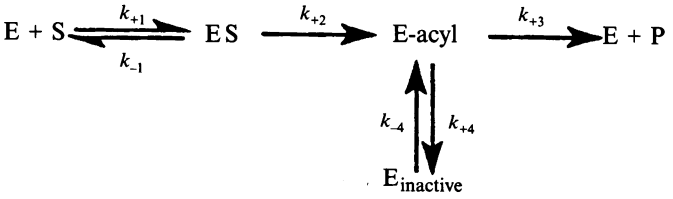


Fig. 3. Dependence of extent of hydrolysis of cloxacillin on the concentration of substrate

Progress curves were measured at pH 7, either spectrophotometrically or in the pH-stat in the presence of 0.1 M-NaCl, with various concentrations of cloxacillin and 20 nM-β-lactamase I; (●), 1 mM-; (○) 5 mM-; (■) 20 mM-; (□) 50 mM-cloxacillin.



Scheme 1.

the change in rate may be ascribed solely to the accumulation of the inactive form of the acyl-enzyme (Waley, 1991). The decreasing concentration of substrate, however, will also decrease the rate. Thus the decline in rate owing to substrate depletion must be corrected for. This can be done by carrying out experiments at decreasing concentration ratios of enzyme to substrate. The parameters found by fitting the results to the 'burst equation' [eqn. (2)] are v_i, v_s and k, which are the initial rate, the steady-state rate, and the rate constant for the change in rate respectively.

$$p = v_s t - (v_s - v_i)(1 - e^{-kt})/k \qquad (2)$$

From these parameters, the rate constant k_{-4} can be found as $k \cdot v_s/v_i$, and the quotient $k_{+3} \cdot k_{-4}/k_{+4}$ from eqn. (3) (Waley, 1991).

$$e_0\left(\frac{1}{v_s} - \frac{1}{v_i}\right) = \frac{k_{+4}(1 - k_{-4}K/k_{+2}s)}{k_{+3}k_{-4}} \approx \frac{k_{+4}}{k_{+3}k_{-4}} \qquad (3)$$

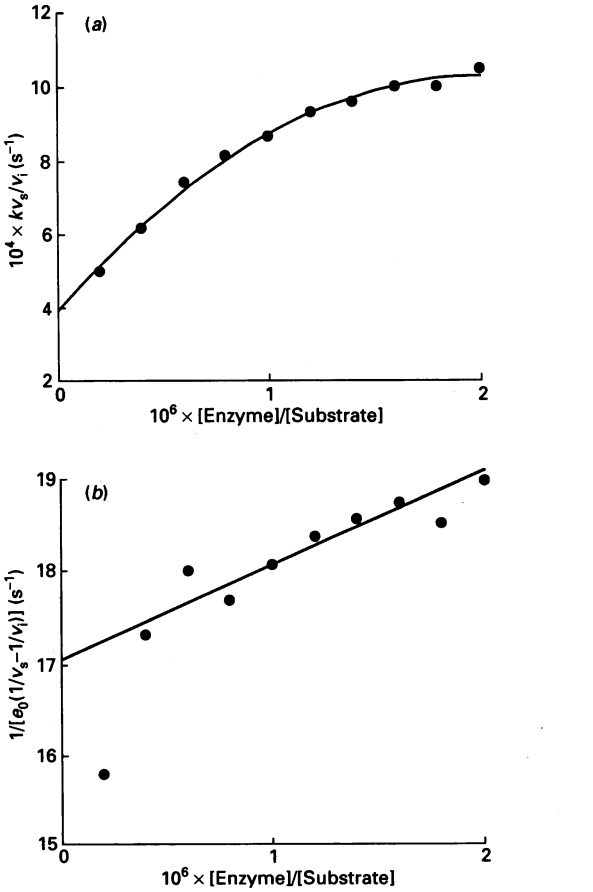


Fig. 4. Dependence of (*a*) k and (*b*) $1/[e_0(1/v_s - 1/v_i)]$ on e_0/s_0

The hydrolysis of 50 mM-cloxacillin by β-lactamase I was measured at pH 7 at 20 °C, and the parameters obtained from fitting the results to eqn. (2) in the text.

Here s is the concentration of substrate and e_0 is the initial concentration of enzyme. The approximation in this equation is likely to hold, since the lower limit for k_{+2} obtained from $k_{cat.}$, and the value for k_{-4} obtained below, show that $k_{-4}/k_{+2} \leqslant 1.4 \times 10^{-6}$. Since k_{-4} has been determined, the k_{+3}/k_{+4} ratio can be found. These are only apparent values, owing to the decrease in substrate concentration, but the corrected values can be found by extrapolation (Fig. 4), as described above. The values thus found were $k_{-4} = (3.9 \pm 0.2) \times 10^{-4}\ s^{-1}$ and $k_{+3}/k_{+4} = 44000 \pm 2500$. A lower limit for k_{+3} is about $300\ s^{-1}$ (from the value of $k_{cat.}$ given in Table 2), and so a lower limit for k_{+4} can be obtained of about $0.007\ s^{-1}$. Moreover, there is a lower limit for k_{+4}/k_{-4} of about 20, so most of the acyl-enzyme partitions into the inactive form.

The above values may be compared with the results of Fink *et al.* (1987). Their value for $k_{inact.}$ of $6.5 \times 10^{-3}\ s^{-1}$ agrees with the lower limit for k_{+4} given above, and it has been shown that the expected value at high concentrations of substrate is approx. $k_{+2}k_{+4}/(k_{+2}+k_{+3})$ (Waley, 1991), which will be similar to k_{+4} unless k_{+3} is much greater than k_{+2}. Their values for K_d and $k_{react.}$ are somewhat (6–10-fold) greater than our values for K_m and k_{-4} (to which it may be shown that they approximately correspond) but the differences are not excessive, considering the approximations in the theory (Waley, 1991) and the imprecision of the experimental values.

Inactivation of wild-type and mutant β-lactamase I

Theoretical treatments for the whole course of a branched-pathway reaction, when substrate depletion has to be taken into account, have yet to be developed. Thus it is convenient to use the Selwyn (1965) test to probe for inactivation. In this test, progress curves are plotted with an abscissa of enzyme concentration × time replacing the usual time axis: the measurements for the experiments with different enzyme concentrations coincide when there is no inactivation. When 10 mM-cloxacillin and about 200–400 nM of enzyme were used there was little or no inactivation of wild-type β-lactamase I, and no inactivation of the disulphide mutant of β-lactamase I nor of the RTEM1 β-lactamase (Fig. 5A). With only 10–20 nM of enzyme inactivation is apparent; the curves are widely separated, and the extent of reaction is only 5%, or less (Fig. 5B). The disulphide mutant is somewhat more prone to inactivation than wild-type β-lactamase I. The RTEM1 β-lactamase is inactivated; it had not been clear from previous work that this enzyme would be inactivated under the present conditions. Thus it is, in retrospect, not surprising that the introduction of the disulphide bond linking residues 77 and 123 into β-lactamase I has not rendered the enzyme resistant to inactivation by cloxacillin when the RTEM1 β-lactamase, containing a similarly situated disulphide bond, is inactivated.

DISCUSSION

Properties of the mutants of β-lactamase I

The mutant containing the disulphide bond between residues 77 and 123 retains activity, but lacks enhanced stability towards cloxacillin. The mutant enzyme lacking the first α-helix has reduced activity, and fails to hydrolyse cloxacillin. To what extent can these findings be rationalized on the basis of the

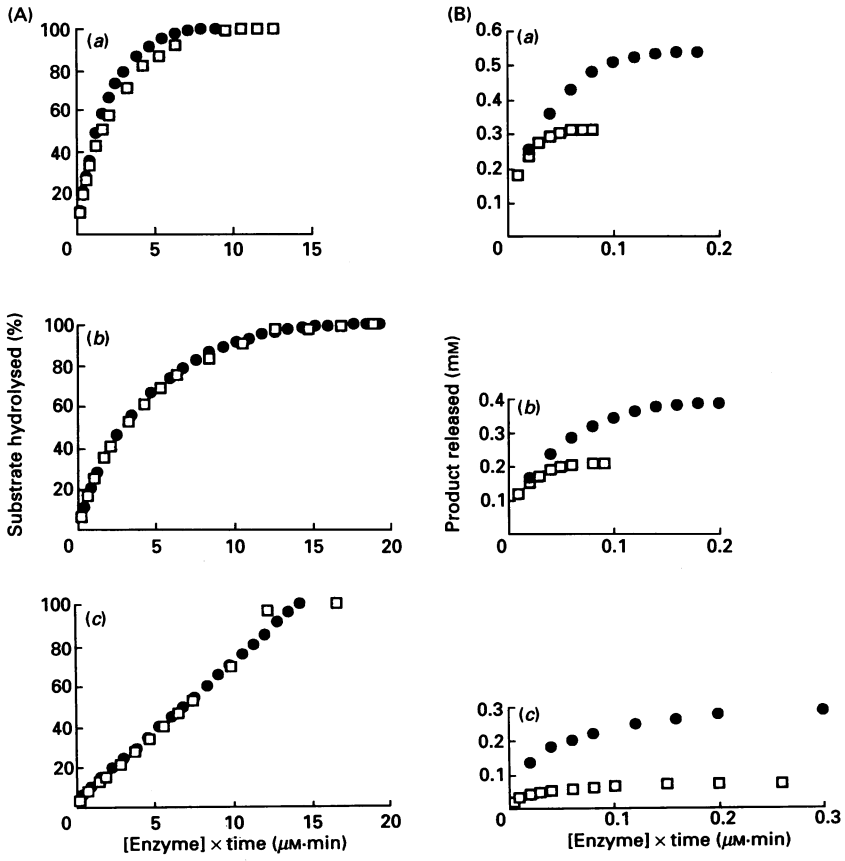


Fig. 5. Progress curves as Selwyn plots for the hydrolysis of cloxacillin

(A) 10 mM-Cloxacillin and 210 nM- (□) or 420 nM- (●) β-lactamase. (*a*) Wild-type β-lactamase I, (*b*) S–S mutant of β-lactamase I, and (*c*) RTEM1 β-lactamase, 189 nM (□) or 378 nM (●). (B) As (A), but with 10 nM- (□) or 20 nM- (●) β-lactamase.

structure? The deletion mutant lacks the *N*-terminal helix which occupies a rather peripheral position in the structure of β-lactamase I (Baguley, 1990). This region contains only one conserved residue, Glu-37, and no active-site groups (Ambler *et al.*, 1991; Waley, 1992). Moreover, variants of other class A β-lactamases that lack this part of the structure are active, albeit less stable (Matagne *et al.*, 1991). Nevertheless, Glu-37 in the homologous β-lactamase from *Bacillus licheniformis* is probably important in positioning the helix containing the active-site serine (Knox & Moews, 1991). So we may surmise that the deletion mutant owes its apparently diminished activity to the absence of such stabilizing interactions. Examination of the crystal structure of β-lactamase I (Baguley, 1990) showed that a disulphide bond in this position would have favourable bond angles and distances and would belong to the right-handed hook class (Richardson, 1981).

Structure of the unstable intermediate

The secondary structure of the protein moeity in the inactive form of the acyl-enzyme cannot be distinguished from that of the active enzyme (Fink *et al.*, 1987), so our working hypothesis is that it is the tertiary structure that is altered. Moreover, the conformational motility, as measured by hydrogen exchange of peptide NH, of β-lactamase I is altered by cloxacillin (Kiener & Waley, 1977); this suggests that the elements of secondary structure are less close-packed in the altered acyl-enzyme. The postulated structure may resemble the structures ascribed to some intermediates in protein folding (Christensen & Pain, 1991). It is not unreasonable that a rather subtle change in structure should lead to pronounced changes in stability. The substrates that bring about inactivation are often aryl amides, and it may be that the energetic barrier to rotation about the Ar–CO bond prevents the acyl-enzyme from adopting a stable conformation (Blanpain *et al.*, 1980). The rigidity of the substrate-derived moeity combined with the flexibility of the protein brings about the inactivation. The presence of antibodies can stabilize the β-lactamase (Pollock, 1964; Zyk & Citri, 1968*a*,*b*): thus, decreasing the flexibility of the protein prevents the inactivation. Nevertheless, the disulphide bond linking residues 77 and 123 fails to stabilize the β-lactamase to cloxacillin.

On the other hand, when the sulphone of cloxacillin (rather than cloxacillin itself) reacts with β-lactamase I the inactivated enzyme shows altered far-u.v. c.d. (Fink *et al.*, 1989); other sulphones also give rise to altered conformations in β-lactamase I, detected by differential scanning calorimetry (Dmitrienko *et al.*, 1985). Here there is a more profound alteration in protein structure.

Substrate-induced inactivation of other β-lactamases

Earlier work by Citri and his colleagues had shown that cloxacillin, and a number of other penicillins, brought about inactivation of the extracellular β-lactamases secreted by Gram-positive bacteria (Zyk & Citri, 1967). The effects seem less marked with the β-lactamase from *B. licheniformis*, the sequence of which is about 50% identical to that of β-lactamase I (Ambler *et al.*, 1991); this enzyme hydrolyses cloxacillin with some inactivation, and the $k_{cat.}$ and K_m are considerably lower (8.5 s^{-1} and 11 μM respectively) (Matagne *et al.*, 1990). The PC1 β-lactamase from *Staphylococcus aureus*, which is less closely related to β-lactamase I, can exist in a distended conformation (Carrey & Pain, 1978), and the deactivation of this enzyme by another penicillin (quinacillin) has some features in common with the results given above (Persaud *et al.*, 1986). Thus the sensitivity of the β-lactamase towards urea is enhanced in the presence of quinacillin and the subtly altered inactive species that accumulates contains a moeity derived from the quinacillin. The structures of these enzymes are known (Samraoui *et al.*, 1986; Baguley, 1990; Herzberg, 1991; Knox & Moews, 1991), but structures for the inactivated enzymes have not been reported. Understanding the substrate-induced inactivation of β-lactamase I awaits determination of the structures of the active and inactive form of the acyl-enzyme.

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