Evidence from a mutant β -lactamase for the mechanism of β -lactamase-catalysed depsipeptide aminolysis

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The Ser-70 $\rightarrow$ Gly mutant of the TEM-1 β -lactamase, where the active-site serine hydroxy group has been lost, does not catalyse the hydrolysis of either benzylpenicillin or *N*-(phenylacetyl)glycyl depsipeptides. This is as would be expected for a double-displacement mechanism where the Ser-70 becomes acylated at an intermediate stage. Further, however, the mutant enzyme, unlike the wild-type, does not catalyse aminolysis of depsipeptides by D-phenylalanine. If the active site is not structurally disrupted by the mutation, this result shows that Ser-70 is necessary for the aminolysis reaction and implies that this reaction, like the hydrolysis, proceeds by way of an acyl-(serine)-enzyme intermediate. Although physical evidence suggests that the mutant enzyme does not have a structure in solution identical with that of the wild-type, the mutant does still bind β -lactam substrates. The latter result suggests sufficient conservation of the active-site structure for the major conclusion above to hold.

INTRODUCTION

The natural substrates of β -lactamases are almost certainly β -lactams. Research in this laboratory has shown, however, that β -lactamases also catalyse the hydrolysis of certain acyclic depsipeptides of general structure 1 [1,2]:

The serine β -lactamases of Classes A and C [3] appear to catalyse these hydrolyses by a double-displacement mechanism involving an acyl-(serine)-enzyme intermediate 2:

We have also shown [2] that the Class A and C enzymes catalyse the aminolysis of depsipeptides, and in particular 3, by specific D-amino acids such as D-Phe (eqn. 1):

This reaction is directly analogous to the transpeptidase reaction catalysed by the bacterial DD-peptidases in cell-wall biosynthesis. Both structural and functional evidence now strongly suggests

that β -lactamases are evolutionary descendents of the DD-peptidases (or, at least, that they share a common ancestor) [2,4–6].

The steady-state kinetics of the reaction of eqn. (1), catalysed by the Class C β -lactamases of *Enterobacter cloacae* P99, are complex and distinctive [7], and also typical of those found for the transpeptidase reaction catalysed by DD-peptidases [8,9]. Analysis of the kinetics of the former reaction suggested that the aminolysis pathway did *not* share a common acyl-enzyme with the hydrolysis reaction, but that the two paths diverged at the stage of a non-covalent complex before amino acid binding [10]. That being so, the question arose as to whether an acyl-enzyme occurs at all as part of the aminolysis reaction, or whether, alternatively, the aminolysis is a simple one-step bimolecular displacement. Although acyl-enzymes have been trapped on reaction of DD-peptidases with acyclic substrates [11–14], there is no direct evidence for the intermediacy of one in aminolysis or transpeptidation. The present paper describes the results of one approach to an answer to this question.

Schimmel and co-workers [15] employed the method of site-specific mutagenesis to change the active-site serine residue (Ser-70, employing the numbering system of Ambler [3]) of the TEM-1 β -lactamase into a glycine residue. The resultant enzyme, not surprisingly, was inactive as a β -lactamase. The question addressed here is whether this mutant, like the wild-type enzyme, is able to catalyse the aminolysis of compounds of the 2 type by D-phenylalanine. Although this might appear to be a decisive

experiment, in the event of a negative result (such as in fact was found) there is the danger (of which there is ample warning in the literature [16–18]) that the mutation might so distort the structure

Abbreviations used: PADAC, pyridine-2-azo-4'-*N,N'*-dimethylaniline)cephalosporin; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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of the active site as to inactive the enzyme towards aminolysis for reasons other than loss of the active-site serine. This point, and the related question as to whether the mutant enzyme binds β -lactams, are also addressed in the present paper.

EXPERIMENTAL

Materials

The DH1 strain of *Escherichia coli* containing the plasmid pUnc, which included the gene coding for the mutant TEM-1 β -lactamase, was generously provided by Dr. Paul Schimmel of the Massachusetts Institute of Technology, Boston, MA, U.S.A. The wild-type TEM-2 β -lactamase was purchased from the PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K., and used as received. Dansylcephalothin [19], dansylpenicillin [20], 6- β -bromopenicillanic acid [21], *m*-[*N*-(phenylacetyl)glycyl]oxybenzoic acid [2] and *N*-(phenylacetyl)glycyl-DL-thiolactic acid (5), the last-named of which has the structure:

were available from previous work in this laboratory [22]. Benzylpenicillin and Nile Red were purchased from Sigma Chemical Co. PADAC [pyridine-2-azo-4'-(*N,N'*-dimethylaniline)cephalosporin] was a gift from Hoechst-Roussel Pharmaceuticals, Somerville, NJ, U.S.A.

Methods

Cells (pUnc/DHI) were grown to late exponential phase at 37 °C in Luria-Bertani media containing tetracycline (10 mg/litre in a New Brunswick Scientific Co. (Edison, NJ, U.S.A.) Microform fermentor (stirred at 100 rev./min with 7 litres/min aeration). The cells (~35 g of paste from 10 litres of medium) were then harvested by centrifugation at 4 °C. The harvested cell paste was suspended in a pH 8.0 buffer containing 50 mM-Tris, 20 mM-EDTA and 20% (w/v) sucrose. Cells were separated by centrifugation at 6500 rev./min (r_{av} 14.6 cm) for 10 min and the wash repeated twice more. The combined wash solution (~1 litre) was dialysed against pH 7.8 buffer containing 50 mM-Tris, 20 mM-EDTA and 0.01% NaN_3 , and then concentrated to ~40 ml by ultrafiltration (Amicon YM-10). The concentrate was dialysed against 25 mM-imidazole buffer, pH 7.4.

Further purification involved chromatofocusing followed by Sephadex G-75 chromatography, as previously described [23,24]. The yield of purified protein, which was homogeneous on SDS/PAGE, was 4 mg. The quantity of protein was estimated spectrophotometrically, with the assumption that the absorption coefficient of the mutant protein was identical with that of the wild-type enzyme ($2.90 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [25]).

C.d. spectra of both wild-type and mutant enzymes were obtained in 20 mM-Mops buffer, pH 7.5, with an Aviv 60 ds spectrophotometer (Instrument Center, Chemistry Department, Yale University, New Haven, CT, U.S.A.); protein concentrations of 33 μM and a cell of path length 0.1 cm were employed for the far-u.v. spectra and 36.4 μM and 1.0 cm respectively for the near-u.v. Fluorescence measurements were made in a 3 mm-path-length cuvette at 25 °C with a Perkin-Elmer MPF-44A spectrofluorimeter. The fluorescence spectra of wild-type and mutant enzymes were obtained on solutions containing 0.64 μM -

protein in 0.1 M-phosphate buffer, pH 7.5. Rates of thermal denaturation were also measured fluorimetrically in this buffer, with protein concentrations of 0.65 μM . All other kinetic and equilibrium binding measurements were obtained in 20 mM-Mops buffer, pH 7.5. A stock solution of Nile Red was prepared in dimethyl sulphoxide, and aliquots of this (1 μl) added to buffered solutions of the proteins (50 μl). The hydrolysis of dansylcephalothin and dansylpenicillin were monitored fluorimetrically at 540 nm, after irradiation at 335 nm. The hydrolysis of PADAC was monitored spectrophotometrically at 570 nm.

Equilibrium measurements of dansylcephalothin binding to the mutant enzyme were obtained by the method of Hirose & Kano [26], where partitioning of the components is achieved by gel exclusion. Thus a solution of dansylcephalothin (5 μl) was added to a mixture of enzyme solution (55 μl) and 50–100 mesh Bio-Gel P-4 (~10 mg, dried *in vacuo* after an ethanol wash). Final concentrations of protein and dansylcephalothin were ~9 μM and 1.4 μM respectively, after vortex mixing. After 15 min incubation at 25 °C, during which period essentially no hydrolysis of the β -lactam occurred, the gel-excluded solution was withdrawn by syringe. Analysis for dansylcephalothin was then performed by fluorescence measurements.

RESULTS

The mutant Ser-70 $\rightarrow$ Gly β -lactamase was purified from the DHI strain of *E. coli* containing the pUnc plasmid [15] as described in the Experimental section. This material behaved during purification by chromatofocusing ($\text{pI} = 5.65$) and gel-filtration chromatography in the same way as the wild-type TEM β -lactamase. SDS/PAGE showed it to be homogeneous and indistinguishable from the wild-type enzyme. Further, the far-u.v. c.d. spectrum (Fig. 1) was essentially identical with that of the wild-type.

The activity of the mutant enzyme against benzylpenicillin ($k_{cat} = 0.0165 \text{ s}^{-1}$) appeared to be 0.0024% of the wild-type value; Toth *et al.* [15] reported 0.002%. This level of activity could be reduced to 0.0005% of the wild-type activity by treatment with 6 β -bromopenicillanate. Since this inactivation was not accompanied by any measurable increase in absorption between 320 and 330 nm (the position of the characteristic chromophore generated on inhibition of the TEM β -lactamase by 6 β -bromopenicillanate [24]), it is probable that most of the residual activity must have been associated with a small amount of a contaminating Ser-70 β -lactamase, possibly the wild-type enzyme. The remaining activity may be that of the Ser-70 $\rightarrow$ Gly mutant active site, although some non-specific activity is also possible.

Catalysis of hydrolysis of depsipeptide 3 (10 mM; wild-type $K_m = 1.8 \text{ mM}$) was $\leq 0.3\%$ of that of the wild-type. The much higher lower limit given for the depsipeptide hydrolysis than for benzylpenicillin hydrolysis is attributable to the higher background hydrolysis rate of the depsipeptide and to the lower specific activity of the enzyme towards this substrate [2]. Since the rate in the presence of the mutant was not distinctly different from the background hydrolysis rate, the catalytic rate due to the mutant, here and in the instances cited below, was assumed to be $\leq 10\%$ of the background rate. Thus, similarly, catalysis of hydrolysis of the thioldepsipeptide 5 (0.9 mM; wild-type $K_m = 17 \text{ mM}$) was $\leq 2.5\%$ as effective as that of the wild-type enzyme.

The mutant enzyme was found to be $\leq 0.2\%$ as effective as the wild-type enzyme in catalysing aminolysis of 3 (10 mM) by D-phenylalanine (75 mM). Under these conditions, some 15% of 3 is aminolysed by the wild-type enzyme. Similarly, the thioldepsipeptide 5 was not aminolysed in the presence of the mutant

Fig. 1. Far-u.v. c.d. spectrum of the mutant Ser-70 $\rightarrow$ Gly β -lactamase in 20 mM-Mops, pH 7.5 (a) and the difference spectrum between the wild-type and mutant enzymes (b)

Molar ellipticities ($[\theta]$; per mean residue; the mean residue weight was taken to be 110) are in units of degrees $\cdot$ cm² $\cdot$ dmol⁻¹.

Fig. 2. Near-u.v. c.d. spectrum of the mutant Ser-70 $\rightarrow$ Gly β -lactamase in 20 mM-Mops buffer, pH 7.5 (a) and the difference spectrum between the wild-type and mutant enzymes (b)

Molar ellipticity units are as for Fig. 1.

($\leq 2.5\%$ wild-type activity). Thus the mutant enzyme showed no catalytic activity toward the depsipeptides at the limit of detection.

The binding of β -lactams to the mutant enzyme was, however, demonstrable. The fluorescence emission spectrum of the mutant (not shown) was very similar to, if not identical with, that of the wild-type enzyme. Addition of benzylpenicillin ($80\ \mu\text{M}$; K_m of the wild-type enzyme is $20\ \mu\text{M}$) to the mutant enzyme produced a small, but reproducible, increase ($\sim 10\%$) in fluorescence of the mutant, which suggested binding of benzylpenicillin to the mutant

Fig. 3. Change in the initial rate of hydrolysis of dansylcephalothin (1.9 mM) catalysed by wild-type TEM-2 β -lactamase (3.1 nM) on addition of the mutant enzyme

The points are experimental (each the average of three determinations) and the line is calculated as described in the text (eqn. 2).

enzyme. The mutant ($20\ \mu\text{M}$), however, had little or no effect on the fluorescence emission spectrum of dansylpenicillin ($120\ \text{mM}$; K_m of wild-type enzyme $77 \pm 12\ \mu\text{M}$) or dansylcephalothin ($170\ \mu\text{M}$; K_m of wild-type enzyme $38 \pm 7\ \mu\text{M}$). These results gave no indication of binding, but it should be recalled that previous studies of these substrates with the Class A *Staphylococcus aureus* PC1 β -lactamase [19,20] showed small (or no) changes in fluorescence on non-covalent binding; large changes in emission intensity were only observed on acyl-enzyme formation, which is precluded, of course, in the mutant TEM-1 β -lactamase.

Two methods were employed to demonstrate the binding of dansylcephalothin to the mutant enzyme. A competitive kinetic experiment showed that the presence of the mutant enzyme decreased the initial rate of hydrolysis of dansylcephalothin by the native TEM-2 β -lactamase. This result was interpreted in terms of Scheme 1 and the derived equation for the initial steady-state rate (eqn. 2):

$$(V_0/V) = 1 + \{ -(S_0 + K_p - P_0) + \sqrt{[(S_0 + K_p + P_0)^2 + 4P_0K_p]} \} / 2K_p \quad (2)$$

In Scheme 1, E represents the active native enzyme, P the mutant, and PS the inert non-covalent complex of P and the substrate S of dissociation constant K_p . In eqn. (2), V_0 represents the initial rate of hydrolysis of S in the absence of P, and S_0 the initial concentrations of S and P respectively. Fig. 2 shows the effect of increasing concentrations of mutant enzyme on the initial rates of hydrolysis of dansylcephalothin. This experiment was done at low substrate concentrations ($k_{\text{cat.}}/K_m$ conditions), but with a concentration of substrate much greater than that of the active β -lactamase and with substrate and mutant enzyme at comparable concentrations, i.e. such that binding by the mutant would deplete free substrate and lead to a decrease in the rate of hydrolysis. The decrease in V with P_0 was fitted to eqn. (2) by a non-linear least-squares procedure [27], yielding the line shown

in the diagram and a value of K_p of $1.6 \pm 0.4 \mu\text{M}$. Similarly, a K_p value of $3.9 \pm 1.1 \mu\text{M}$ was obtained for dansylpenicillin. A similar experiment with PADAC (concentrations of wild-type enzyme, mutant enzyme and PADAC were 3.1 nM, 18.6 μM and 3.05 μM respectively) indicated that the mutant enzyme had no effect on the initial rate of PADAC hydrolysis. This indicates a K_p value for PADAC of $> 25 \mu\text{M}$; the K_m of PADAC as a substrate of the wild-type enzyme was $83 \pm 14 \mu\text{M}$.

Independent measurements of the binding of dansyl-cephalothin to the mutant β -lactamase were made by the gel-exclusion method of Hirose & Kano [26]. Two separate experiments yielded K_p values of $1.5 \pm 1.1 \mu\text{M}$ and $3.3 \pm 1.8 \mu\text{M}$. These values are in good agreement with that obtained from the kinetic competition method.

Several other experiments bearing on the structure of the mutant enzyme were performed. A PAGE experiment, under non-denaturing conditions (Tris/glycine buffer, pH 8.3), showed mutant enzyme to run slightly faster than the wild-type (R_F values of 0.46 and 0.42 respectively were obtained). This result suggests a more compact protein structure. Perhaps the previously hypothesized [28] 'closed' conformation is favoured in the mutant. Rates of denaturation of the wild-type and mutant enzymes at 60 °C were measured by monitoring changes in protein fluorescence. This procedure generated what appeared to

very similar to that of the TEM enzyme, but which lacks tryptophan residues [3], suggests that much of the measured ellipticity of the latter enzyme derives from tryptophan. Fig. 3 also shows the difference near-u.v. c.d. spectrum between wild-type and mutant β -lactamases. This indicates that, in the mutant, at least one aromatic amino acid residue, probably tryptophan in view of the position of the peak observed in the difference spectrum, had moved with respect to its surroundings. The extent of this movement cannot be inferred, but it could represent only a simple side-chain rotation of one of the tryptophan residues.

DISCUSSION

The unadorned result of these experiments was that the Ser-70 $\rightarrow$ Gly mutant TEM-1 β -lactamase did not catalyse either the hydrolysis or aminolysis of the depsipeptides **3** and **5**, just as it did not catalyse the hydrolysis of β -lactams. At face value this suggests that the serine hydroxy group is just as necessary for the aminolysis reaction as it is for hydrolysis, which, in turn, implies that an acyl-enzyme may occur as an intermediate in aminolysis. A more complete reaction scheme for hydrolysis and aminolysis together, expanding on that derived previously [10], would then

Scheme 2

be first-order curves. The mutant enzyme was more labile than the wild-type by a rate-constant factor of 3.7-fold.

The effects of the wild-type enzyme and the mutant on the fluorescence spectrum of Nile Red were determined. Nile Red has been used as polarity-sensitive probe of hydrophobic protein surfaces [29]. Its emission in water (665 nm), on excitation at 550 nm, shifts to lower wavelength and becomes more intense in less-polar solvents and when bound to the hydrophobic surfaces of proteins. When the β -lactamases (final concn. 6.4 μM) were added to Nile Red (43 nM) in 20 mM-Mops buffer at pH 7.5, an emission peak at 610 nm appeared (results not shown), similar to that observed by Sackett & Wolff [29] for β -lactoglobulin. The intensity of this peak was significantly greater (close to 2-fold) in the presence of the mutant β -lactamase. This suggests stronger binding of Nile Red to the mutant than to the wild-type enzyme, or greater access of Nile Red to hydrophobic surfaces. The latter could signify either a different structure or a more mobile structure. The emission at 610 nm generated by the mutant enzyme was not affected by addition of benzylpenicillin (70 μM).

Finally, as an independent indication of the state of the aromatic residues, the near-u.v. c.d. spectra of the wild-type and mutant enzymes were obtained (Fig. 3). The near-u.v. c.d. spectrum is a sensitive probe of the tertiary structure adjacent to aromatic chromophores [30,31]. Comparison of the present spectrum with that of the *Staphylococcus aureus* PCI β -lactamase [32], another Class A β -lactamase with an amino acid sequence

be that of Scheme 2, where D, A, P, H and Q represent depsipeptide, D-phenylalanine, phenacetate, *m*-hydroxybenzoate and the amide product **4** respectively. The E-D₁ species represent acyl-enzymes. Interconversion of E-D₁ and E-D₁D₂, and of E-D₁ and E-D₁A, may also occur.

The above conclusion would hold, however, only if the active-site structure of the mutant enzyme is essentially the same as that of the native enzyme, except for the absence of the serine hydroxymethyl group. For proteins in general there is mixed evidence on this point. The serine-to-glycine conversion would produce a 'deletion' mutant [33], where a cavity is generated in the protein. This might be expected to give rise to only local changes to the structure [34] (a "nondisruptive deletion" [33]), although there are certainly cases known where more widespread structural changes are apparently triggered by single-site mutations [35–39]. The creation of a cavity, in general, would be destabilizing to the native structure [40]. It might also give rise to a more mobile (and thus perhaps more labile) protein [41,42], unless perhaps the cavity were filled with a water molecule [43]. The change to glycine, specifically, would also likely lead to a less stable protein through destabilization of the α_2 -helix or, perhaps equivalently, through stabilization of the denatured form through its greater configurational entropy [44]. Thus the mutant used in the present work might be expected to be less stable and more labile than the original, but not necessarily to have a significantly different structure.

The structural evidence presented here does suggest that the Ser-70 $\rightarrow$ Gly mutant has a somewhat different structure in solution than the wild-type enzyme. The changes in electrophoretic behaviour under non-denaturing conditions, in the fluorescence of Nile Red and in the near-u.v. c.d. spectrum indicate this. The greater thermal lability may also support this conclusion, although, as noted above, this result can be explained alternatively in terms of the stability of the unfolded form. On the other hand, the far-u.v. c.d. evidence suggests that there is little change in the extent and gross arrangement of secondary structure, i.e. that the changes may be largely in the position and mobility of amino acid side chains. The protein fluorescence emission spectrum suggests, by contrast perhaps with the Nile Red results and the near-u.v. c.d. spectrum, little change in the environment of tryptophan residues; the solvent exposure of the tryptophan residues may therefore not have changed significantly, although the orientation of one or more of them may have.

Perhaps the most interesting property of the mutant protein is its ability to bind β -lactams. The evidence presented indicates that both dansylpenicillin and dansylcephalosporin are quite tightly bound, probably more tightly in fact than by the wild-type enzyme (from a comparison of the binding constants of the mutant with the K_m values for the wild-type enzyme). This tight binding suggests that the β -lactamase active site is still largely intact. That the binding may be even tighter in the mutant suggests that the hydroxymethyl group of serine in the wild-type enzyme interacts unfavourably, presumably through steric or van der Waals repulsion, with the substrate in the non-covalent Michaelis complex. This strain could be relieved on formation of the acyl-enzyme. Alternatively, the binding could be promoted by the entropy of release of a trapped water molecule. None of the structural results, however, suggest that a water molecule has replaced the hydroxymethyl group of the active-site serine residue, since such a replacement would seem to better preserve the structure, stability and activity of the enzyme [43]. There seems no obvious reason for the relatively poorer binding of PADAC to the mutant. It might be noted, however, that the dansyl groups of dansylpenicillin and dansylcephalosporin are not likely to occupy the same space in the complex. A final caveat is that the tight β -lactam binding in the mutant may occur at a site other than the active site. There is certainly evidence for a second depsipeptide and peptide binding area on the P99 β -lactamase [10], although its occupancy by β -lactams is yet to be demonstrated.

On balance, the results presented here suggest that, although the mutant is in some way different from the native enzyme in solution, the difference does not destroy the active site, and thus the straightforward conclusion probably holds, namely that the serine hydroxy group is necessary for the aminolysis reaction, which therefore may involve an acyl-enzyme intermediate as depicted in Scheme 2. This conclusion would likely hold for D-alanyl-D-alanine carboxypeptidase/transpeptidase also.

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