Site-directed mutagenesis of β-lactamase I: role of Glu-166

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Two Glu-166 mutants of β-lactamase I from *Bacillus cereus* 569/H were constructed: one with a lengthened side chain (E166Cmc, the *S*-carboxymethylcysteine mutant) and the other with the side chain shortened and made non-polar (E166A). Their kinetic properties were studied and compared with those of the wild-type and the E166D mutant (with a shortened side chain) previously made by Gibson, Christensen and Waley (1990) (Biochem. J. **272**, 613–619). Surprisingly, with good penicillin substrates, K_m, $k_{cat.}$ and $k_{cat.}/K_m$ of the two conservative mutants (E166Cmc and E166D) are similar to those of the non-conservative mutant E166A. Their $k_{cat.}$ values are 3000-fold lower than that of the wild-type enzyme, showing that Glu-166 is a very important residue. The acylenzyme intermediate of E166A and a good substrate, penicillin V, was trapped by acid-quench and observed by electrospray ionization mass spectrometry, suggesting that Glu-166 is more important in catalysing the deacylation step than the acylation step. The β-lactamase I E166A mutant is about 200-fold more active than the *Bacillus licheniformis* E166A mutant with nitrocefin or 6β-furylacryloylamidopenicillanic acid as substrate. This suggested that other groups in the active site of the β-lactamase I mutant may activate the catalytic water molecule for deacylation.

INTRODUCTION

β-Lactamases are bacterial enzymes that are a major cause of antibiotic resistance (Waley, 1988). They catalyse the hydrolysis of the four-membered β-lactam ring of penicillins and cephalosporins, thus inactivating the antibiotic. On the basis of their primary structures, three classes of active-site-serine β-lactamases, A, C and D, have been identified. Their mechanism is usually represented by Scheme 1. The formation and breakdown of an acylenzyme intermediate in the pathway has been well established (Waley, 1992).

β-Lactamases differ from DD-peptidases in that they catalyse the deacylation step very efficiently (Ghuysen, 1991). It has been proposed by Gibson et al. (1990) that the active-site residue Glu-166 is required for both the acylation and deacylation in the hydrolysis of benzylpenicillin (penicillin G) by β-lactamase I from *Bacillus cereus*. However, the same residue has recently been shown to be mainly involved in catalysing the deacylation step in the *Bacillus licheniformis* enzyme (Escobar et al., 1991; Knox et al., 1993) and the *Escherichia coli* RTEM-1 enzyme (Adachi et al., 1991; Strynadka et al., 1992). It has been suggested from site-directed mutagenesis and protein crystallography that Glu-166 has nothing to do with the acylation step in the interaction between the RTEM-1 enzyme and penicillin G (Strynadka et al., 1992).

Previous experiments showed that shortening the side chain of Glu-166 of β-lactamase I by replacing the glutamate residue with aspartate through site-directed mutagenesis decreased activity (Gibson et al., 1990). Two questions may be posed: What happens if we lengthen it? What happens if we remove the carboxylate functional group by replacing Glu-166 with alanine?

The side chain of Glu-166 could be lengthened by carboxymethylation of a cysteine residue using the thiol-selective reagent iodoacetic acid (IAA). The mature wild-type β-lactamase I contains no cysteine residues (Thatcher, 1975; Madgwick and Waley, 1987; Amber et al., 1991). We made use of this advantage of first replacing the Glu-166 residue with cysteine by site-directed mutagenesis and then chemically modifying the thiol group by reaction with IAA, turning the cysteine mutant E166C into the *S*-carboxymethylcysteine mutant E166Cmc (Scheme 2).

The kinetic properties of these two mutants (E166A and E166Cmc) and the E166D mutant previously constructed and studied by Gibson et al. (1990) were compared with each other and those of the wild-type enzyme.

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. The 'ARISTAR' grade of guanidinium chloride used was from BDH Chemicals Ltd., Poole, Dorset, U.K. IAA (Phase Separations Ltd.) was recrystallized from carbon tetrachloride by K. W. Fung (Dyson Perrins Laboratory, University of Oxford) before use. Potassium 6β-iodopenicillanate (6β-IPA) was a gift from Leo Pharmaceutical Products.

Bacterial strains and plasmids

E. coli strain RZ1032 {HFr KL16 PO/45 [*lysA*(16–21)], *dut-1*, *ung-1*, *thi-1*, *relA1*, *zbd-279*::Tn*10*, *supE44*} was obtained from P. Handford (Sir William Dunn School of Pathology), and XL1-

Abbreviations used: 6β-IPA, 6β-iodopenicillanic acid; e.s.i.-m.s., electrospray ionization mass spectrometry; IAA, iodoacetic acid; FAP, 6β-furylacryloylamidopenicillanic acid; 6-APA, 6-aminopenicillanic acid.

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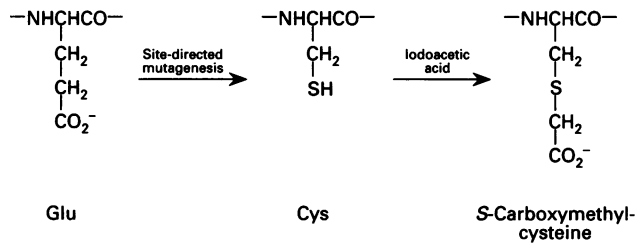
|| Deceased.

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

Scheme 1



Scheme 2

Blue {*endA1*, *hsdR17* (*rk*$^-$, *mk*$^+$), *supE44*, *thi-*, λ-, *recA1*, *gyrA96*, *relA1*, (*lac*), [F′, *proAB*, *lacI*^{q}, *lacZ*ΔM15, Tn*10*Tc]} was from Stratagene, Cambridge, U.K. Bacteria were grown in 2× TY broth [16 g/l Tryptone T (Oxoid, Basingstoke, Hants., U.K.), 10 g/l yeast extract and 5 g/l NaCl]. Plasmids pSG2penPC and pSG703 were from S. Thornewell (Sir William Dunn School of Pathology). The mutagenic oligonucleotides were synthesized by K. Gould (Sir William Dunn School of Pathology).

Construction of site-directed mutants

β-Lactamase I is encoded by the penPC gene. This gene was subcloned into pKS$^-$ cat from pSG2penPC by digestion with *Xba*I and *Sac*I. Site-directed mutagenesis was performed by using the uracil-template method (Kunkel, 1985; Kunkel et al., 1987) with the following 20-base-long oligonucleotides: for E166A, ATCGCTTTGCAACAGAATTA and for E166C, ATCGCTTTTGTACAGAATTA. Specific base changes (underlined) were confirmed by dideoxy sequencing (Sanger et al., 1977), with the aid of Sequenase (U.S. Biochemicals). The efficiency of mutagenesis for both mutants was 17%.

Expression and purification of mutants

E166A and E166C mutant genes were cloned into the integration plasmid pSG703 and were overexpressed by the *B. subtilis* prophage expression system φ105MU331 (Thornewell et al., 1993). About 200 and 250 μg of E166A and E166C mutant proteins respectively were produced per ml of culture supernatant 7 h after thermoinduction. The mutant enzymes were isolated from the culture medium of *B. subtilis* by a simplified method based on the procedures of Davies et al. (1974). As judged by SDS/PAGE, the protein band corresponding to β-lactamase I represented more than 80% of the total secreted protein present in the culture supernatant about 5 h after thermoinduction. For the E166C mutant, 3 mM 2-mercaptoethanol and 0.1 mM EDTA were present throughout the purification procedure. Purification of the wild-type enzyme was followed by Western blots (Burnette, 1981) and enzyme activity. However, for the mutants, the β-lactamase activities were too low for routine assay of the fractions during the purification, and so the enzyme-containing fractions were detected by SDS/PAGE with the wild-type protein as a molecular-mass standard. The concentration of the pure enzymes (> 95%) was determined from ϵ_{280} 29000 $M^{-1} \cdot cm^{-1}$. Purified wild-type and mutant enzymes could be stored at 4 °C in 50 mM sodium phosphate buffer, pH 7, over a period of weeks with little loss of activity. For more extended periods, solutions were dialysed against 20 mM NH_4HCO_3 and then lyophilized before storage at −20 °C.

N-Terminal amino acid sequence analysis

Wild-type β-lactamase I was expressed in *B. subtilis* φ105MU331. At 3 h after thermoinduction, 10 μl of culture supernatant was loaded directly on to an SDS/12.5% polyacrylamide gel and the proteins of different sizes were separated by electrophoresis. The gel was stained with Coomassie Brilliant Blue to show the proteins of individual bands. The three most strongly stained bands corresponding to different processed forms of β-lactamase I of similar size were cut out directly from the gel, and the N-terminal sequences of these three forms of proteins were determined by stepwise Edman degradation using a gas-phase sequencer (Applied Biosystems, model 470A).

Electrospray ionization mass spectrometry (e.s.i.-m.s.)

Electrospray ionization (e.s.i.) mass spectra were measured on a VG BIO Q triple-quadruple atmospheric-pressure mass spectrometer as described previously (Thornewell and Waley, 1992).

Carboxymethylation of the thiol group in the E166C mutant

The wild-type and E166C mutant of β-lactamase I (1.8 mg) were dissolved in 6 M guanidinium chloride to give a final concentration of denaturant of 5.7 M. The unfolding reaction for each protein was performed in the presence of dithiothreitol (final concentration, 1 mM). The reaction mixtures were incubated at 30 °C for 30 min to allow the unfolding of the protein. A tenfold molar excess of IAA was added (final concentration 10 mM), the pH was adjusted to 7–8, and the reaction mixtures were incubated for 20 min at 20 °C to allow carboxymethylation of the thiol group:

$$E \cdot CH_2\text{-}S^- + I\text{-}CH_2\text{-}COO^- \rightarrow E \cdot CH_2\text{-}S\text{-}CH_2\text{-}COO^- + I^-$$

The solutions containing the modified proteins were diluted 5-fold with 10 mM NH_4HCO_3 to reduce the concentration of denaturant and allow refolding of the β-lactamases, then dialysed against 10 mM NH_4HCO_3, and lyophilized.

Active-site titration

The concentrations of active enzyme were measured by allowing each mutant enzyme to react with 6β-IPA, a site-specific inhibitor of serine β-lactamases (Knott-Hunziker et al., 1979). After reaction, the sample was analysed by e.s.i.-m.s. to detect the formation of covalently modified enzymes.

Estimation of steady-state kinetic parameters

The hydrolyses of four penicillins [benzylpenicillin (penicillin G), phenoxymethylpenicillin (penicillin V), 6β-furylacryloylamidopenicillanic acid (FAP) and 6-aminopenicillanic acid (6-APA)] and one cephalosporin (nitrocefin) were studied. Rate constants were determined in 0.05 M sodium phosphate buffer, pH 7, containing 0.5 M NaCl and 0.1 mM EDTA at 20 °C. The

parameters were determined 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 Stanislawski (1991). Substrate hydrolysis was monitored by using the spectrophotometric assay method of Waley (1974) at 232 nm for penicillin G and penicillin V, 330 nm for FAP, 240 nm for 6-APA and 500 nm for nitrocefin. Initial rates were measured in a Varian Cary model 3 spectrophotometer at various substrate concentrations. For the slow hydrolysis of 6-APA by the mutants, K_m was found by inhibition of the hydrolysis of nitrocefin. The K_i found gives the K_m accurately when the $k_{cat.}/K_m$ of the inhibiting substrate is less than 5% of the monitoring substrate (Waley, 1983). Deviations from Michaelis–Menten kinetics were not detected. The presence of lysozyme [0.1 mg/ml (Persaud et al., 1986)] increased the reproducibility of steady-state experiments with wild-type β-lactamase I.

Trapping the acylenzyme

The experiment was carried out using the Biologic QFM5 quenched-flow apparatus. One syringe of the quenched-flow apparatus contained the mutant enzyme E166A (70 μM), the second contained the buffer (2 mM triethanolamine, pH 7), the third contained the substrate penicillin V (70 μM) and the fourth syringe contained 90% (v/v) formic acid. All of the solutions in the syringes were allowed to equilibrate at 20 °C for 15 min. The depression of the pistons were controlled by stepping motors, which allowed 50 μl of the enzyme to mix with 50 μl of the substrate for between 5 ms and 1 s before the reaction was halted by mixing with 5 μl of the 90% (v/v) formic acid [final concentration, 4% (v/v)]. Each sample was then collected and stored on ice before being analysed by e.s.i.-m.s.

RESULTS

N-terminal processing and isolation of mutant enzymes

N-Terminal amino acid sequence analysis of wild-type β-lactamase I expressed in *B. subtilis* φ105MU331 showed that there were three forms of the enzyme 3 h after thermoinduction (Figure 1). The largest form, starting with Lys-16, was completely processed to the two shortened forms, starting with His-24 and Ala-28, by the action of secreted proteases from *B. subtilis* 6 or 7 h after thermoinduction. *In situ* detection of β-lactamase activity on SDS/polyacrylamide gel containing the three forms showed that all of them were active (results not shown). A control band on this SDS/polyacrylamide gel containing a degraded form of β-lactamase I starting with Ala-48, i.e. lacking the α1-helix, did not show any detectable β-lactamase activity, which is in agreement with results shown by Matagne et al. (1991) and Thornewell and Waley (1992). The two mature forms of both the E166A and E166C mutants were processed to give the same N-terminal sequences as those for the wild-type enzyme, as indicated by their measured masses (Table 1). The fact that the larger form starts at His-24 and the smaller form starts at Ala-28 and yet they are both equally active, as judged from *in situ* activity detection, suggested that the residues preceding the N-terminus of Ala-28 in the α1-helix are expendable. This has also been reported by Matagne et al. (1991) for the *B. licheniformis* and the *Streptomyces albus* G enzymes.

About 58 mg of E166A and 36 mg of E166C mutant proteins were purified from 880 ml culture supernatants, obtained 7 h after thermoinduction when the Lys-16 form had been completely cleaved to the His-24 and Ala-28 forms. The two forms of both mutant proteins were more than 95% pure as judged by SDS/PAGE. E.s.i.-m.s. showed that the purified E166A and E166C mutants contained about 80% of the larger form and 20% of the smaller form. Active-site titration with 6β-IPA indicated that both forms of the two mutants were fully active (Figures 2a and 2b). If there had been appreciable amounts of denatured protein present, the e.s.i.-m.s. would have shown the presence of free enzyme. The kinetic properties of the mixture would not be expected to be significantly different from those of the isolated forms, by analogy with the results of Matagne et al. (1991) for four of the other class-A β-lactamases. 6β-IPA is a mechanism-based inactivator of class-A, -C and -D β-lactamases. It inactivates β-lactamase I stoichiometrically (Cohen and Pratt, 1980; Knott-Hunziker et al., 1980). The inactivation is characterized by the rearrangement of the penicilloyl moiety of the acylenzyme into a dihydrothiazine chromophore (Orlek et al., 1980). The attachment of the inactivator to the active site of

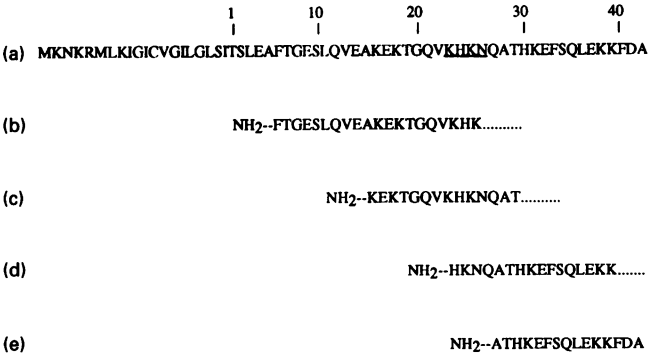


Figure 1 N-Terminal amino acid sequences of β-lactamase I

(**a**) Amino acid sequence of the first 62 residues of β-lactamase I predicted from DNA sequence data. Previously known N-termini of processed forms of the enzyme (Thatcher, 1975; Ambler, 1980) are underlined. (**b**) N-Terminus and sequence following for β-lactamase I purified from *E. coli* (Mézes et al., 1985). (**c**) The largest form with Lys-16 as the N-terminus. It was processed to the two shortened forms. The N-terminus of the larger form is His-24 (**d**) and that of the smaller form is Ala-28 (**e**). This observation suggests that, during the secretion of β-lactamase I in *B. subtilis*, a specific signal peptidase cleaves the translation product at Phe-6, but, in the presence of the *Bacillus* proteases, the product is cleaved rapidly to the form with Lys-16 as the N-terminal residue, which is then further processed to the two shortened forms. This post-translational N-terminal processing also occurs in the natural host *B. cereus*. Numbering is based on the ABL numbering scheme (Ambler, 1980; Ambler et al., 1991).

Table 1 Comparison of the calculated and measured masses of different forms of each mutant

The cysteine and alanine mutants of β-lactamase I are shown as E166C and E166A respectively. The unique thiol group of the E166C mutant was modified by reaction with IAA, and this *S*-carboxymethylcysteine mutant is shown as E166Cmc. The calculated mass of the larger form of each mutant was obtained from the amino acid sequence by assuming that the N-terminus was His-24 and that of the smaller form was Ala-28.

Mutant	Mutant form	Calculated mass	E.s.i.-m.s.-measured mass
E166C	Larger	28787.4	28790.3 ± 2.4
	Smaller	28279.9	28285.4 ± 3.8
E166A	Larger	28755.3	28760.3 ± 2.1
	Smaller	28247.5	28250.5 ± 3.5
E166Cmc	Larger	28845.4	28847.8 ± 3.4
	Smaller	28337.9	28340.6 ± 4.1

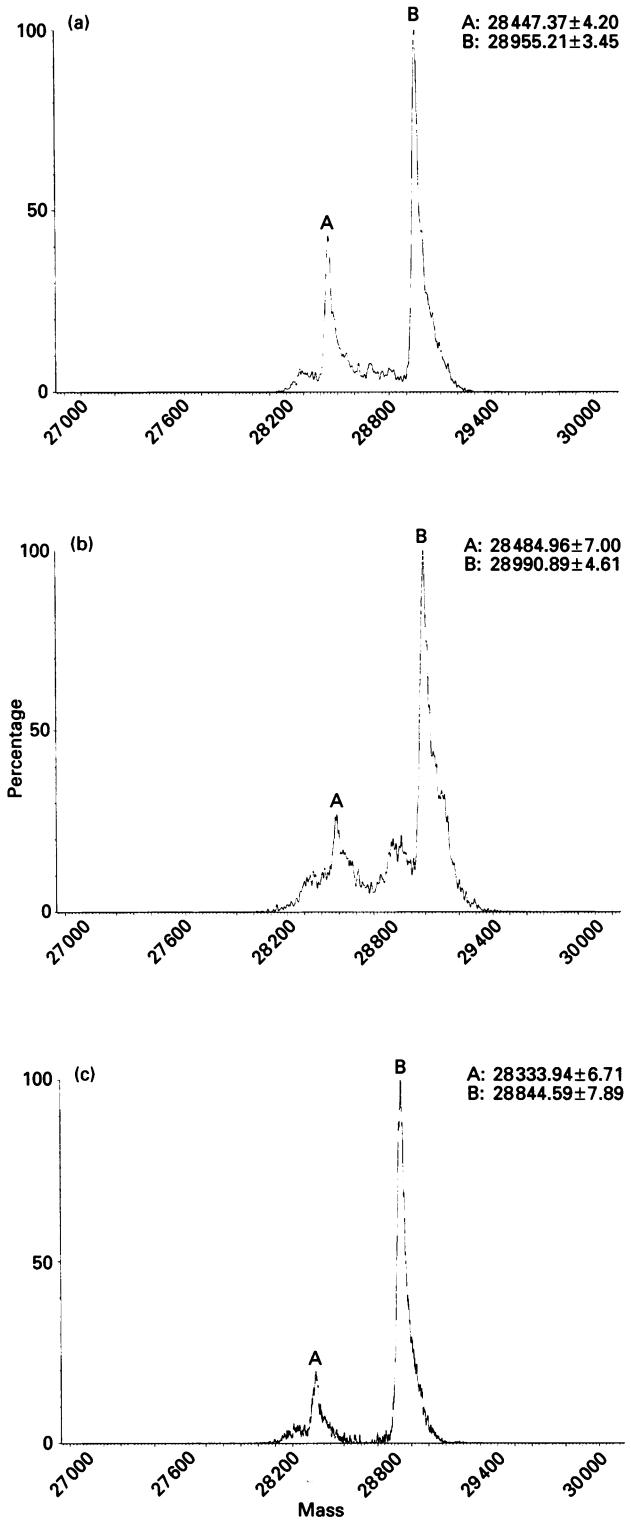


Figure 2 E.s.i. mass spectra showing the covalently modified enzymes of mutant forms

(**a**) Purified E166A was allowed to react with 6β-IPA in 1:3 ratio for 1 min at room temperature. (**b**) Purified E166C was allowed to react with 6β-IPA in 1:1 ratio for 1 min at room temperature. For both mutants, more than 90% of the free enzyme of each form was converted into covalently modified enzyme, indicating that both forms of the two mutants were fully active. (**c**) No covalently modified enzyme was formed when E166Cmc was allowed to react with 6β-IPA, even when the ratio at reaction was 1:10 and the reaction was for 3 min. The masses of the two peaks were the same as those of the free-enzyme forms, indicating no interaction between E166Cmc mutant and 6β-IPA. The highest peak in each spectrum is treated as 100%.

the mutant was predicted to add 199 Da to the mass of the modified protein. This was shown to be the case by an increase in the molecular mass of both species by 199 Da.

Chemical modification

E.s.i.-m.s. showed that the modification was nearly 100% (Figure 3). Both forms of the E166C mutant were converted into E166Cmc and the measured masses were in agreement with the calculated masses for this modification (Table 1). Active-site titration of E166Cmc with 6β-IPA was carried out under the same conditions as for the E166A and E166C mutants. Surprisingly, e.s.i.-m.s. indicated that there was no reaction, even when a 10-fold excess of 6β-IPA was added (Figure 2c). The e.s.i.-m.s.-measured masses of the two forms of E166Cmc after treatment with 6β-IPA indicated no change from the free E166Cmc proteins (Table 2). This was probably not due to improper refolding of the protein after denaturation because the wild-type enzyme was fully active after exactly the same treatment. The lengthening of the side chain at the 166 position might somehow prevent the entry of 6β-IPA but not some other substrate molecules into the active site.

Kinetic studies

The measured steady-state kinetics parameters for hydrolysis of β-lactams by mutant enzymes are shown in Table 3. The K_m values of the four penicillin substrates were lowered more than those of nitrocefin for all the mutants. E166D has a similar K_m value to that of wild-type enzyme when interacting with nitrocefin (Gibson et al., 1990). As has been pointed out by Christensen et al. (1990), we could not say much about the substrate affinity or binding by just looking at the change in K_m values. This is because substrate affinity or binding depends on K_s, the dissociation constant of the non-covalent enzyme–substrate complex ES (i.e. k_{-1}/k_{+1}), whereas in the acylenzyme mechanism of Scheme 1, K_m is given by:

$$K_m = \left(\frac{k_{-1}+k_{+2}}{k_{+1}}\right)\left(\frac{k_{+3}}{k_{+2}+k_{+3}}\right)$$

and so it depends on all of the four rate constants.

$k_{cat.}$ and $k_{cat.}/K_m$ values were greatly lowered by the mutations. With penicillin G or FAP as substrate, $k_{cat.}$ values of the mutants were between 0.01 and 0.04% of those of wild-type. With nitrocefin as substrate, the E166D mutant showed higher K_m, $k_{cat.}$ and $k_{cat.}/K_m$ values than all the other mutants, although $k_{cat.}$ and $k_{cat.}/K_m$ were still 162-fold and 229-fold lower than those of the wild-type respectively.

The specificity profile was not drastically altered, the best substrates, penicillin G, penicillin V and FAP, also being efficiently hydrolysed by the wild-type β-lactamase I.

As a whole, all of the mutants showed a few thousand-fold decrease in $k_{cat.}$ and $k_{cat.}/K_m$ values. This indicated that Glu-166 is a very important residue in β-lactamase I action.

Trapping the acylenzyme of the E166A mutant

Previous workers (Adams et al., 1980; Goto and Fink, 1989) have shown that β-lactamase from *Staphylococcus aureus* and β-lactamase I adopt a non-native partially folded conformation at low pH in aqueous solutions. Acylenzyme intermediate formed under steady-state conditions at subzero temperature has been trapped by acid-quenching (Cartwright et al., 1989). Quenching the reaction with acid resulted in stabilization of the intermediate;

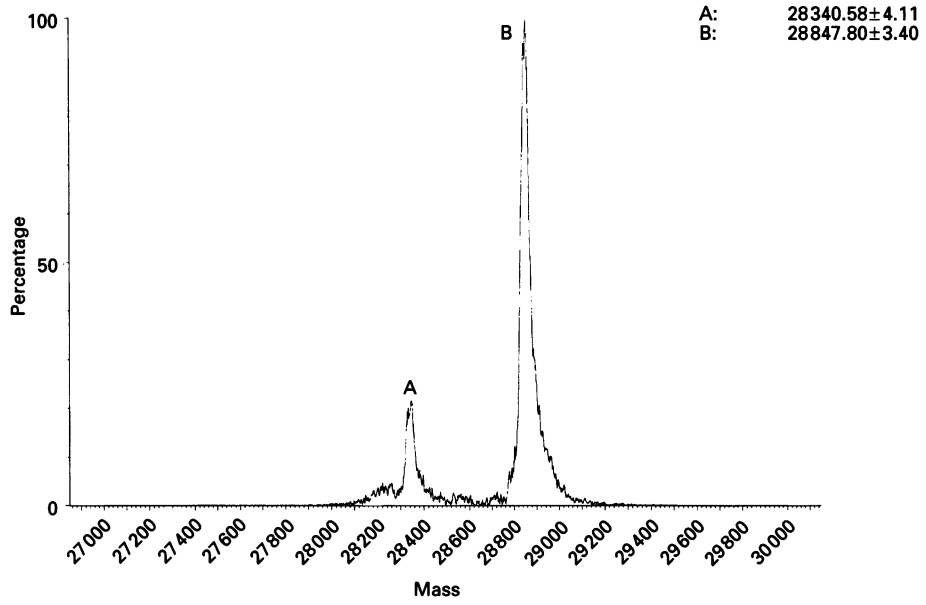


Figure 3 E.s.i. mass spectrum of E166Cmc

E166C was treated with IAA under denaturing conditions as mentioned in the text. About 20 μg of the carboxymethylated product (E166Cmc) was analysed by e.s.i.-m.s. The e.s.i. mass spectrum shows that the carboxymethylation reaction was complete. The reaction was also very specific as there was no evidence in the spectrum of multiple carboxymethylation. The highest peak is treated as 100%.

Table 2 Active-site titration

Each mutant was allowed to react with 6β-IPA before being analysed by e.s.i.-m.s. The calculated mass of the covalently modified enzyme of each mutant form was obtained by adding 199 Da (mass of the dihydrothiazine moiety) to the mass of each free mutant form.

Mutant	Mutant form	Calculated mass of covalently modified enzyme	E.s.i.-m.s.-measured mass
E166C	Larger	28986.4	28990.9±4.6
	Smaller	28478.9	28485.0±7.0
E166A	Larger	28954.3	28955.2±3.5
	Smaller	28446.5	28447.4±4.2
E166Cmc	Larger	29044.4	28844.6±7.9
	Smaller	28536.9	28333.9±6.7

the rate of acid-induced conformational change was greater than the rate of deacylation.

Acylenzyme intermediates of the E166A mutant were detected by a new combination of two powerful techniques: quenched-flow at room temperature and neutral pH and e.s.i.-m.s. with a good substrate (Figure 4). The fact that we could trap acylenzyme intermediate in the interaction between E166A mutant and penicillin V indicated that the deacylation rate constant k_{+3} must have been decreased more than that of the acylation rate constant k_{+2} by replacing the Glu-166 residue with an alanine residue. In this sense, Glu-166 is more important in catalysing the deacylation step than the acylation step, which is in agreement with the results of Escobar et al. (1991) and Strynadka et al. (1992). In marked contrast, analysis of the products of the reaction of penicillin V with wild-type β-lactamase I after 2.5 ms at 5 °C showed no acylenzyme and complete hydrolysis of penicillin V. The reaction with wild-type enzyme is too fast for the present technique.

Acylenzyme intermediates were also observed when penicillin G was used as the substrate but the mass of the smaller-form acylenzyme was similar to that of the free larger form of β-lactamase I so that the two peaks in the e.s.i.-m.s. charge state series overlapped. To facilitate the observation of formation of acylenzyme by each enzyme form, another good substrate, penicillin V, of slightly higher molecular mass was therefore used.

DISCUSSION

The primary object of the present work was to determine whether lengthening the side chain of Glu-166 in β-lactamase I had the same effect as shortening or removing it. The acylation and deacylation rate constants k_{+2} and k_{+3} of the E166D mutant of β-lactamase I, the mutant with a shortened side chain, have been previously determined using penicillin G as the substrate, and both of them were found to be decreased by about 2000-fold (Gibson et al., 1990). This led to the conclusion that Glu-166 was equally important in acylation and deacylation. Although k_{+2} and k_{+3} values for the E166A mutant have not yet been determined, the fact that we could trap and observe its acylenzyme intermediate with penicillin V or penicillin G as substrate by acid-quench and e.s.i.-m.s. means that both k_{+2} and k_{+3} must be decreased considerably compared with the wild-type, and k_{+3} must be decreased more than k_{+2}. This indicates that Glu-166 in β-lactamase I is also more important in catalysing deacylation than acylation, as reported for the *B. licheniformis* (Escobar et al., 1991) and RTEM-1 enzymes (Strynadka et al., 1992).

However, there are also some quantitative differences. In the case of the E166A mutant of the *B. licheniformis* enzyme, a burst was reported corresponding to formation of acylenzyme when

Table 3 Kinetic parameters for hydrolysis of β-lactams by mutant enzymes

The steady-state kinetic parameters were determined from initial rates. The reactions were carried out in 50 mM sodium phosphate buffer, pH 7, containing 0.5 M NaCl and 0.1 mM EDTA at 20 °C. ND, Not determined.

	K_m (μM)				$k_{cat.}$ (s^{-1})			$k_{cat.}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)			
Substrate	Wild-type	E166A	E166C	E166Cmc	Wild-type	E166A	E166C	E166Cmc	Wild-type	E166A	E166C	E166Cmc
Penicillin G	118.4±5.4	18.7±2.5	18.4±2.9	17.0±3.3	4515±61	1.62±0.06	0.818±0.031	0.863±0.036	38.1±2.25	0.0866±0.0147	0.0444±0.00862	0.0507±0.0121
Penicillin V	132.9±11.3	17.0±1.6	20.3±2.1	10.3±1.3	4525±109	0.923±0.021	0.340±0.008	0.362±0.008	34.1±3.72	0.0544±0.00652	0.0167±0.00216	0.0352±0.00514
FAP	40.9±2.2	5.59±0.31	10.2±1.2	10.6±1.5	1491±28	0.356±0.0037	0.145±0.0031	0.170±0.0042	36.4±2.65	0.0637±0.0042	0.0142±0.00196	0.0160±0.0027
6-APA	1604±148	101±16	127±17	219±26	227.4±8.2	ND	ND	ND	0.142±0.0182	ND	ND	ND
Nitrocefin	95.4±7.1	59.7±3.5	64.6±8.7	84.7±10.0	49.2±1.27	0.0151±0.0003	0.0102±0.00042	0.0086±0.00034	0.516±0.0517	0.00025±0.00002	0.00016±0.00003	0.00010±0.00002

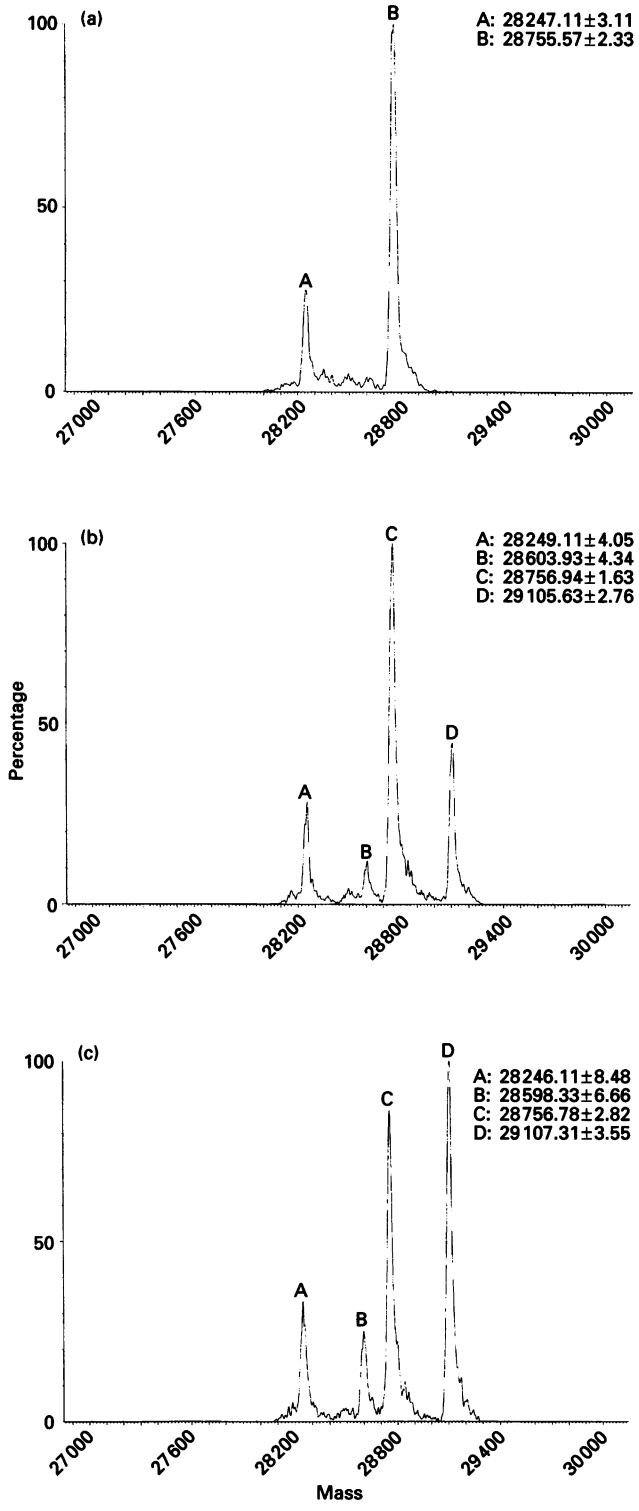


Figure 4 E.s.i. mass spectra showing the time course of formation of acylenzyme intermediate

E166A mutant enzyme (50 μl of a 70 μM solution) was mixed with 1 molar proportion of penicillin V inside the quenched-flow apparatus. Three experiments were carried out, and 5 μl of 90% (v/v) formic acid was added to stop the reaction at 5 ms, 200 ms and 1 s. The three samples were each analysed by e.s.i.-m.s. (**a**) At 5 ms, the spectrum contains only the two free E166A enzyme species. (**b**) At 200 ms, the e.s.i.-m.s. indicates formation of the acylenzyme. (**c**) At 1 s, the e.s.i.-m.s. indicates increased acylenzyme formation compared with the free enzymes. The highest peak in each spectrum is treated as 100%.

Table 4 Comparison of steady-state kinetic parameters of E166A mutants from *B. cereus* and *B. licheniformis* enzymes

The numbers in parentheses are parameters of wild-type enzymes. Assay buffer conditions were, for β-lactamase I, 50 mM sodium phosphate, pH 7, containing 0.5 M NaCl and 0.1 mM EDTA (20 °C) and, for the *B. licheniformis* enzyme (Escobar et al., 1991), 50 mM potassium phosphate, pH 7, containing 0.5 M KCl (30 °C). ND, Not determined.

	E166A of β-lactamase I from *B. cereus* (pH 7, 20 °C)		E166A of β-lactamase from *B. licheniformis* (pH 7, 30 °C)	
	FAP	Nitrocefin	FAP	Nitrocefin
K_m (μM)	5.6 (41)	60 (95)	≪ 1 (63)	≪ 1 (41)
$k_{cat.}$ (s^{-1})	3.6×10^{-1} (1500)	1.5×10^{-2} (49)	1.4×10^{-3} (990)	$< 1 \times 10^{-4}$ (1100)
$k_{cat.}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)	6.4×10^{-2} (36)	2.5×10^{-4} (5.2×10^{-1})	ND (16)	ND (27)

the mutant enzyme was added to a solution of either nictrocefin or FAP at pH 7.0, 30 °C (Escobar et al., 1991). We did not observe such an accumulation of the acylenzyme with our β-lactamase I E166A mutant under similar conditions. In fact, for both substrates, the value of $k_{cat.}$ for our mutant was still about 200-fold higher than that of the *B. licheniformis* E166A mutant (Table 4). Our acylenzyme intermediate was much shorter lived, indicating that k_{+3} was still quite high and may not be represented by $k_{cat.}$. That the E166A mutant of β-lactamase I exhibited a higher activity than the equivalent alanine mutants of the *B. licheniformis* and RTEM-1 β-lactamases indicated that other groups in the active site of the β-lactamase I mutant can somehow activate the catalytic water molecule for deacylation. This is not surprising because even when the Ser-70 residue of the *S. albus G* β-lactamase was replaced with alanine, the enzyme still retained a significant activity against at least two good substrates of the wild-type enzyme, penicillin G and ampicillin (Jacob et al., 1991). This led to the suggestion that other groups in the active site may be able to activate a water molecule to perform a direct attack on the β-lactam amide bond.

Acylenzyme with good substrates could also be trapped at steady state by using the quenched-flow apparatus and then measured spectrophotometrically at 282 nm by the penamaldate reaction (Christensen et al., 1990). However, data collected from such studies could only be used to determine the k_{+2} and k_{+3} values. We are now trying a pre-steady-state approach which involves quenched-flow and e.s.i.-m.s. to measure all of the four rate constants for the E166A mutant.

The *E. coli* RTEM-1 cysteine β-lactamase, constructed by replacing the active-site serine with a cysteine residue (Sigal et al., 1982), was found to be inactivated by IAA at pH below 7 in the native state of the protein (Knap and Pratt, 1991). However, when we treated the E166C mutant of β-lactamase I in the same way as the RTEM-1 cysteine enzyme with IAA, there was no change in activity when the attempted modification was carried out at pH 5 or pH 8.5 with or without addition of the reducing agent dithiothreitol (results not shown). To make sure that the unique thiol group in the active site of the E166C mutant was available for reaction with IAA, the mutant protein was unfolded in 5.7 M guanidinium chloride before IAA was added. After reaction, the protein was allowed to fold back to the native state. The wild-type β-lactamase I was treated in the same way as a control and was found to have recovered its activity fully, suggesting the right conditions for refolding had been found. Kinetic studies with several substrates indicated that E166Cmc was active (Table 3), which further supports the notion that the protein had folded back to its native conformation. However, it is quite disappointing that the chemical modification of the E166C mutant with IAA did not generate a mutant with catalytic activity near to the wild-type levels. This may be due to the added carboxylate group pointing away from the active site. The change from Glu-166 to E166Cmc could then have a minimal impact on the active-site geometry. The added carboxylate group prevented reaction between 6β-IPA and the enzyme. This indicated that the group may be pointing in a direction which blocks the entry of 6β-IPA.

Another explanation for the lack of increase in activity of E166Cmc is that the length of the side chain at the 166 position has to be exactly that of glutamate in order to give optimal catalytic activity. Even though the functional group is kept unchanged (here a carboxylate group), shortening or lengthening the side chain may disturb the hydrogen-bonding network between the carboxylate oxygen atoms and the other groups in the active site such as Lys-73 and the catalytic water molecule. Hydrogen bonds cannot form if the distances between the two groups concerned are too long or too short. The requirements of a precisely located carboxylate group are also supported by the fact that E166A, E166D and E166Cmc all have very similar activities to each other. This implies that if the group is not correctly located, there is no difference whether it is present or not.

Another example showing that the precise positioning of Glu-166 is needed for its carboxylate group to function was reported by Herzberg et al. (1991). This crytallographic study of a D179N mutant of the *Staph. aureus* β-lactamase showed a mispositioned Glu-166, severely disordered by virtue of floppiness in the 163–175 loop; this was thought to be the cause of a lowered deacylation rate in the mutant enzyme.

With benzylpenicillin, the $k_{cat.}$ values of the E166A and E166D mutants of β-lactamase I were reduced to the same extent (about 3000-fold). This is quite surprising because, in the case of the *B. licheniformis* and RTEM-1 mutants, E166A was much less active than E166D, which led to the suggestion that some of the catalytic function of Glu-166 in deacylation remained because of the positioning of the aspartate carboxylate either via a conformational change or via a bridging water molecule (Escobar et al., 1991).

However, with nitrocefin, the E166D mutant did exhibit a higher $k_{cat.}$ value than E166A. The difference was about 14-fold. However, on comparison with the *B. licheniformis* and RTEM-1 E166A mutants, the $k_{cat.}$ value for the β-lactamase I E166A was still 200-fold higher and no acylenzyme intermediate accumulation was observed under normal conditions.

In the serine proteases, acylation and deacylation are similar, involving essentially the same mechanism, with His-57 functioning as a general base catalyst in both steps (Carter and Wells, 1988). In serine β-lactamases, Glu-166 is probably more important in the catalysis of the deacylation step than the

acylation step. The enormous rate differences between the wild-type β-lactamase I and the Glu-166 mutants demonstrate the importance of Glu-166 in the catalytic mechanism, but its exact role remains to some extent enigmatic.

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