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Site-Directed Mutagenesis of β-Lactamase Leading to Accumulation of a Catalytic Intermediate†

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Received January 30, 1991; Revised Manuscript Received August 19, 1991

ABSTRACT: Site-specific mutation of Glu-166 to Ala in β-lactamase causes a millionfold reduction in catalytic activity toward both penicillin and cephalosporin substrates and results in the stoichiometric accumulation of a normally transient acyl-enzyme intermediate. Kinetic analysis indicated that substitution of Glu-166 by Ala leads to negligible effect on the acylation half of the reaction but effectively eliminates the deacylation reaction. Such differential effects on the rates of formation and breakdown of an enzyme–substrate intermediate have not been previously reported. Thus, unlike the situation for most transfer enzymes, e.g., the serine proteases, acylation and deacylation in β-lactamase catalysis are not "mirror" images and must involve different mechanisms. The results suggest an explanation for the different catalytic activities between the β-lactamases and the penicillin-binding proteins involved in bacterial cell-wall synthesis.

The production of β-lactamases is the principal means by which bacteria achieve β-lactam antibiotic resistance. These enzymes catalyze the hydrolysis of penicillins and cephalosporins to their biologically inactive products. Due to the widespread use of these antibacterial agents, there has been a strong selection for microorganisms which are resistant to β-lactams. In order to overcome this trend to resistance much research has been carried out to determine the mechanisms of these enzymes. Thus, an understanding of the interactions between β-lactamases and their substrates is critical to the design of inhibitors of potential application as antibiotics.

The β-lactamase from *Bacillus licheniformis* 749C is a class A β-lactamase, exhibiting both penicillinase and cephalosporinase activities, and characterized by the presence of an active

† This research was supported in part by a grant from the National Science Foundation.
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0006-2960/91/0430-10783$02.50/0

site serine residue (Ambler, 1980; Pollock, 1965; Joris et al., 1988). Recently, high-resolution crystal structures have been reported for two class A β-lactamases (Herzberg & Moult, 1987; Moews et al., 1990). The structural information from these crystallographic studies, as well as the results from inhibition, mutagenesis, and homology investigations, indicate that at least four residues, Ser-70, Lys-73, Lys-234, and Glu-166, play a central role in catalysis. Although Ser-70 has been implicated as a nucleophilic catalyst in the catalytic reaction (Fisher et al., 1981; Cohen & Pratt, 1980; Cartwright & Coulson, 1980), the function of the other residues remains unclear.

Glu-166 has been postulated to act as a general base in catalysis (Herzberg & Moult, 1987; Moews et al., 1990), possibly in a manner analogous to histidine in the catalytic triad of serine proteases, or, perhaps in a more limited fashion, participating only in acylation or deacylation (Herzberg & Moult, 1987). Madgwick and Waley (1987) have shown that conversion of Glu-166 to Gln leads to a dramatic reduction in the ampicillin resistance of an *Escherichia coli* strain in which the gene for the *Bacillus cereus* β-lactamase had been inserted. To investigate the role of this residue in catalysis, we have specifically replaced Glu-166 with alanine and characterized the kinetic properties of the resulting mutant.

Experimental Procedures

The *Bacillus amyloliquefaciens* subtilisin promoter and signal sequence were used to express the *B. licheniformis* β-lactamase in *Bacillus subtilis* (using the shuttle vector pSS5-βLac) (Ellerby et al., 1990). Mutagenesis was accomplished via the Kunkle method (Kunkle, 1985), in conjunction with restriction purification (Wells et al., 1986) to enhance mutation efficiency. The sequence of the oligonucleotide used to generate E166A was 5′-GAA-CGA-TTC-(GCG)-CCA-GAG-CTC-AAT-GAA-GTG-3′. This sequence replaces Glu-166 with Ala (in parentheses) and introduces a *Sac*I site (underlined). Thus, digestion of the mutant sequence with *Sac*I allows for restriction purification. The E166A sequence was confirmed by dideoxy sequencing (Sanger et al., 1977), with the aid of Sequenase (U.S. Biochemicals). The primer sequence used was 5′-TT-GGC-GGA-CCT-GAA-AGT-TTG-3′ and was designed to sit 50 bp upstream from the mutagenesis site.

The acyl-enzyme burst reaction of E166A β-lactamase with nitrocefin under steady-state conditions was monitored at 482 nm. A 10 μM nitrocefin solution (0.05 M potassium phosphate, 0.5 M KCl, pH 7.0) was prepared and incubated at 30 °C. E166A was added to give final concentrations of 2.0 or 4.5 μM.

HPLC of ((furylacryloyl)amido)penicillin- and nitrocefin-labeled E166A β-lactamase was carried out as follows. Substrates and enzyme were mixed for 5 s at 0 °C. The reaction mixture was immediately loaded onto a minigel column (DEAE-Sephadex G25, 1.3 × 5 cm) and spun at 4000 rpm at 0 °C for 1 min to remove excess substrate. The column had previously been equilibrated with 0.05 M potassium phosphate, pH 7.0 buffer. The eluent was collected in an Eppendorf vial and placed on ice. The enzyme–substrate mixture was analyzed using a Beckman gradient HPLC with a Model 165 detector and a C3 ultrapore RPSC column (4.6-mm diameter × 7.5 cm) operated at a flow rate of 1 mL/min. Before use the column was equilibrated with 5% (v/v) acetonitrile. The following program was used: (a) 5% acetonitrile, 2 min; (b) 5–45% (v/v) acetonitrile, 15 min; (c) 45% (v/v) acetonitrile, 8 min; (d) reversal of gradient, 15 min. The FAP[1]-E166A mixture was monitored at 305 and 280 nm to assay for the presence of FAP and E166A, respectively. Both traces showed a single peak with coincident retention times of 14.7 min. Similar results were seen for the nitrocefin experiment monitored at 385 nm (nitrocefin) and 280 nm (E166A). The coincident peaks had a retention time of 15 min. Controls were done to check for E166A contribution to signal at 305 and 385 nm. Both controls indicated negligible contributions to absorbance at wavelengths used to assay for substrates.

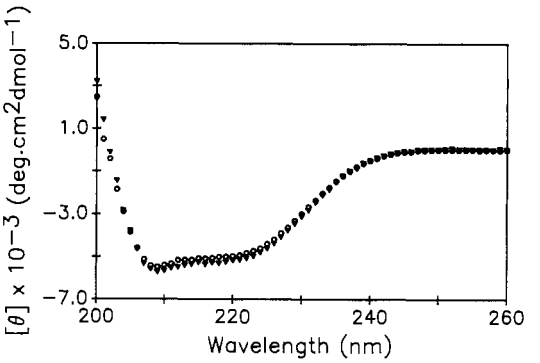


FIGURE 1: Far-UV circular dichroism spectra of E166A (circles) and wild-type β-lactamase (inverse triangles) in 0.05 M potassium phosphate, pH 7.0, 30 °C.

Stopped-flow kinetics experiments were performed with a Biologic SFM-2 instrument. The reaction of β-lactamase (final concentration 4.0 μM) and nitrocefin (final concentration 160 μM) was monitored at 482 nm, at pH 7.0 (50 mM phosphate buffer), 20 °C.

CD spectra were obtained with an AVIV Model 62DS spectrometer using a 0.1-cm path length cell with 10.2 μM protein for E166A, and 0.2-mm path length and 25.2 μM protein for the wild type.

Proteolysis experiments were carried out as follows. All solutions were prepared in sodium phosphate buffer, 0.05 M, pH 7.0. Stock solutions of nafcillin (252 mM), nitrocefin (500 μM), and FAP (1 mM) were prepared. Nine microliters of E166A (45 μM) was preincubated with nafcillin (7 μL) for 4 min at 25 °C and with nitrocefin (7 μL) and FAP (7 μL) for 30 s. A control involved preincubation with 7 μL of buffer alone. After the prescribed times, α-chymotrypsin (Worthington Biochemical Corp.) (4 μL of 2.5 μM) was added to the solutions, the solutions were mixed (final concentrations: β-lactamase 20 μM, α-chymotrypsin 0.5 μM), and the digestion was allowed to proceed for 4 and 20 min, at which times the solutions were centrifuged in DEAE-Sephadex minigel tubes for 1 min, the eluant being quenched in 5 μL of 50% TCA. The precipitated protein was then taken up in SDS running buffer and loaded onto a 10–15% Phastsystem SDS–polyacrylamide gel. Both Coomassie blue and silver staining were used to visualize the samples.

Results and Discussion

Structural Effects of the Mutation. The close proximity of Lys-73 and Glu-166 (3.2 Å) in β-lactamase suggests the presence of an ionic interaction between these residues. Since ion pairs often contribute to the stability of proteins, we investigated whether the structure of E166A was perturbed from that of the wild type. The thermal stability of the wild type and E166A mutant were determined at pH 7.0 by tryptophan fluorescence. Both proteins had identical T_m's of 64 ± 1 °C. As shown in Figure 1, the far-UV circular dichroism spectra

[1] Abbreviations: FAP, 6-((2-furylacryloyl)amido)penicillin; T_m, midpoint of thermal denaturation transition.

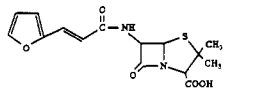
Table I: Kinetic Parameters for Wild-Type and E166A β-Lactamase[a]

	nitrocefin			FAP		
enzyme	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
wild type	1088	41.0	2.65×10^7	982	62.8	1.56×10^7
E166A	$<1 \times 10^{-4}$	$\ll 1$ μM	ND	1.4×10^{-3}	$\ll 1$ μM	ND

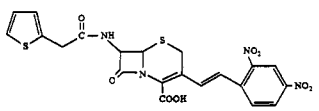
[a] Conditions: 30.0 °C, pH 7.0 (0.05 M potassium phosphate buffer, 0.5 M KCl). Kinetic constants were determined from initial velocity measurements and Eadie–Hofstee analysis. Experiments were done in triplicate. Substrate concentrations: FAP, 2–54 μM; nitrocefin, 2–62 μM. Enzyme concentrations: wild type, 1.35 nM; E166A, 0.33–1.3 μM. The FAP hydrolysis was monitored at 330 nm, nitrocefin at 482 nm. ND = not determined. Units are s^{-1} for k_{cat}, μM for K_m and Ms^{-1} for k_{cat}/K_m.

are superimposable within experimental error. In addition, the tryptophan fluorescence spectra (not shown) were superimposable for the wild type and the E166A mutant, confirming negligible effects of the mutation on secondary and tertiary structure. Further corroboration came in the form of experiments with α-chymotrypsin, which indicated similar slow rates of proteolysis for both wild-type and E166A β-lactamases.

Kinetic Effects of the Mutation. The kinetic characteristics of the wild-type and mutant enzymes with both penicillin and cephalosporin substrates were investigated. Nitrocefin was chosen as a cephalosporin substrate because of the very large Δε associated with cleavage of its β-lactam bond, which facilitated monitoring the catalytic reaction. FAP, 6-((2-furylacryloyl)amido)penicillin, was chosen as a representative penicillin substrate since it has a distinctive chromophore in the 300–350-nm region. For both substrates the value of k_{cat}



6-β-Furylacryloylamido-penicillanic acid



Nitrocefin

was reduced at least a millionfold in the mutant compared to wild type (Table I). Because the values of K_m for the mutant were so low ($\ll 1$ μM), it was not possible to determine their values due to limitations on the sensitivity of the assay. Similarly, it was not possible to accurately determine the value of k_{cat} for nitrocefin because the rate was so slow. Thus, only an upper limit could be measured. A greater decrease in the cephalosporinase activity was noted. Corroboration of the very low values of K_m was obtained in experiments measuring the concentration of acyl-enzyme with the mutant enzyme (see below); stoichiometric amounts of acyl-enzyme were produced with enzyme and substrate concentrations of the order of 1 μM. The decrease in k_{cat} is of the same order of magnitude as that seen with the replacement by alanine of serine or histidine from the active site of subtilisin (Carter & Wells, 1988) and clearly indicates a central role for Glu-166 in the chemistry of β-lactam catalysis.

Accumulation of the Acyl-enzyme. There have been several reports (Virden et al, 1990; Cartwright et al., 1989; Pratt et al., 1988) of the formation of an acyl-enzyme intermediate by class A β-lactamases, indicating that the catalytic mechanism may be represented by a two-step mechanism (eq 1), where EA represents the covalent acyl-enzyme intermediate.

$$E + S \overset{K_s}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P \qquad (1)$$

When the E166A mutant enzyme was added to a solution of nitrocefin at pH 7.0, 30 °C, an immediate increase in

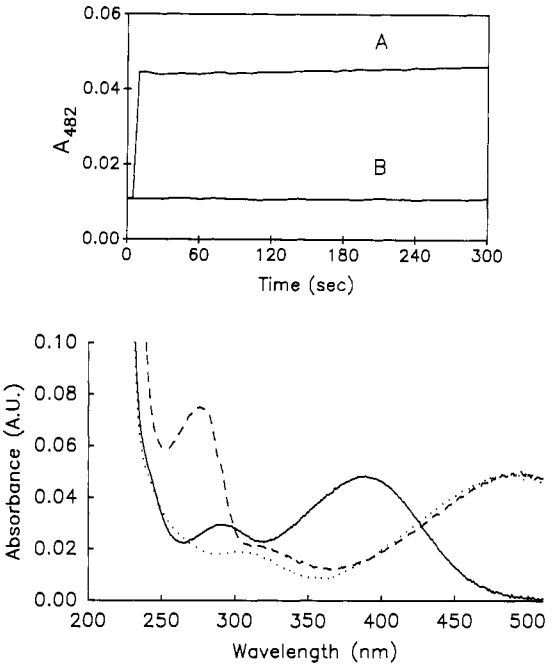


FIGURE 2: The reaction of E166A β-lactamase with nitrocefin under steady-state conditions. The upper panel shows the time-dependent changes monitored at 482 nm. Curve A shows the reaction of E166A with nitrocefin: the immediate increase of the signal at 482 nm is followed by slow turnover of substrate. The burst is equivalent to the formation of 2 μM product indicating that the reaction is stoichiometric (1:1). Curve B shows the rate of spontaneous hydrolysis of nitrocefin solution alone. A control in which E166A was added to the same buffer without nitrocefin gave a negligible change in signal. The lower panel shows the spectra of nitrocefin (—), the acyl-enzyme formed as in curve A (---), and hydrolyzed nitrocefin (···). Conditions: nitrocephin concentration 2.5 μM, temperature 1 °C, pH 7.0.

absorbance was observed at 482 nm. The intensity indicated a 1:1 stoichiometry between nitrocefin and the enzyme (assuming the same extinction coefficient for the acyl-enzyme as for the product cephalosporinoic acid) (Figure 2). This suggests that rapid formation of the acyl-enzyme occurred, followed by very slow turnover. The spectrum of the putative acyl-enzyme is also shown in Figure 2 and is very similar to that of hydrolyzed nitrocefin in the visible region. With FAP a burst was also seen corresponding to formation of the acyl-enzyme, with essentially a 1:1 stoichiometry assuming similar extinction coefficients for acyl-enzyme and product at 330 nm.

Corroboration that the acyl-enzyme intermediate accumulated rapidly was obtained in HPLC experiments. Analysis of mixtures of E166A β-lactamase and a small excess of substrate at pH 7.0 and 0 °C showed the substrate chromophore coeluting with the enzyme in a 1:1 stoichiometry (Figure 3). Similar behavior indicative of the accumulation of the acyl-enzyme was observed with both FAP and nitrocefin. Stopped-flow experiments were carried out in order to directly measure the rate of acylation of E166A β-lactamase with nitrocefin. These experiments indicated that the acylation

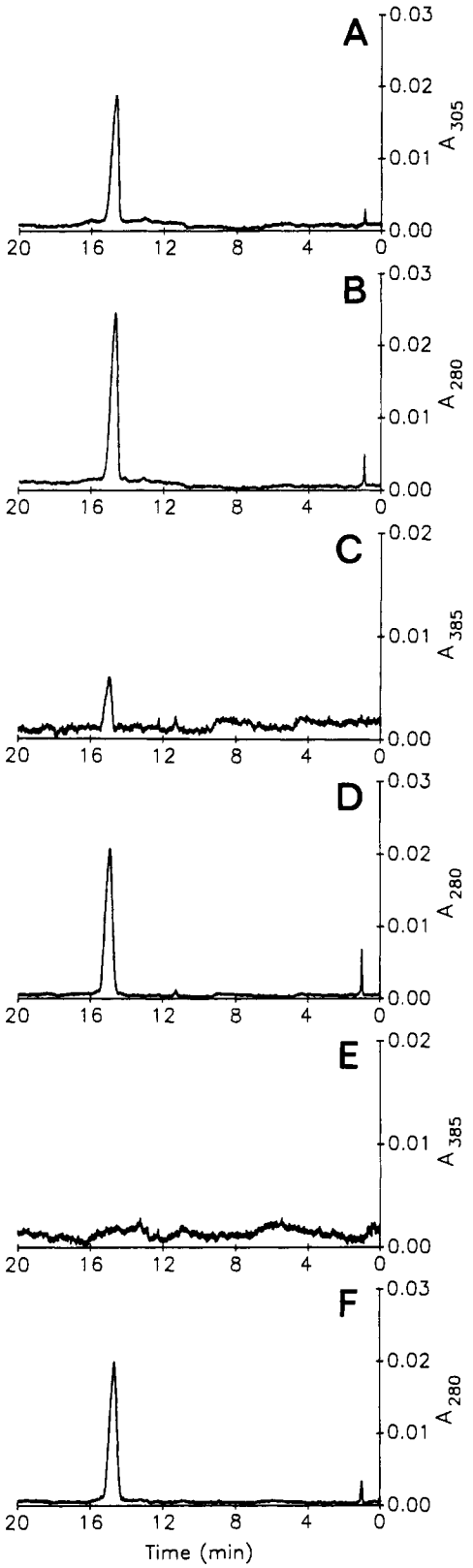


FIGURE 3: HPLC traces of ((furylacryloyl)amido)penicillin- and nitrocefin-labeled E166A β-lactamase. The FAP–E166A mixture was monitored at (A) 305 nm and (B) 280 nm to assay for the presence of FAP and E166A, respectively. Both traces show a single peak with coincident retention times of 14.7 min. Similar results were seen for the nitrocefin experiment monitored at (C) 385 nm (nitrocefin) and (D) 280 nm (E166A). The enzyme-only control is shown (E) at 385 nm and (F) at 280 nm.

reaction was essentially complete in the dead time of the apparatus, allowing estimation of a minimum first-order rate constant of 1000 s^{-1} for the acylation reaction at 20 °C, pH 7.0. Confirmation of the long-lived nature of the acyl-intermediate was obtained from experiments in which the stability of the acyl-enzyme was investigated by HPLC. Analysis by HPLC at several time points indicated the gel-filtration-purified acyl-enzymes underwent hydrolysis to release the penicilloic acid product on a time scale of days at 4 °C.

The accumulation of the acyl-enzyme in the present study indicates that deacylation is rate-limiting for E166A β-lactamase for both substrates. Thus, k_{cat} represents the value of k_3, the deacylation rate constant. The dramatic decrease in the rate of deacylation with the E166A mutant is consistent with Glu-166 acting as a general base catalyst to activate water in the deacylation step.

Values of k_2, the acylation rate constant, for good substrates of the class A β-lactamases are typically of the order of (1–6) × 10^3 s^{-1} (Martin & Waley, 1988). For rate-limiting deacylation

$$K_m = k_3K_s/(k_2 + k_3)$$

It has previously been shown for other class A β-lactamases with FAP as substrate (Virden et al., 1990; Cartwright et al., 1989) that the rate-limiting step is deacylation. We estimate that for the wild-type enzyme K_s is of the order of 10^{-4} M. The results from the stopped-flow experiments indicate that K_s is similar or larger for the mutant and that value of k_2 for the mutant must be similar to that of the wild type. Thus, there is relatively little effect of the mutation on the rate of acylation, and the substitution of Ala for Glu-166 leads to a very large differential effect on the rates of acylation and deacylation. This behavior by the E166A enzyme is in contrast to that of the corresponding aspartate mutant (E166D) in which the acylation rate appears to be comparable to that of deacylation, since no accumulation of the acyl-enzyme occurs (Gibson et al., 1990; W. Escobar and A. Fink, unpublished results). It is likely that the replacement of Glu-166 by aspartate leads to some of the catalytic function of Glu-166 in deacylation remaining (by positioning of the aspartate carboxylate either via a conformational change or via a bridging water molecule). For benzylpenicillin, both acylation and deacylation are reduced about 2000-fold with E166D (Gibson et al., 1990); it is possible that nitrocefin is an unusual substrate, due to its highly activated β-lactam bond, and for less activated substrates Glu-166 also participates as a catalytic group for acylation. Very recently, Adachi et al. (1991) have reported that mutants of the RTEM-1 β-lactamase in which Glu-166 is replaced by Asn, Gln, or Ala have drastically reduced deacylation rates and thus accumulate covalent acyl-enzyme intermediates with benzylpenicillin. They also observe that with E166D the rate of deacylation is significant, although much slower than for the wild type.

The possibility that the E166A mutation resulted in the substrates acting as type A substrates (Citri et al., 1976) and that the lack of deacylation resulted from substrate-induced deactivation was investigated as follows. We have previously shown that inactivation by type A substrates leads to conformational changes resulting in a stabilized acyl-enzyme which is more susceptible to proteolysis (Fink et al., 1987). Thus, the susceptibility of E166A, nafcillin-inactivated E166A (nafcillin is a type A substrate), and the acyl-enzymes of E166A with nitrocefin and FAP to proteolysis by chymotrypsin was studied. Only the nafcillin-inactivated enzyme showed enhanced proteolysis, indicating that the formation of the stable acyl-enzyme between E166A and nitrocefin and FAP was not due to their acting as type A substrates. This is in contrast to a recent report in which hydrolysis of cephalosporin C by the K73R mutant of *B. cereus* β-lactamase exhibited type

A behavior, although the corresponding E166D mutant showed normal behavior (Gibson et al., 1990).

Mechanistic Implications. The stoichiometric accumulation of an enzyme–substrate intermediate is a novel result. This stable acyl-enzyme offers an excellent opportunity for structural studies on what is normally a transient, and productive, catalytic intermediate. The data indicate that Glu-166 is an essential catalytic residue only in the deacylation step of the catalytic reaction and that its function in acylation appears to be minimal. This is in marked contrast to the serine proteases, which catalyze a similar reaction but for which acylation and deacylation involve essentially the same catalytic mechanism with His-57 functioning as a general base catalyst in both steps. In the E166A mutant it is likely that solvent catalysis (HO^-) is responsible for the observed very slow deacylation.

If Glu-166 is not involved in acylation the question arises as to whether there is another active site group which may act as a general base to activate the hydroxyl of Ser-70 for nucleophilic attack on the lactam carbonyl. Examination of the active site structure of *B. licheniformis* β-lactamase (Moews et al., 1990) reveals that in the free enzyme there are no side chains sufficiently close to Ser-70 to act in this fashion. Thus, either major conformational changes occur on binding of substrate to reposition a suitable group or the nucleophilic attack by Ser-70 is unassisted. Interestingly, Glu-166 would be the most likely candidate for the former proposal but is ruled out by the present results. However, there is evidence to suggest that general base-catalyzed assistance of the nucleophilic attack by Ser-70 on the substrate lactam carbonyl might not be necessary. For example, the reactivity of penicillin β-lactam rings is intrinsically greater than that of normal amides (Page, 1984, 1987). Structural studies, combined with model building of the substrate in the active site, suggest that the β-lactam carbonyl will be polarized by hydrogen bonding to two backbone amides (Ser-70, Thr-237), increasing the reactivity of the group to nucleophilic attack (Herzberg & Moult, 1987; Moews et al., 1990). In addition, the hydroxyl of Ser-70 will be activated by the effect of the helix dipole moment from helix a2 (Moews et al., 1990).

The present results support the proposal of Moult and Herzberg (1987) that Glu-166 is involved in activating water in deacylation and not in the acylation reaction. It is likely that the factors mentioned above are sufficient to permit the nucleophilic attack by serine in the acylation reaction. It has been suggested that Lys-73 is involved in facilitating proton transfer to the β-lactam nitrogen (Herzberg & Moult, 1987); however, it is also possible that the proton comes from a solvent molecule. The replacement of Glu-166 by Ala will also disrupt the salt bridge between Glu-166 and Lys-73. Thus, it is possible that the mutation may have indirect effects due to changes in the position and pK of the side chain of Lys-73. However, the present results role out such possibilities if Lys-73 acts as a catalyst in the acylation half of the reaction, and it is difficult to conceive reasonable roles for Lys-73 which involve only the deacylation step.

The pH-rate profile of β-lactamase catalysis shows an acidic pK in the vicinity of 5, which is also detected by titration/modification of the thiol group of the S70C mutant (Knap & Pratt, 1991). The most likely residue responsible for this pK is Glu-166. It is possible that Glu-166 plays a variable role in catalyzing acylation, depending on the nature of the substrate; nitrocefin, for example is known to be a highly activated β-lactam, and might require little assistance in formation of the acyl-enzyme. Less activated substrates may benefit from potential catalysis by Glu-166.

Comparison of the structures of β-lactamase from *B. licheniformis* and the penicillin-binding protein DD-peptidase from *Streptomyces* R61 reveals that the residue in the equivalent position to Glu-166 of the β-lactamase is Phe-164 (Knox & Kelly, 1989). Thus, a simple explanation why a stable acyl-enzyme is formed by the DD-peptidases and related penicillin-binding proteins involved in bacterial cell-wall synthesis is that the Phe-164 prevents the positioning and general base catalysis of the water molecule necessary for deacylation. Consequently it is the potential for catalysis by Glu-166 which leads to the breakdown of the acyl-enzyme in the β-lactamases. This is supported by the findings in the present study in which replacing Glu-166 by alanine prevents deacylation.

Acknowledgments

We thank Jim Wells and Eugenio Ferrari for the *Bacillus* strains used and generous help with transformation procedures.

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