Site-Directed Mutagenesis of Glutamate-166 in β-Lactamase Leads to a Branched Path Mechanism†

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ABSTRACT: Glutamate-166 of the *Bacillus licheniformis* β-lactamase was specifically mutated to aspartate and cysteine in order to probe the function of this residue in catalysis. In both cases, a large decrease in activity (k_{cat}/K_m was 3.5×10^{-5} smaller for E166C and 1×10^{-3} smaller for E166D than for the wild-type) was observed, although the kinetics for the two mutants were very different. The pH–rate profiles for E166D and E166C reflected the ionization characteristics of the new residue at site 166. This result indicates that the ionization of Glu-166 is responsible for the acidic limb of the k_{cat}/K_m–pH profiles, and suggests that the function of Glu-166 is that of a general base catalyst. The kinetics of the E166C mutant were investigated in detail. An initial burst was observed, whose amplitude was stoichiometric with the enzyme concentration, suggesting rate-limiting deacylation of the acyl-enzyme intermediate. However, further study revealed that in the presence of 0.5 M sodium sulfate, which stabilizes the native conformational state, the magnitude of the burst corresponded to 2 equiv of enzyme. This observation, in conjunction with the limited effect of the mutation on K_m, indicated that the mutation resulted in a change in the kinetic mechanism from the linear, acyl-enzyme pathway to one with a branch leading to an inactive form of the acyl-enzyme. This change in mechanism is attributed to a substantial decrease in the rate of hydrolysis of the normal acyl-enzyme. On the basis of a variety of observations, we propose that the branched pathway is characteristic of β-lactamase catalysis when the deacylation rate is slow. The results indicate that both acylation and deacylation are decreased by several orders of magnitude in the E166C mutant, indicating that Glu-166 acts as a general base catalyst in both formation and hydrolysis of the acyl-enzyme intermediate.

β-Lactamases are a clinically important group of enzymes since their hydrolysis of β-lactam antibiotics is the major source of bacterial resistance to penicillins and cephalosporins. Moreover, since the genes encoding β-lactamases are often located on plasmids, this resistance phenotype is readily passed from one bacterial cell to another (Matthew & Hodges, 1976). This is evidenced by trends to resistance in several pathogenic microbes such as *Gonococci* and *Staphylococci* (Neu, 1992; Aral & Holmes, 1991; Abraham, 1983). In order to counteract this trend, attempts to determine the molecular interactions which occur between β-lactamases and their chemotherapeutic substrates are currently underway. It is hoped that an understanding of the β-lactamase mechanism will result in the rational design of β-lactam antibiotic derivatives which will be unaffected by β-lactamases. Although β-lactamases are known to be a heterogeneous group of enzymes, several classes have been identified which share a high degree of homology and contain similar catalytic machinery. The present study concerns the class A β-lactamase (Ambler, 1980; Pollock, 1965; Joris et al., 1988) from *Bacillus licheniformis*.

As a first approximation to describing the mechanism for these enzymes, an attempt has been made to compare β-lactamases to other well-studied systems. For example, several lines of evidence indicated that the catalytic reaction of these enzymes was similar to that of serine proteases. The active-site serine residue [Ser-70 in the numbering system of Ambler (1980)] is involved in nucleophilic attack on the β-lactam amide bond, leading to the formation of an acyl-enzyme intermediate. If β-lactamases do have a reaction pathway analogous to that of the serine proteases, then one would also expect to find the catalytic triad characteristic of these proteases. Herzberg and Moult (1987) compared the structure of the catalytic site for these two classes of enzymes by a least-squares fit algorithm. They found that although a lysine was approximately positioned to substitute for histidine of the triad there was no appropriately placed carboxyl group to take the role of aspartate. This raised the question of how serine was activated for attack on the carbonyl carbon of the lactam ring, and the related question of how water was activated to attack the acyl-intermediate in the process of deacylation. From the active-site structure, two likely candidates for increasing the nucleophilic reactivity of Ser-70 are lysine-73 and glutamate-166. There is considerable controversy regarding the possible role of these residues (Fink, 1993).

Glutamate-166 has been implicated in the mechanism of class A β-lactamases. It is highly conserved and is found in several penicillin binding proteins outside of the class A family. X-ray crystallography (Herzberg & Moult, 1987; Knox & Moews, 1991; Moews et al., 1990; Strynadka et al., 1992) has demonstrated that the carboxyl group of this residue is located within the catalytic site close to Ser-70 (3.5 Å) and Lys-73 (3.2 Å). Furthermore, site-directed mutagenesis studies (Madgwick & Waley, 1987; Gibson et al., 1990; Adachi et al., 1991; Escobar et al., 1991, Delaire et al., 1991) have directly implicated this group in the mechanism since specific mutations at site 166 resulted in a large decrease in activity.

Could Glu-166 be participating as a general base in the reaction? The possibility that this residue is responsible for the acidic limb of the pH profile of these enzymes would support this hypothesis. To act as a general base, the enzyme would become active as the carboxyl group ionized with increasing pH. Work by Cartwright et al. (1989) indicates this may be the case. It was noted that the acidic pK_a of the

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pH profile for β-lactamase was insensitive to changes in temperature. This is characteristic of groups displaying small heats of ionization such as the carboxyl group. We have previously shown that conversion of Glu-166 to Ala leads to more than a millionfold decrease in catalytic activity: however, this mutant enzyme is so inactive it was not technically feasible to determine its pH-dependence. To further explore its role in the catalytic mechanism, we have specifically mutated Glu-166 to Cys and Asp. The E166D mutant would be expected to have a pH–rate profile analogous to that of the wild-type since the pK_a values of glutamate (4.1) and aspartate (3.9) are similar. It has been suggested that the Ω loop which contains Glu-166 may be relatively mobile (Herzberg & Moult, 1987). If this site is mobile, then one might expect that shifting the carboxyl group of Glu-166 by one methylene group, ~1.5 Å, as in the E166D mutant, should not appreciably alter catalysis. However, if Glu-166 is responsible for the acidic limb of the pH profile, then replacing Glu-166 with Cys should dramatically alter the pH profile. That is, we would expect the acidic pK_a to reflect the ionization properties of the incorporated cysteine residue with an anticipated pK in the vicinity of 9.

An interesting aspect of catalysis by class A β-lactamases is that they exhibit a phenomenon of reversible inactivation with certain substrates [known as type A substrates (Citri et al., 1976)]. This has been shown to be due to a branched pathway in which the initially-formed acyl-enzyme undergoes a conformational change into an inactive form (Kiener et al., 1980; Frère, 1981; Persaud et al., 1986; Fink et al., 1987). This inactivation is normally only seen with relatively slowly hydrolyzed substrates at very high concentration.

Here we show that mutations at Glu-166 not only reveal the role of this residue as a general base in acylation and deacylation but also that the mutations lead to a branched pathway, where partitioning to the inactive acyl-enzyme is strongly favored.

EXPERIMENTAL PROCEDURES

Materials. Benzylpenicillin and phenoxymethylpenicillin was purchased from Sigma. Nitrocefin was a gift from Glaxo. Sequenase used for sequencing mutants was purchased from U.S. Biochemicals. Restriction enzymes, Klenow fragment, and ligase used for mutagenesis were obtained from New England Biolabs.

Mutagenesis. E166C and E166D mutant β-lactamases where made as described in Escobar et al. (1991). Mutations were introduced via the Kunkle method (Kunkle, 1985) and sequenced with Sequenase (U.S. Biochemicals). The primers used for mutagenesis were 5′-GAA-CGA-TTC-(XXX)-CCA-GAG-CTC-AAT-GAA-GTG-3′ where (XXX) was (TGT) for the cysteine mutant and (GAC) for the aspartate mutant. These primers were designed to introduce a *Sac*I site (underlined) to allow for restriction purification (Wells et al., 1986).

Purification Procedure. The wild-type and mutant enzymes were purified as follows. *Bacillus licheniformis* β-lactamase (wild-type, E166C, and E166D) was expressed and purified from *Bacillus subtilis*. Fresh streaks were inoculated into 6 L of rich media (Ellerby et al., 1990) and grown on a 37 °C shaker for 12–14 h. Cells were removed by centrifugation (8000 rpm for 30 min), and the cell-free supernatant was dialyzed against 0.02 M sodium acetate, pH 4.8 (exchanged twice in 30 L of buffer). CM-Sepharose matrix (CL-6B from Sigma) was preequilibrated with the same buffer. The protein was equilibrated with CM-Sepharose matrix (15 mL of gel) for 1 h by gently swirling on a Shaker at room temperature. This enzyme–matrix mixture was batch-loaded onto a CM-Sepharose column (50 mL of gel in a 2.5 × 19.5 cm column) and eluted with a linear salt gradient of 0–0.25 M NaCl. Fractions containing β-lactamase activity were pooled and exchanged via ultrafiltration with an Amicon YM10 filter into 0.02 M Tris, pH 7.2, buffer. This was equilibrated with DEAE-Sepharose (0.02 M Tris, pH 7.2, for 1 h; 10 mL of gel), batch-loaded onto a DEAE-Sepharose column (same size as above), and eluted with a linear salt gradient of 0–0.25 M NaCl. Fractions containing β-lactamase activity were pooled and exchanged into 0.05 M potassium phosphate, pH 7.0, buffer by ultrafiltration with a YM10 filter. All proteins were purified to homogeneity as determined by SDS-PAGE using the Pharmacia PhastSystem.

Kinetics. The pH–rate profiles for E166C were determined by initial velocities using Hanes–Woolf analysis (Segal, 1975). Above pH 9, the relatively high rate of spontaneous hydrolysis and exceedingly low K_m for nitrocefin (whose structure is shown below) permitted only the determination of k_{cat}. The nitrocefin–wild-type and the phenoxymethyl–E166D pH profiles were obtained from complete progress curves and analyzed by the method of Koerber and Fink (1987). All points were determined in triplicate. Substrate hydrolysis was measured at 30 °C with a Perkin-Elmer 320 spectrophotometer using a 1-cm path-length cell. All buffers contained 0.5 M KCl and were brought to a final concentration of 50 mM buffer. The buffers used for the pH profiles were sodium acetate for pH 4.0–5.5, potassium phosphate for pH 6.0–7.5, Tris-HCl for pH 8.0–8.5, CHES for pH 9.0–9.5, and CAPS for pH 10.0–11.0.

Burst Kinetics with E166C. Nitrocefin (45–120 μM final concentration) in 50 mM potassium phosphate/0.5 M KCl at pH 7.0 was preequilibrated to 18.5 °C. E166C was added to a final concentration of 3–10 μM. The reaction was monitored at 482 nm with a Perkin-Elmer Model 320 spectrophotometer. Experiments in the presence of sodium sulfate or urea were done with the appropriate concentration of cosolute in the buffer. Stopped-flow kinetic experiments utilized an Applied Photophysics instrument, Model DX17mV, with a mixing ratio of 1:1. The final enzyme concentration was 7.0 μM. Nitrocefin concentrations were 20–150 μM. Branched pathway kinetics parameters were determined from fitting the data as described by Waley (1991).

Circular Dichroism Measurements of the Mutant Proteins. The far-UV circular dichroism (CD)[1] spectra of the mutant proteins were measured at pH 4.0, 7.0, and 9.0 using an AVIV Model 60DS instrument at 30 °C. Protein concentrations (final) were 9.1 μM for the E166D mutant and 8.7 μM for the E166C mutant. The cell path length was 0.1 cm. The buffers used were 50 mM potassium phosphate for pH 7.0, 50 mM sodium acetate for pH 4.0, and 50 mM CHES for pH 9.0. All these buffers contained 0.5 M KCl. The far-UV CD spectrum of wild-type was also obtained at pH 7.0, and the final concentration was 8.2 μM.

Thermal Stability Measurements. A Perkin-Elmer MPF-4 spectrofluorometer was used to measure the thermal unfolding transitions of the mutant proteins by tryptophan fluorescence. The excitation and emission wavelengths used were 280 and 330 nm, respectively. Proteins were brought to a final concentration of 0.12 μM (E166C) and 0.43 μM (E166D) in the following buffers: 50 mM sodium acetate/0.5 M KCl, pH 4.0; 50 mM potassium phosphate/0.5 M KCl, pH 7.0,

[1] Abbreviation: CD, circular dichroism.

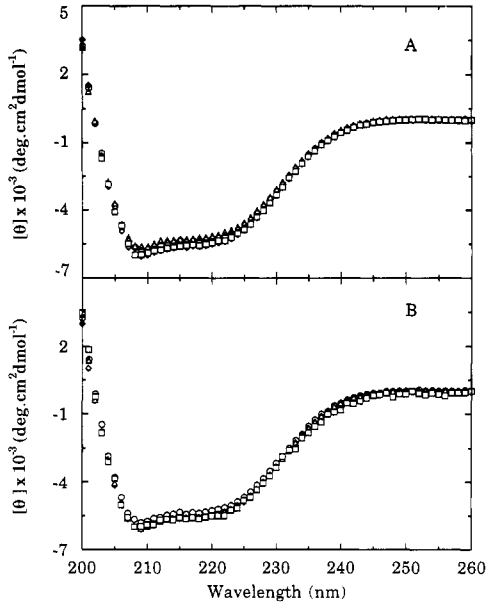


FIGURE 1: Comparison of the structures of wild-type and the E166C and E166D β-lactamase mutants as a function of pH by far-UV CD spectra. Panel A shows the spectra for E166C, and panel B those for E166D. The symbols are as follows: circles, native wild-type at pH 7.0; diamonds, pH 4.0, E166C or E166D; triangles, pH 7.0, E166C or E166D; squares, pH 9.0, E166C or E166D.

Table 1: Thermal Denaturation Midpoints (T_m) for Wild-Type, E166C, and E166D β-Lactamases

	T_m (°C)		
pH	wild-type	E116C	E166D
4.0	52.2 ± 1	46.4 ± 1	43 and 60 ± 1[a]
7.0	65.0 ± 1	64.0 ± 1	60.0 ± 1
9.0	56.5 ± 1	60.2 ± 1	55.6 ± 1

[a] Biphasic transition.

and 50 mM CHES/0.5 M KCl, pH 9.0. The fluorescence cell (1-cm path length) was stirred throughout the experiment to maintain a uniform temperature which was increased linearly from 15 to 85 °C at a rate of 0.4 °C/min. Linear extrapolations of the native and denatured curves were used to determine the fraction of unfolded protein as a function of temperature.

RESULTS AND DISCUSSION

Structure of the Mutant Proteins. Circular dichroism studies as well as thermal stability measurements indicate that removal of the carboxyl group at site 166, as in the E166A enzyme, does not significantly perturb the secondary or tertiary structure of this enzyme (Escobar et al., 1991). This is supported by the high-resolution crystallographic map obtained for E166A which deviates by 0.25 Å (rms) in backbone atoms from the wild-type structure (Knox et al., 1993). Similarly, mutation of Glu-166 to cysteine or aspartate has minimal effects on secondary structure as evidence by their CD spectra (see Figure 1). CD spectra for the mutants at pH 4, 7, and 9 (Figure 1A,B) were all superimposable on that of the native wild-type spectrum (pH 7.0) within experimental error.

Thermal denaturation of the mutant proteins was also carried out to measure their relative stability. The results are given in Table 1. At pH 7.0, the E166C mutant had the same thermostability as the wild-type, but E166D was somewhat less stable. At pH 9.0, the E166D mutant was as stable as the wild-type, and the E166C mutant was more stable. At pH 4.0, both mutants were less than the wild-type enzyme; the E166D mutant showed a markedly biphasic unfolding transition.

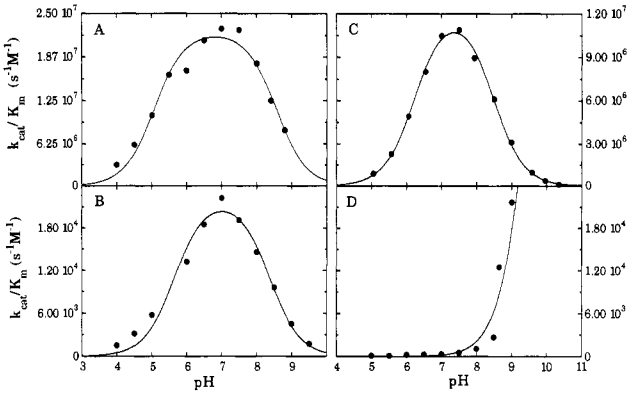


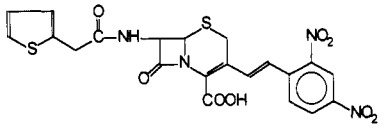
FIGURE 2: k_{cat}/K_m vs pH profiles for the mutants and wild-type β-lactamase. Panel A, wild-type with phenoxymethylpenicillin; panel B, E166D with phenoxymethylpenicillin; panel C, wild-type with nitrocefin; panel D, E166C with nitrocefin. All reactions monitored at 30 °C. The corresponding pK's are listed in Table 2.

Table 2: Ionization Constants (pK_a) for Wild-Type, E166C, and E166D β-Lactamases

	substrate: nitrocefin				substrate: phenoxymethylpenicillin			
	wild-type		E166C		wild-type		E166D	
	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}
pK_{a1}	6.2	5.3	9.7	ND	5.0	4.5	5.7	4.4
pK_{a2}	8.5	7.5	ND[a]	ND	8.6	8.1	8.4	8.4

[a] Not determined.

Wild-Type Kinetics. The pH–rate profile for the wild-type enzyme was determined with phenoxymethylpenicillin and nitrocefin (whose structure is shown below). These are both good substrates for this enzyme with turnover numbers in the thousands per second for the wild-type (Ellerby et al., 1990).



NITROCEFIN

Nitrocefin was chosen because of the large Δε associated with the hydrolysis of this cephalosporin. This is ideal for studying the kinetics of mutants displaying very low catalytic activity, such as E166C. Phenoxymethylpenicillin is relatively stable in acidic pH and is thus suitable for studies at low pH values. As can be seen in Figure 2, the k_{cat}/K_m–pH profiles for both of these substrates with the wild-type enzyme have the characteristic bell-shaped curve seen with many enzyme systems. Values from these curves were fit to eq 1in order to

$$k_{obs} = k_{lim}/(1 + [H^+]/K_1 + K_2/[H^+]) \quad (1)$$

determine K_1 and K_2, the ionization constants corresponding to the acidic and basic limbs, respectively. The pK_a's measured for the wild-type and mutant enzymes are given in Table 2. The values of pK_1 and pK_2 for k_{cat}/K_m correspond to the pK's of the key ionizing groups in the *free* enzyme. The unusual dependence of k_{cat}/K_m on pH for E166C is discussed in detail

Table 3: Kinetic Constants for Wild-Type, E166C, and E166D β-Lactamases at pH 7.0, 30 °C

	substrate: nitrocefin			substrate: phenoxymethylpenicillin		
	wild-type	E166C	k_{mut}/k_{wt}[a]	wild-type	E166D	k_{mut}/k_{wt}[a]
k_{cat} (s^{-1})	1685	1.41×10^{-2}	8.4×10^{-6}	2.33×10^{3}	1.35	5.8×10^{-4}
K_m (M)	1.61×10^{-4}	3.83×10^{-5}	0.24	1.0×10^{-4}	6.1×10^{-5}	0.61
k_{cat}/K_m (M^{-1} s^{-1})	1.05×10^{7}	3.68×10^{2}	3.5×10^{-5}	2.3×10^{7}	2.23×10^{4}	9.7×10^{-4}

[a] Ratio of mutant/wild-type value.

Scheme 1

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P$$

below. Interestingly, pK_1 for the wild-type enzyme with nitrocefin is higher than that observed with most substrates.

The reaction catalyzed by class A β-lactamases has been shown to proceed through a covalent acyl-intermediate. Thus, as with other acyl-transfer systems, the reaction can be described by a two-step mechanism (Scheme 1), where EA represents the acyl-enzyme and k_2 and k_3 the acylation and deacylation rate constants, respectively. During acylation, Ser-70 attacks the carbonyl carbon of the lactam ring to form a covalent penicilloyl–enzyme intermediate. Water then attacks the ester bond of the intermediate and releases the hydrolyzed product in the deacylation step.

E166D Kinetics. The effect of mutating Glu-166 to Asp on the kinetic parameters over the pH range 4.0–9.5 was examined using phenoxymethylpenicillin. The K_m values were similar to those for the wild-type (Table 3) in the pH range studied (Figure 3). The k_{cat} values for E166D were significantly lower (at least 1500-fold lower, see Table 3) than those of the wild-type in the observed pH range. However, the shape of the k_{cat} vs pH profile was very close to the one obtained for the wild-type (Figure 4). The pK_1 and pK_2 for E166D from the k_{cat}–pH profile were comparable to the values for the wild-type enzyme with this substrate (Table 2). The plot of k_{cat}/K_m vs pH (Figure 2) reflects the pK value(s) of the essential residue(s) of the free enzyme involved in catalysis. The k_{cat}/K_m values were approximately 1000-fold lower than those obtained for the wild-type (Table 3). The pK_1 value obtained from the acidic limb of Figure 2 is 5.7, which is 0.7 pH unit higher than than the wild-type value. The basic limb, pK_2 = 8.4, approximates the wild-type value of 8.6. Decreased values of k_{cat} and k_{cat}/K_m indicate the catalytic efficiency of E166D has been disrupted. Figure 4 (k_{cat} vs pH) and Figure 2 (k_{cat}/K_m vs pH) demonstrate that it is predominantly the acidic limb which is affected by the mutation. The higher pK_1 values for E166D reflect the environment in which the carboxyl group residues. Since the side chain of Asp is one methylene group shorter than that of Glu, it is likely the carboxyl group finds itself in a region of decreased polarity which would increase the pK_a.

The 1500-fold decrease in k_{cat} is attributable, in part, to the increased distance between the Asp-166 carboxylate and the water molecule involved in deacylation (assuming k_3 becomes rate-limiting in the mutant, which is likely since a stoichiometric burst of product is observed).[2] In addition, it is likely that the substitution of Asp for Glu-166 leads to a rearrangement in the position of the key water molecule involved in deacylation, possibly even to the presence of an additional water to fill the space created by the missing methylene. The fact that K_m is not significantly affected by the Glu to Asp substitution implies that both k_2 and k_3 are decreased to similar extents, if K_m remains unaffected by the mutation. The conservative nature of this mutation should not significantly alter the electrostatic potential of the catalytic site, and therefore E166D should maintain the binding properties which are dependent on this potential (Ellerby et al., 1990).

[2] Even if a branched pathway is involved (see Scheme 2), it is a consequence of the low value of k_3).

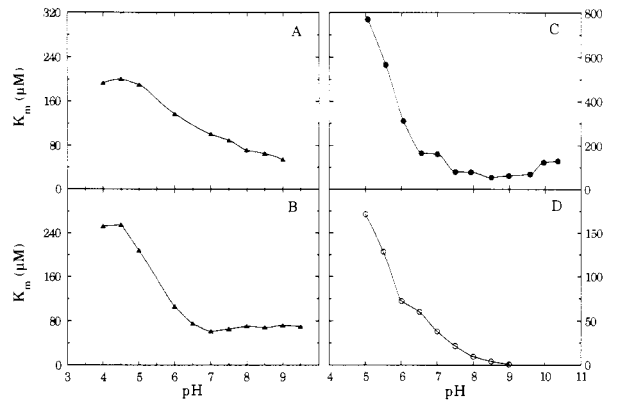


FIGURE 3: K_m–pH profiles for the mutants and wild-type β-lactamase. Panel A, wild-type with phenoxymethylpenicillin; panel B, E166D with phenoxymethylpenicillin; panel C, wild-type with nitrocefin; panel D, E166C with nitrocefin. All reactions monitored at 30 °C.

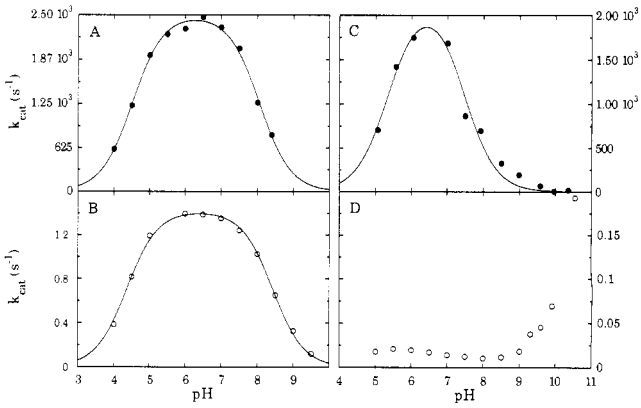


FIGURE 4: k_{cat}–pH profiles for the mutants and wild-type β-lactamase. Panel A, wild-type with phenoxymethylpenicillin; panel B, E166D with phenoxymethylpenicillin; panel C, wild-type with nitrocefin; panel D, E166C with nitrocefin. All reactions monitored at 30 °C. The E166C data (panel D) were not fit to a standard ionization curve since the shape of the profile was not appropriate for this type of analysis.

The 1000-fold decrease in k_{cat}/K_m is similar to that observed by the substitution of Glu-43 by Asp in staphylococcal nuclease, a catalytic system in which the glutamate acts as a general base, directly activating a water molecule for attack on the phosphodiester (Hibler et al., 1987). The effects of k_{cat} and k_{cat}/K_m are considerably larger than those observed by Gibson et al. (1990) for the substitution of Glu-166 by Asp in the class A β-lactamase from *B. cereus* at pH 7. The implications for the catalytic mechanism are discussed below.

E166C Kinetics. In contrast to the bell-shaped pH profiles for the wild-type and E166D β-lactamases, the k_{cat}/K_m vs pH and k_{cat} vs pH profiles of the E166C mutant are very different

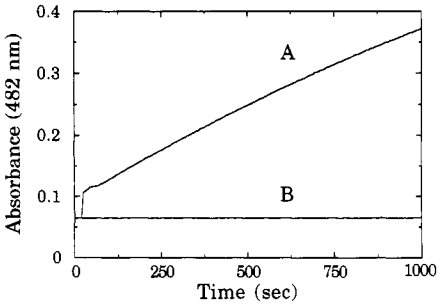


FIGURE 5: Burst seen upon incubation of E166C with nitrocefin. The reaction was monitored at 482 nm and 30 °C. The concentrations of E166C and nitrocefin were 2.41 and 45 μM, respectively. The initial rapid increase in absorbance (curve A) corresponds to a stoichiometric production of acyl-enzyme (2.41 μM), indicating that acylation is rapid and that it is followed by a much slower deacylation step. Line B represents the reaction in the absence of enzyme.

(Figures 2 and 4). For the E166C mutant, the k_{cat}/K_m profile shows a dramatic increase above pH 8. This increase could reflect the ionization of cysteine-166, or it could reflect enzyme-assisted hydroxide catalysis. If we fit the E166C k_{cat}/K_m–pH profile with eq 2, we find there is an ionization constant of pK_a

$$k_{obs} = k_{lim}/(1 + [H^+]/K_1) \quad (2)$$

9.7 (for k_{lim} of 1×10^5) for the free enzyme (Figure 2). This pK_a value correlates well with that seen for thiols of polar aliphatic groups (Friedman, 1973), and is consistent with the ionization of Cys-166. Attempts to directly determine the ionization constant of Cys-166 were unsuccessful due to technical problems.

At neutral pH, the value of k_{cat} for E166C is 10^{-5} that of the wild-type. Values of k_{cat} are very low at all pH values below pH 9 and appear to have a slight maximum around pH 5.5. The value of k_{cat} begins to increase dramatically above pH 9.0, reflecting either thiolate or hydroxide catalysis.

To investigate the possibility of hydroxide ion-assisted catalysis, we examined the activity of the enzyme as a function of hydroxide ion concentration. Technical difficulties prevented acquisition of data above pH 10.5. Although there is a linear dependence on the hydroxide ion concentration above pH 8.5, the slope is 0.6, not 1, as would be expected if hydroxide ion were responsible for the catalysis. It is possible that enzyme-mediated hydroxide catalysis would have such a dependence on hydroxide concentration due to the participation of enzyme groups in facilitating the hydroxide catalysis.

On the basis of the available data, it is not possible to unambiguously assign the increase in catalytic activity observed at high pH to thiol or hydroxide catalysis, although it is more consistent with catalysis by the latter. If Cys-166 does participate in catalysis, its shorter side chain means that additional water molecules acting as a bridge would be necessary to intervene between the thiol anion and the water molecule involved in deacylation.

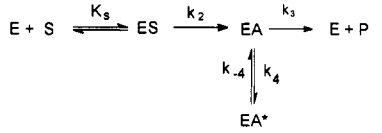
Burst Experiments and the Branched Pathway Mechanism. The major reduction in k_{cat} for both mutations suggested that the deacylation rate constant might be correspondingly reduced and that the acyl-enzyme intermediate might accumulate and be detectable in burst experiments. As shown in Figure 5, when nitrocefin was reacted with micromolar concentrations of E166C a burst was indeed observed prior to the steady-state turnover reaction. Assuming that the extinction coefficient for the acyl-enzyme is similar to that for the product penicilloic acid, the calculated stoichiometry for the observed burst with nitrocefin was 1:1 (Table 4). Similar data were

Table 4: Magnitude of Nitrocefin Burst as a Function of Solvent for E166C[a]

conditions	burst amplitude[b]
pH 7.0	1.0 ± 0.1
pH 7.0, 0.5 M sodium sulfate	2.0 ± 0.1
pH 7.0, 2 M urea	1.2 ± 0.1

[a] The reaction was monitored at 482 nm. [b] Burst magnitude is given as the ratio of the burst at time zero to the enzyme concentration, assuming the Δε for the burst is the same as for the hydrolysis of nitrocefin.

Scheme 2



obtained for the E166D mutant (not shown). In terms of the kinetic pathway shown in Scheme 1, this burst indicates a relatively rapid acylation rate (complete within the dead time of manual mixing) followed by a slower deacylation process. However, this interpretation suffers from some apparent inconsistencies in the steady-state kinetic parameters: for example, if the low value of k_{cat} reflects k_3, the deacylation rate constant, then the value of K_m should be correspondingly decreased, but K_m is only decreased 4-fold in E166C at pH 7 [at high pH (Figure 3), K_m does become very small].

Thus, we considered whether the burst, in spite of the 1:1 stoichiometry, might reflect a branched pathway (Scheme 2). It is well-known that wild-type class A β-lactamases react with certain substrates by such a mechanism (Citri et al., 1976; Kiener et al., 1980; Frère, 1981; Persaud et al., 1986; Fink et al., 1987), and it has been reported that the K73R mutant of *B. cereus* β-lactamase reacts with cephalosporin C in such a manner (Gibson et al., 1990). In all previous reports of this type of behavior, one of the hallmarks has been the fact that the partitioning of the acyl-enzyme was very much in favor of the *product*, rather than the inactive EA* species, reflected in a burst with a magnitude corresponding to many times that of the enzyme concentration (i.e., $k_3 \gg k_4$). If the bursts observed in the reaction of E166C or E166D with nitrocefin are due to a branched pathway, the magnitude of the burst, corresponding stoichiometrically to the enzyme concentration, indicates that the partitioning favors the inactive acyl-enzyme species (i.e., $k_3 \leq k_4$).

If the burst were due to a branched pathway, previous studies have indicated that the inactive EA* species is conformationally different from the native enzyme (Pain & Virden, 1979; Kiener & Waley 1980; Persaud et al., 1986; Fink et al., 1987); thus, it is possible that the presence of stabilizing agents might hinder the interconversion from EA to EA*, and thus decrease the partitioning to EA*. If this were so, then a larger burst would be expected in the presence of a kosmotrope such as sodium sulfate. This was indeed observed: in the presence of 500 mM sodium sulfate, the size of the burst doubled (Table 4). Interestingly, the presence of urea had little effect. Previous studies have shown that the EA* form is more sensitive to denaturant.

Combined steady-state and pre-steady-state kinetic data for the reaction of E166C β-lactamase with nitrocefin were analyzed in accord with the detailed kinetic analyses for branched path schemes reported by Waley (1991). A typical stopped-flow trace is shown in Figure 6A. The rapidity of the inactivation reaction means that there are insufficient data to provide meaningful initial phase parameters. The expressions for the steady-state parameters, plus those for the magnitude

Table 5: Steady-State Kinetic Parameters for Linear and Branched Path Mechanisms (Schemes 1 and 2, Respectively)

	linear pathway	branched pathway
burst amplitude	$[k_2/(k_2 + k_3)]^2E_0/[1 + k_3K_s/S(k_2 + k_3)]^2$	$\{k_2k_3k_4E_0S[S(k_2 - k_{-4}) - k_{-4}K_s]\}/\{S[k_{-4}(k_2 + k_3) + k_2k_4] + k_3k_{-4}K_s\}^2$
burst rate constant	$\{S(k_2 + k_3) + k_3K_s\}/(S + K_s)$	$\{S[k_{-4}(k_2 + k_3) + k_2k_4] + k_3k_{-4}K_s\}/\{S(k_2 + k_3 + k_4) + K_s(k_3 + k_{-4})\}$
k_{cat}	$k_2k_3/(k_2 + k_3)$	$k_2k_3k_{-4}/\{k_{-4}(k_2 + k_3) + k_2k_4\}$
K_m	$k_3K_s/(k_2 + k_3)$	$k_3k_{-4}K_s/\{k_{-4}(k_2 + k_3) + k_2k_4\}$
k_{cat}/K_m	k_2/K_s	k_2/K_s

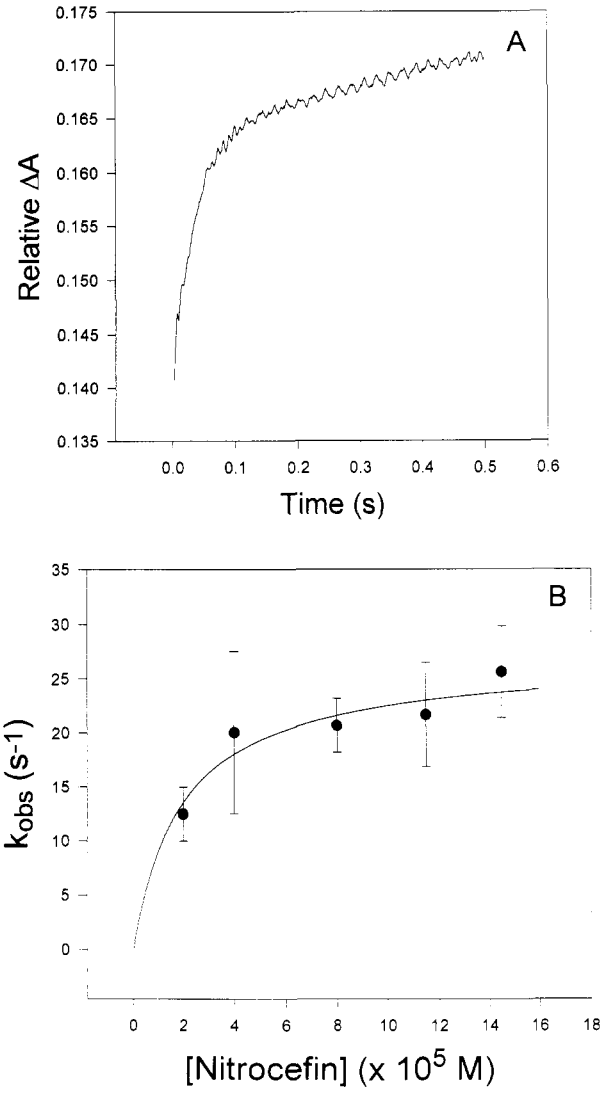


FIGURE 6: Stopped-flow kinetic studies of E166C β-lactamase with nitrocephin. Panel A: Stopped-flow trace of the reaction of 80 μM nitrocefin with 7μM enzyme, pH 7.0, monitored at 482 nm. The data were fit to the equation $P = V_s t - (V_s - V_i)(1 - e^{-kt})/k$, to yield V_i = 2.0 mM s^{-1}, V_s = 0.015 mM s^{-1}, and k = 20 s^{-1} (Waley, 1991). Panel B: The burst rate constant, k, as a function of nitrocefin concentration. Conditions as for panel A. The error bars represent the experimental error for the average of 4–6 independent experiments. The curve was drawn according to the kinetic expression given in Table 5.

and rate of the burst for the branched path shown in Scheme 2, are given in Table 5, along with the corresponding expressions for the nonbranched path (Scheme 1). The fact that the observed burst amplitude is essentially stoichiometric with enzyme concentration means that $k_3 < k_4$. The rate of the burst as a function of substrate concentration was measured in an attempt to obtain values for individual rate constants (Figure 6B). Over the accessible concentration rate (20–150 μM; limited by substrate solubility), the rate showed a small increase. This is consistent with K_s being of the order of $\leq$1 mM. Since there is no evidence that residue 166 is directly

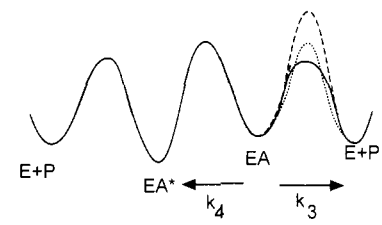


FIGURE 7: Schematic diagram of the free energy barriers for the interconversion of EA and EA* (Scheme 2). The solid line is the case for the wild-type enzyme and good substrates (type S; Citri et al., 1976). The dotted line represents the case for the wild-type enzyme with type A substrates. The dashed line represents the E166 mutants with all substrates. The figure shows a constant height for the energy barrier between EA and EA*, although this may also change.

involved in binding of the substrate, it is reasonable to assume that K_s is similar for the mutant and wild-type enzymes. Thus, the decrease in k_{cat}/K_m of 3.5 × 10^{-5} in the mutant suggests that k_2 has also decreased by a comparable amount in the mutant. Using a value of 5 × 10^3 s^{-1} as an upper limit for the wild-type k_2, this means a mutant value in the vicinity of 2 × 10^{-1} s^{-1}. If $k_3 \approx k_4$, the value for k_{cat} reduces to that of k_{-4}, i.e., k_{-4} = 1.4 × 10^{-2} s^{-1}. It was not possible to directly estimate values for k_3 and k_4, but it is likely that they are similar to k_2. Since most of the enzyme is in the form of the EA* species, the k_4/k_{-4} ratio must be of the order of 10^2, leading to an estimate of around 1 s^{-1} for k_3 and k_4.

Since this analysis indicates that both k_2 and $k3$ are decreased by 3 orders of magnitude or more in the mutant, this means that Glu-166 is involved in both the acylation and deacylation reactions. It is likely that a branched pathway also operates for the E166A mutant, which also shows a burst with a 1:1 stoichiometry (Escobar et al., 1991).

We believe that the determining factor for the change in kinetic mechanism, from a linear to a branched path, is the reduced deacylation rate, k_3. The concentration of EA*, the inactive form of the acyl-enzyme, is proportional to k_4/k_3; if this ratio is very low, then there will be no significant buildup of the inactive form. EA* is an alternate conformation of the acyl-enzyme which appears to be of comparable, or even greater, stability than the "normal" acyl-enzyme, EA. Thus, as shown in Figure 7, if a mutation in the enzyme leads to a slow deacylation rate, k_3, corresponding to a high free energy barrier to products, the alternate path to EA* becomes competitive. In the case of E166C, the height of the energy barrier separating EA from products is higher than that of EA*. In the case of the wild-type enzyme with type A (Citri et al., 1976) substrates, a similar situation must exist, with the exception that the partitioning to EA* is much less favorable, indicating that the energy barriers are similar, as shown in Figure 7. It is not clear whether the reactivation reaction involves the reverse pathway, as shown in Scheme 2 (k_{-4}), or whether EA* undergoes very slow deacylation to yield the products directly, although preliminary experiments with wild-type enzyme and a type A substrate, in which the rate of disappearance of EA* and the rate of appearance of the penicilloic acid were measured independently (A. K. Tan

and A. L. Fink, unpublished results), are most consistent with Scheme 2.

Thus, it seems likely that any class A β-lactamase/substrate combination in which the rate of deacylation is relatively slow, due either to mutation or to substrate structure, will exhibit the branched kinetic pathway of Scheme 2. β-Lactamase seems to be unique in having an alternate conformation for a catalytic intermediate which is inactive, and separated by a barrier of similar free energy to that of the products. What is particularly unusual in the present case is that the k_4/k_3 ratio is very large compared to previously reported situations, and as a consequence the size of the burst is stoichiometric with the enzyme concentration.

Proposed Catalytic Mechanism. The results of this study support the role of Glu-166 in class A β-lactamases as a general base in both acylation and deacylation, and are consistent with this residue being responsible for the ionization reflected in the acidic limb of the pH profile for catalysis. Mutations at this site to cysteine and aspartate resulted in a large decrease in activity, a result previously seen for other mutations at this site in other class A β-lactamases by several workers (Adachi et al., 1991; Gibson et al., 1990; Madgwick & Waley, 1987), demonstrating the central function of this residue in catalysis. Evidence indicating that this loss of activity did not result from conformational changes comes from the CD and thermal stability studies, and is supported by previous investigations with *B. licheniformis* (Escobar et al., 1991; Knox et al., 1993).

Although E166D activity is down 3 orders of magnitude, the shape of the pH profiles for this mutant approximate those of the wild-type, an expected result, given the similarities in the intrinsic pK_a's of aspartate and glutamate. In contrast, the pH profiles for E166C are very different than those of the wild-type. Moreover, we observe a large decrease in activity with this mutant (at pH 7.0, the optimum for the wild-type k_{cat}/K_m is decreased by 10^{-5} with nitrocefin). In the only other report concerning the pH-dependence of a Glu-166 mutant, Delaire et al. (1991) also noted that the pH profiles for the E166Y mutant of RTEM β-lactamase also have an appreciably modified shape in comparison to the wild-type profiles, as well as decreased activity. Thus, it is clear that Glu-166 plays a critical role in catalysis by the wild-type enzyme.

Lys-73 is highly conserved throughout the class A β-lactamase family as well as other classes of serine β-lactamases and penicillin binding proteins (Joris et al., 1988). Moreover, since replacement of lysine with arginine results in a large decrease in activity (Gibson et al., 1990; Madgwick & Waley, 1987) Lys-73 is thought to participate in the catalytic mechanism of these enzymes. There are several different hypotheses concerning its contribution to the mechanism. It has been suggested that Lys-73 polarizes the Ser-70 hydroxyl group so as to direct transfer of its proton onto the secondary amine produced upon acylation of the lactam carbonyl carbon. (Herzberg & Moult, 1987). It is also possible this lysine is acting as a general base and activating Ser-70 for attack onto the lactam ring (Strynadka et al., 1992). In this case, the lysine ammonium group would have to be deprotonated under physiological conditions, and its side-chain pK_a should appear in the acidic limb, not the alkaline limb, of the wild-type pH profiles. In light of the present results, as well as the observation that the K73R mutant is quite active (Gibson et al., 1990), the possibility that Lys-77 acts as a general base can be eliminated (Fink, 1993).

The present results are most consistent with a mechanism similar to those proposed by Lamotte-Brasseur et al. (1991) and Juteau et al. (1992), in which the acylation step involves activation of Ser-70 by shuttling of the γOH proton via a water molecule to the carboxylate of Glu-166 to form the tetrahedral transition state or intermediate. Subsequent transport of the proton from Glu-166 to the lactam nitrogen leaving group occurs via a water molecule, Lys-73 and Ser-130. Deacylation again involves Gly-166 acting as a general base, in this case to activate a water molecule to form the transient tetrahedral species, which collapses with transfer of the proton from Glu-166 via a bridging water molecule to the Ser-70 hydroxyl.

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