The Role of Lysine-234 in β-Lactamase Catalysis Probed by Site-Directed Mutagenesis†

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ABSTRACT: Lys-234 has been postulated to participate in β-lactamase catalysis by acting as an electrostatic anchor for the C3 carboxylate of penicillins [Herzberg, O., & Moult, J. (1987) *Science 236*, 694–701]. To test this hypothesis, site-directed mutagenesis was used to convert the Lys-234 in *Bacillus licheniformis* β-lactamase into Glu-234 or Ala-234. The wild-type, Glu-234, and Ala-234 β-lactamases have been expressed in *Bacillus subtilis* and purified to homogeneity. The wild-type, K234E, and K234A enzymes have virtually identical circular dichroism and fluorescence spectra, similar thermal stabilities at neutral pH, and the same susceptibilities to proteolysis, indicating the lack of significant structural perturbation caused by the mutation. At acidic and basic pH the mutant enzymes have the same native circular dichroism as the wild-type enzyme but the thermal stability is significantly different. The mutations cause perturbations of the pK values of the ionizing groups responsible for the pH dependence of the catalytic reaction in both the free enzyme and the E·S complex. As expected, conversion of Lys-234 to Ala or Glu decreased substrate binding (K_m) by 1–2 orders of magnitude for several penicillin and cephalosporin substrates at neutral and higher pH. However, at low pH, K_m is essentially the same for the K234E and K234A enzymes as for the wild-type enzyme. Furthermore, decreases of 2–3 orders of magnitude in k_{cat} were also observed, indicating substantial effects on the transition-state binding, as well as on ground-state binding. Surprisingly, changing the C3 carboxylate of phenoxymethylpenicillin to a hydroxymethyl group led to little difference in kinetic properties with the K234E or K234A enzyme. The results of this investigation indicate the Lys-234 is an important active-site residue involved in both ground-state and transition-state binding.

The susceptibility of β-lactam antibiotics to β-lactamases as a major source of resistance by pathogenic organisms has made elucidation of the mechanism of action of β-lactamases an important goal in mechanism-based antibiotic design. However, the current understanding of the catalytic mechanism of these enzymes is limited. Four major classes of β-lactamases have been identified, of which the class A enzymes have been characterized in the most detail. From studies of conserved residues from related enzymes, chemical modification experiments, and recent structural data, the following groups are believed to be important for catalysis in class A β-lactamases (Herzberg & Moult, 1987): Ser-70, which acts as a nucleophile in attacking the β-lactam carbonyl to form a covalent penicilloyl-enzyme intermediate (the acyl-enzyme); Lys-73, which may help stabilize the oxyanion tetrahedral intermediate (Carroll & Richards, 1990) or which may facilitate the transfer of a proton from Ser-70 to the β-lactam nitrogen (Moews et al., 1990); Glu-166, which may act to facilitate the transfer of protons in the acylation and deacylation steps (Madgwick & Waley, 1987); and Lys-234, which was first postulated to serve as an electrostatic anchor for the substrate carboxylate by Knox (personal communication) based on the analogous residue in the R61 D,D-peptidase (Kelly et al., 1986). The catalytic mechanisms of the cell-wall D-Ala-D-Ala peptidases must also be similar based on the high degree of sequence and three-dimensional homology with the β-lactamases (Kelly et al., 1987; Joris et al., 1988).

The first detailed X-ray structure of a class A β-lactamase (*Staphylococcus aureus*) was reported by Herzberg and Moult (1987). Very recently, a high-resolution structure of *Bacillus licheniformis* β-lactamase at 2.0 Å was described (Moews et al., 1990). These structures show that the aliphatic portion of the side chain of the Lys-234 residue is buried below the floor of the active-site depression, with the ammonium group penetrating the floor of the active-site cavity at the closed end. Model building studies based on the requirements of electrostatic, hydrophobic, and shape complementarity confirm that the only good candidate for electrostatic interaction with the substrate carboxylate is Lys-234 (Herzberg & Moult, 1987). Consequently, the postulated role of this conserved residue[1] is to facilitate substrate binding and orientation by formation of a salt bridge with the substrate carboxyl group. In order to determine whether the postulated role of Lys-234 is correct, we have used site-directed mutagenesis to convert this residue into either an alanine or glutamate residue. Site-specific mutations were introduced into β-lactamase from *B. licheniformis* and the mutant enzymes were secreted from *Bacillus subtilis*. If Lys-234 is important in substrate binding, these changes should lead to major effects on K_m. Alternatively, if its role is to stabilize the transition state, these changes should lead to substantial perturbations of the catalytic mechanism.

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[1] The tripeptide around Lys-234 is conserved in all the class A and class C β-lactamases, as well as the DD-peptidases, although in some of these Lys-234 is replaced by His (Kelly et al., 1987; Joris et al., 1988).

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Experimental Procedures

Materials. Trypsin, thermolysin, benzylpenicillin, phenoxymethylpenicillin, and ampicillin were purchased from Sigma. PADAC[2] and FAP were obtained from Calbiochem. Corp. Nitrocefin was a gift from Glaxo. Benzyl[^{14}C]penicillin was purchased from Amersham. Oligonucleotides were synthesized and purified by the Organic Chemistry Department at Genentech. Enzymes for DNA manipulation were from New England Biolabs or Bethesda Research Labs, except for *Escherichia coli* DNA polymerase I large fragment (Boehringer Mannheim).

Expression and Mutagenesis of B. licheniformis β-Lactamase. The integration plasmid, pac-SLJHL101, used to secrete *B. licheniformis* β-lactamase from *B. subtilis* I168, was kindly provided by Dan Yansura and Dr. Dennis Henner (Genentech). This plasmid was constructed by ligation of three fragments: fragment 1 contained the 1.5-kb *Eco*RI–*Bam*HI fragment from pBAS105 (Yansura & Henner, 1984) harboring the lac operator, *B. licheniformis* β-lactamase promoter, signal sequence, structural gene, and terminator; fragment 2 contained the 347-bp *Sau3*A fragment from the *B. subtilis trp E* gene [nucleotides 681–1028, Henner et al. (1985)]; and fragment 3 was the 5-kb *Eco*RI–*Bam*HI fragment from JH101 (Ferrari et al., 1983) containing the pBR322 origin of replication *E. coli* β-lactamase gene and the Gram-positive chloroamphenicol transacetylase gene. The ligation restored the *Bam*HI site between fragments 1 and 2. Subsequently, Cys +1 (just after the signal peptidase cleavage site) was mutated to Ser +1 (C1S) by site-directed mutagenesis as described (Kroyer & Chang, 1981), which created another *Bam*HI site at nucleotide 213 in fragment 1. Plasmid DNA from *E. coli* was transformed (Anagnostopoulos & Spizizen, 1961) into *B. subtilis* I168 and transformants were selected on TBAB plates (Miller, 1972) containing 5 μg/mL chloramphenicol. Chromosomal DNA was prepared (Maniatis et al., 1982) from transformants cultured in LB media (Miller, 1972) plus 5 μg/mL chloramphenicol. Chromosomal DNA was used to transform *B. subtilis* I168 again, and transformants were selected first on TBAB plates containing 10 and subsequently 25 μg/mL chloramphenicol. This procedure allowed amplification of the stably integrated pac-SLJH101 plasmid in the *trp E* locus in *B. subtilis*.

The K234E mutant was prepared by standard procedures (Zoller & Smith, 1982; Carter et al., 1985). Briefly, the K234E mutant was constructed on an M13mp18 single-stranded template using the following synthetic mutagenic oligonucleotide containing a *Nhe*I restriction site: 5′-GTG-
* * * *
GCT-GAT-GAA-ACT-GGA-GCG-GCT-AGC-TAT-G-GA-ACC-3′, where asterisks show the location of mismatches and the underlined sequence shows the position of an introduced restriction site. The heteroduplex was used to transfect *E. coli* Mut L (Kramer et al., 1984) and the resultant phage supernatant was used to transfect *E. coli* JM101 cells. Potential mutant clones were identified by hybridization of the plaques with ^{32}P-labeled mutagenic primers. Positive clones were confirmed by restriction analysis and dideoxy sequencing (Sanger et al., 1977). Mutants were subcloned back into the original pacSLJH101 plasmid by a three-way ligation using the 6.0-kb *Bgl*II–*Cla*I vector fragment and the 440-bp *Bgl*II–*Nhe*I and 730-bp *Nhe*I–*Cla*I fragment, both from the mutant RF DNA. The ligation mix was used to transform competent *E. coli* D1210 cells and positive subclones were identified by restriction analysis. Plasmid DNA from mutant clones was then used to transform competent *B. subtilis* I168 cells, and successful integrants were amplified on chloramphenicol-containing plates as described above.

The exo-large form of the *B. licheniformis* β-lactamase gene was placed under the control of the *Bacillus amyloliquefaciens* subtilisin promoter and signal sequence (Wells et al., 1983) by cloning into the free replicating *E. coli–B. subtilis* shuttle vector pSS.5 (B. Cunningham, D. Powers, and J. Wells, unpublished results). The pSS.5 plasmid is a derivative of pB0180-SUBT (Cunningham & Wells, 1987) that also contains an origin of replication for the phage f1. This permits production of single-stranded DNA in the presense of K07 helper phage (Vieira & Messing, 1987). The 1.3-kb *Bam*HI fragment from pac-SLJH101 containing the entire mature *B. licheniformis* β-lactamase structural gene was cloned into the *Bam*HI site of pSS.5 (after the terminator of the subtilisin gene). Single-stranded DNA was prepared from transformants identified by restriction analysis to contain the 5′-end of the β-lactamase gene linked to the 3′-end of the subtilisin gene (tandem orientation). A 31-mer oligonucleotide was synthesized having the sequence 5′-C-ACA-TCC-TCT-GCC-
* *
CAA-GCT-TCG-CAA-CCT-GCC-3′, where asterisks represent mismatches with the template and the underlined sequence is a unique *Hin*dIII site. The primer was used for site-specific mutagenesis to delete the pro-subtilisin gene and join the GCT alanine codon (−78) at the end of the subtilisin signal sequence (Vasantha & Thompson, 1986) to the TCG serine codon (+35), which is the first residue of the exo-large form of *B. licheniformis* β-lactamase (Izui et al., 1980). Isolation of the correct construction (pSS.5-βlac) was facilitated by *Hin*dIII restriction–purification (Wells et al., 1986) and selection for ampicillin resistance in *B. subtilis* I168. Dideoxy sequencing (Sanger et al., 1977) confirmed the correct junction between the subtilisin signal and exo-large portion of the β-lactamase structural gene. This plasmid (pSS.5-βlac) could be propagated in both *E. coli* MM294 and *B. subtilis* I168. The plasmid produced β-lactamase in *B. subtilis* at levels nearly as high as for the integrated construction but without the need for several steps of amplication. The K234A mutation was introduced by site-directed mutagenesis using the primer
* * *
5′-GAA-GTG-GCT-GAT-GCA-ACT-GGA-GCG-GCT-
* * *
AGC-TAT-GGA-ACC-C-3′, where asterisks represent mismatches with the wild-type template and the underlined sequence is a new *Nhe*I site. Restriction–purification on the *Nhe*I site allowed enrichment for the mutant clones, which were confirmed by dideoxy sequencing.

β-Lactamase Purification. The secreted *B. licheniformis* wild-type, K234E, and K234A enzymes were purified from *B. subtilis* as follows. Cell cultures inoculated from fresh overnight culture plates were grown at 37 °C in 3 L of media containing 30.0 g of tryptone, 15.0 g of yeast extract, 15.0 g of NaCl, 15.0 g of KH_2PO_4, pH 7.4, 30.0 mL of a 20% glucose solution, and 75 mg of chloramphenicol (30 mg of chlor-

[2] Abbreviations: PADAC, [2-[[*p*-(dimethylamino)phenyl]azo]-pyridinio]cephalosporin; FAP, 6-β-[(furylacryloyl)amido]penicillanic acid; TBAB, tryptone–blood agar base; RF, replicative form; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; Tris-bis, tris(hydroxymethyl)aminomethane-*N*,*N*′-methylene bis(acrylamide); FPLC, fast protein liquid chromatography; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; TFA, trifluoroacetic acid; pH*, apparent protonic activity, i.e., apparent pH of aqueous–organic solvent system, as measured by a glass electrode; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography.

amphenicol for the K234A enzyme) in a Lab-Line fermenter. The cell growth was monitored by turbidity and the production of β-lactamase was monitored by SDS–PAGE and catalytic activity. The cells produced 25 μg/mL wild-type, and 10–20 μg/mL K234E and K234A β-lactamases.

The cell-free culture supernatant (harvested by centrifugation at 10 000 rpm at 4 °C) was acidified with acetic acid to pH 4.5 and Amberlite CG-50 was suspended in the solution for 2–3 hours at 4 °C until all the β-lactamase was bound. The Amberlite resin was then collected and washed with water. To remove the β-lactamase, the Amberlite was suspended in a minimum volume of ammonium acetate buffer and titrated with 6 N NaOH until the pH reached 8–8.5. The protein was concentrated by ultrafiltration (or by lyophilization) and applied to a Sephadex G-100–125 column equilibrated against Tris-bis buffer (0.022 M, pH 6.2) at room temperature. The protein was eluted from the column with Tris-bis buffer (0.022 M, pH 6.2). The β-lactamase fractions from the Sephadex column were pooled and concentrated with a Amicon YM5 ultrafiltration membrane.

The β-lactamase was then purified by chromatofocusing using a Mono P HR 5/20 FPLC column (Pharmacia) equilibrated with start buffer (0.022 M Tris-bis, pH 6.2). The sample was applied by first running 1 mL of Polybuffer 74 (17:1 dilution, pH 4.0) onto the column, followed by the protein sample, and then switching back to the Polybuffer 74. The majority of K234E β-lactamase eluted at pH 4.8; 17% eluted at pH 4.9; all of the K234A enzyme eluted at pH 4.9, while the major species of the wild-type enzyme eluted at pH 5.05. Polybuffer 74 was removed by chromatography with FPLC Superose 12 equilibrated against sodium phosphate buffer (0.05 M, 0.15 M KCl, pH 7.0). The β-lactamase solutions were homogeneous (>95%) as judged by analytical isoelectric focusing and SDS–PAGE. A total of 30–35 mg for the wild-type β-lactamase and 5–20 mg for the mutant enzymes was typically recovered from 3 L of bacterial culture.

Enzyme Kinetics. β-Lactamase activity toward various substrates was determined spectrophotometrically with a Perkin-Elmer 320 spectrophotometer using a 1.0, 0.4, or 0.2 cm path length cell. β-Lactam hydrolysis was monitored at 30 °C in 50 mM sodium phosphate, 0.15 M KCl, pH 7.0, unless stated otherwise. The extinction coefficients used were as follows: for benzylpenicillin (240 nm) $\epsilon = 820\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 570\ M^{-1}\ cm^{-1}$; ampicillin (235 nm) $\epsilon = 1889\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 820\ M^{-1}\ cm^{-1}$; ampicillin (240 nm) $\epsilon = 1060\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 538\ M^{-1}\ cm^{-1}$; nitrocefin (482 nm) $\epsilon = 1405\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 17\,400\ M^{-1}\ cm^{-1}$; PADAC (466 nm) $\epsilon = 9450\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 9590\ M^{-1}\ cm^{-1}$; FAP (340 nm) $\epsilon = 3460\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 1730\ M^{-1}\ cm^{-1}$; and phenoxymethylpenicillin (240 nm) $\epsilon = 845\ M^{-1}\ cm^{-1}$ and $\Delta\epsilon = 524\ M^{-1}\ cm^{-1}$ at pH 7.0. The values of $\Delta\epsilon$ were 593, 524, and 511 $M^{-1}\ cm^{-1}$ at pH 4.0, 7.0, and 9.0, respectively, for phenoxymethylpenicillin; $\Delta\epsilon$ values were 600, 570, and 557 $M^{-1}\ cm^{-1}$ at pH 4.0, 7.0, and 9.0, respectively, for benzylpenicillin. The buffers (0.05 M) used for pH profiles were sodium formate for pH 3.0–4.0, sodium acetate for pH 4.0–5.5, sodium phosphate for pH 6.0–7.5, sodium pyrophosphate for pH 8.0–9.0, CHES for pH 9.5–10.0, and CAPS for pH 10.5. All buffers for the pH profiles contained 0.5 M KCl. In the regions of buffer pH overlap, the same results were obtained when either buffer or a mixture of the two buffers was used. The high salt concentration used masked any specific interactions that the different buffers gave at lower ionic strength.

Values for K_m and k_{cat} were obtained by two different methods: (1) initial velocity measurements (three to five determinations) from a minimum of five different substrate concentrations, analyzed with an Eadie–Hofstee plot, or (2) complete progress curves, fitted to the Michaelis–Menten equation using substrate concentrations at least 5× K_m. The k_{cat}/K_m ratio was determined from either of the above methods or directly from measurements of the first-order rate of hydrolysis under conditions of substrate concentration much less than K_m. Low substrate conditions were used for the K234E and K234A β-lactamases in order to complete k_{cat}/K_m vs pH profiles when K_m became too high (pH >8.5) to be accurately determined via the initial velocity method. The concentration of β-lactamase was determined by using $\epsilon_{280} = 23\,430\ M^{-1}\ cm^{-1}$. The extinction coefficient was determined by methods described in Van Iersel et al. (1985).

Circular Dichroism Measurements. Circular dichroism spectra were obtained with an AVIV Model 60DS instrument at 30 °C. Protein concentrations were 25.2 μM for wild-type, 13.1 μM for the K234E, and 11.1 μM for the K234A enzyme. The buffer used was 0.05 M sodium phosphate, pH 7.0; the cell path length was 0.2 mm. Spectra were also collected for all three samples at pH 9.5 (0.05 M CHES, 0.15 M KCl) and pH 4.0 (0.05 M sodium acetate, 0.15 M KCl).

Proteolysis Experiments. Solutions of the enzyme (0.87 μM, 0.2 mL, 50 mM sodium phosphate, pH 7.0) were treated with or without thermolysin (10 μL of 21.4 μM solution) at 55 °C. The rate of inactivation was monitored by assaying aliquots with benzylpenicillin at 30 °C. Similar experiments were carried out with trypsin at temperatures of 25, 40, and 50 °C.

Thermal Stability Measurements. Fluorescence was measured with a Perkin-Elmer MPF-4 instrument equipped with stirrer. The thermal unfolding of the proteins (0.32 μM) was observed by fluorescence (excitation 280 nm, emission 335 nm) in sodium acetate (pH 5.0), sodium phosphate (pH 6.0, 7.0) or sodium pyrophosphate (pH 9.0) buffer with a temperature ramp of 0.3 °C/min (15–90 °C). Linear extrapolations of the temperature dependence of the fluorescence of the native and denatured states were used to calculate the fraction unfolded as a function of temperature. The T_m was measured as the temperature at which 50% unfolding had occurred.

Determination of Rate-Limiting Step: Low-Temperature Acyl-Enzyme Trapping Experiments. To determine whether deacylation was the rate-limiting step, the rates of K234E or K234A β-lactamase catalyzed hydrolysis of phenoxymethylpenicillin or FAP were monitored spectrophotometrically in the presence of nucleophilic competitors, methanol (1–5 M) or hydroxylamine (2.5–25 mM). The reaction mixture contained enzyme (0.5 μM) and substrate at 30 °C (pH 8.0 or 5.0).

In addition, the following procedure was used to isolate the acyl-enzyme: enzyme and substrate (FAP or [^{14}C]benzylpenicillin) were mixed at low temperature and quenched after 5 s with TFA (Cartwright et al., 1989). The cryosolvent used was 50% methanol, 20% ethylene glycol, 30% aqueous buffer (0.05 M sodium formate), pH* 5.5. The enzyme (100 μL, 70 μM) was added to cryosolvent (700 μL) at 4 °C. The solution was then cooled to −40 °C for the wild-type and −15 °C for the K234E enzyme. At this point, FAP (100 μL of cryosolvent, 196 mM) was added to the solution and quenched within 4 s with TFA (0.5 M, 250 μL). The excess substrate was removed by centrifuging the sample on a DEAE column (5 mL, 0.4 M formic acid). The sample was applied to and eluted from a silica-based reverse-phase C3 ultrapore RPSC (Beckman) column and monitored at 280 and 305 nm as

Table I: Thermal Unfolding of Wild-Type β-Lactamase from *B. licheniformis* and the K234E and K234A Mutants[a]

pH	T_m °C		
	wild type[b]	K234E	K234A
5.0	65.0	67.8	67.9
6.0	64.7	66.0	ND
7.0	65.0	62.2	69.2
9.0	56.5	49.0	61.7

[a] ND, not determined. [b] Standard deviation, ±0.4 °C.

previously described (Fink et al., 1987). The same procedure was used for ^{14}C-labeled benzylpenicillin except the volume was reduced by 10-fold and HPLC was used to obtain separation of excess substrate from the enzyme.

Characterization of Wild-Type, K234E, and K234A β-Lactamases with 3α-(Hydroxymethyl)-2,2-dimethyl-6β-(phenoxyacetamido)penam. 3α-(Hydroxymethyl)-2,2-dimethyl-6β-(phenoxyacetamido)penam (replacement of the C3 carboxyl of phenoxymethylpenicillin with hydroxymethyl) was prepared by the method of Balsamo, et al. (1984) and the physical properties were the same as reported. The kinetics of wild-type, K234E, and K234A β-lactamases with the hydroxymethyl derivative of the penicillin were carried out in the presence of 20% methanol. Methanol was necessary to increase the solubility of the substrate. The buffers (0.04 M) used for the pH profiles were described above and contained 0.4 M KCl. The $\Delta\epsilon$ for the hydrolysis of 3α-(hydroxymethyl)-2,2-dimethyl-6β-(phenoxyacetamido)penam was assumed to be the same as that for phenoxymethylpenicillin.

Results

K234E and K234A Expression. The rate and level of expression, by *B. subtilis*, of the wild-type and K234E β-lactamases (10–20 μg/mL) is similar when the growth media are maintained at pH 7.4 with phosphate buffer (described in purification section). However, if the final pH was above 8.0, the peak level for expression of the K234E enzyme occurred before that of wild-type enzyme. The transient expression in these cultures correlates with a slightly decreased thermostability of the K234E enzyme at higher pH (see Table I).

The precursor form of *B. licheniformis* β-lactamase (exo-large) is processed quantitatively into the exo-small form for both the wild-type and K234E enzymes as shown by gel electrophoresis and amino-terminal sequence analysis (unpublished results). The K234A mutant was expressed in the exo-small form only and shows a greater propensity to proteolysis than wild-type or K234E β-lactamase in culture supernatant at pH 7.4.

Structural Comparison of Wild-Type, K234E, and K234A Mutant Proteins. Far-UV circular dichroism was used to compare the secondary structures of the wild-type, K234E, and K234A proteins. As shown in Figure 1, the spectra are virtually superimposable, indicating no differences in secondary structure. Moreover, the spectra of all three proteins at pH 9.5 and 4.0 (not shown) were superimposable with the spectra at pH 7.0 (Figure 1), indicating the absence of differential structural changes among these proteins at these pH extremes. Similarly, the tryptophan fluorescence emission spectra (not shown) were identical for all three proteins, indicating similar tertiary structures. As further corroboration, the susceptibility to proteolysis was investigated. Both wild-type and K234E enzymes were resistant to trypsin for >24 h at temperatures up to 50 °C. At higher temperatures some digestion occurred, but the simultaneous thermal denaturation of trypsin precluded accurate analyses. With thermolysin an identical first-order rate constant (4.7×10^{-4} s^{-1} at 55 °C) for loss of catalytic

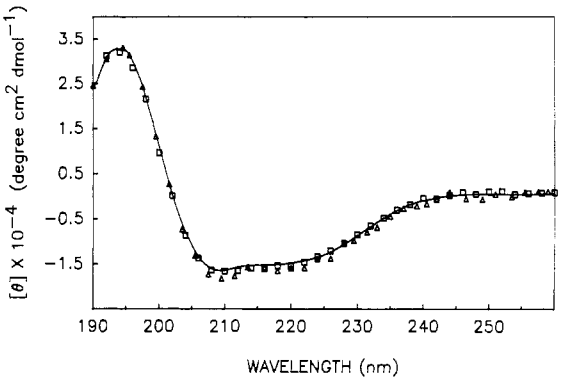


FIGURE 1: Far-UV circular dichroism spectra of wild-type (solid line), K234E mutant (□), and K234A mutant (△) β-lactamases.

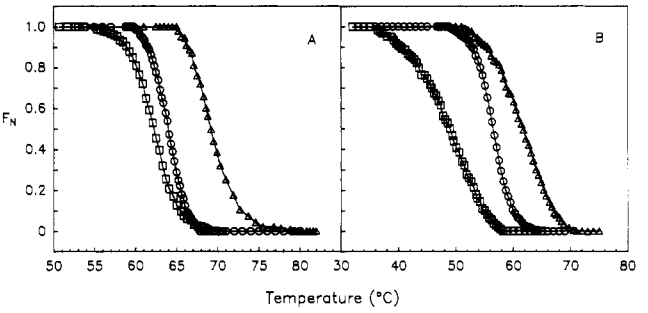


FIGURE 2: Thermal denaturation curves for wild-type (○), K234E mutant (□), and K234A mutant (△) proteins. Panels A and B are at pH 7.0 and 9.0, respectively. The reaction was followed by tryptophan fluorescence emission at 335 nm (see Experimental Procedures for details).

activity was found for wild-type, K234A, and K234E proteins.

The stability toward thermal denaturation at pH 7.0 was decrease by 2.8 °C for the K234E mutant with respect to the wild-type enzyme, while the K234A mutant stability was enhanced by 4 °C (Figure 2, Table I). It is noteworthy that the K234E mutant was significantly less stable at pH 9.0 than the wild type (Figure 2, Table I), while at pH 5.0 the mutant became slightly more stable than the wild type. The thermal unfolding of these proteins was fully reversible at pH 9.0 and 7.0. However, at pH 5.0, which is near the isoelectric point (p*I*) of the protein, the thermal unfolding is irreversible due to aggregation.

Several modes of amino-terminal processing occur for the enzyme β-lactamase in *Bacillus*; chromatofocusing allowed resolution of at least three major forms and several minor forms. The three major processed forms of wild-type β-lactamase examined had identical catalytic properties. The three major forms of K234E enzyme examined also had identical catalytic properties. Experiments were carried out with samples containing a mixture of these exo-small forms. For the K234A β-lactamase, which was expressed under the control of the subtilisin promoter, only one exo-small processed form was obtained (data not shown). The isoelectric points were 5.0, 4.8, and 4.9 for the most abundant exo-small form of the wild-type, K234E, and K234A enzymes, respectively, as determined by analytical IEF.

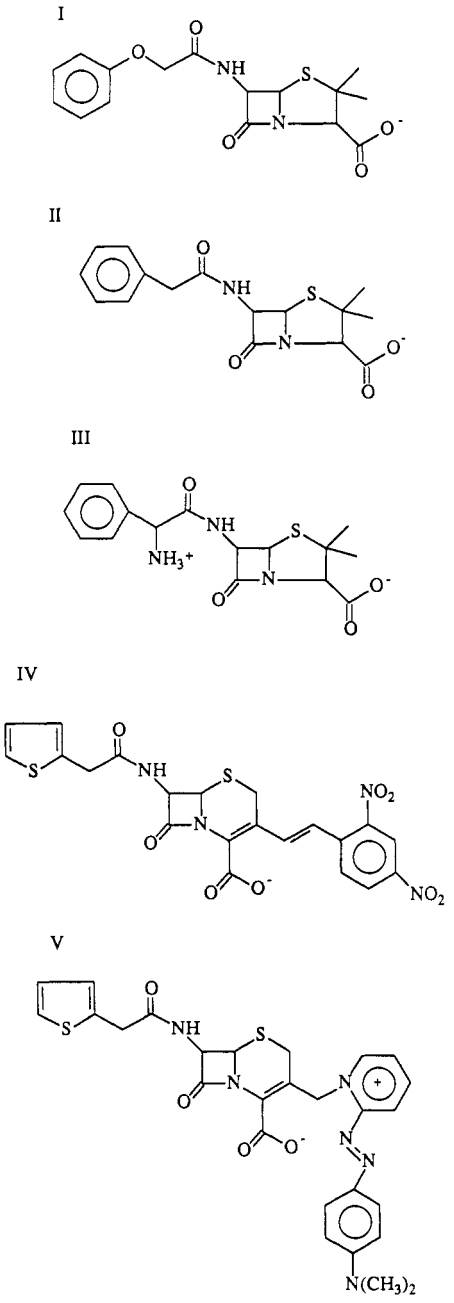
Substrate Specificity Profiles. Substrate specificity of the wild-type, K234E, and K234A β-lactamases at pH 7.0 was determined by comparison of the ratio of k_{cat} to K_m for a number of penicillin and cephalosporin substrates (Table II). Substrates included phenoxymethylpenicillin (I), which has a neutral side chain on C6 and which is stable at low pH, and ampicillin (III) which has a positively charged side chain (see Chart I). The mutations at Lys-234 cause significant changes in the catalytic efficiency toward different substrates. Both

Table II: Substrate Specificity Profiles of Wild-Type β-Lactamase from *B. licheniformis* and the K234E and K234A Mutants[a]

substrate	wild type			K234E				K234A	
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	rel[b]	k_{cat}/K_m	rel[b]
phenoxymethylpenicillin (I)	2552	0.10	2.48×10^7	31.7	1.5	2.1×10^4	8.5×10^{-4}	9.3×10^3	3.8×10^{-4}
benzylpenicillin (II)	2650	0.12	2.15×10^7	49.6	8.1	6.1×10^3	2.8×10^{-4}	2.3×10^3	1.1×10^{-4}
ampicillin (III)	2241	0.21	1.06×10^7	21.1	13.7	1.5×10^3	1.4×10^{-4}	8.9×10^2	8.4×10^{-5}
nitrocefin (IV)	1088	0.041	2.64×10^7	24.7	3.3	7.6×10^3	2.9×10^{-4}	3.9×10^3	1.5×10^{-4}
PADAC (V)	667	0.14	4.63×10^6	3.2	1.2	2.7×10^3	5.8×10^{-4}	3.2×10^2	6.9×10^{-4}

[a] Units are s^{-1} for k_{cat}, mM for K_m, and M^{-1} s^{-1} for k_{cat}/K_m. Conditions were 0.15 M KCl, pH 7.0, 30 °C. Standard deviation, <±6.0%. [b] Ratio of k_{cat}/K_m of the mutant relative to wild type.

Chart I



K234E and K234A enzymes exhibited substantial reductions in k_{cat}/K_m [(8.5×10^{-4})- to (8.4×10^{-5})-fold decrease] compared to the wild-type enzyme. Interestingly, the mutant enzymes catalyzed the hydrolysis of phenoxymethylpenicillin (I) 4 times more efficiently than benzylpenicillin (II) whereas the catalytic efficiency for these two substrates, which differ by only a single atom, was the same for wild-type enzyme. The individual k_{cat} and K_m values for the K234A mutant enzyme were not measured because the K_m values were too high to measure accurately at $I \cong 0.2$ M. At pH 7.0, values of K_m were increased by 1–2 orders of magnitude by the mutation, and the values of k_{cat} were decreased by a comparable amount.

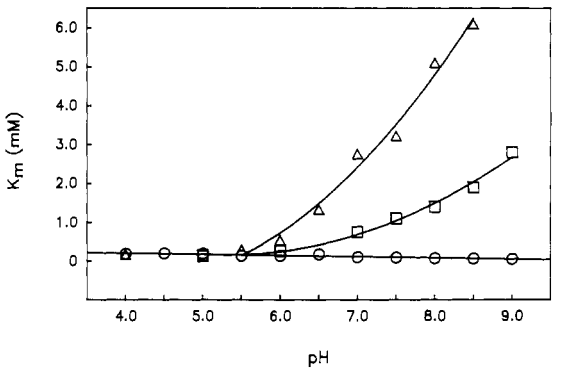


FIGURE 3: Effect of pH on K_m for the β-lactamase-catalyzed hydrolysis of phenoxymethylpenicillin for wild-type (○), K234E (□), and K234A (△) enzymes at $I \cong 0.55$ M.

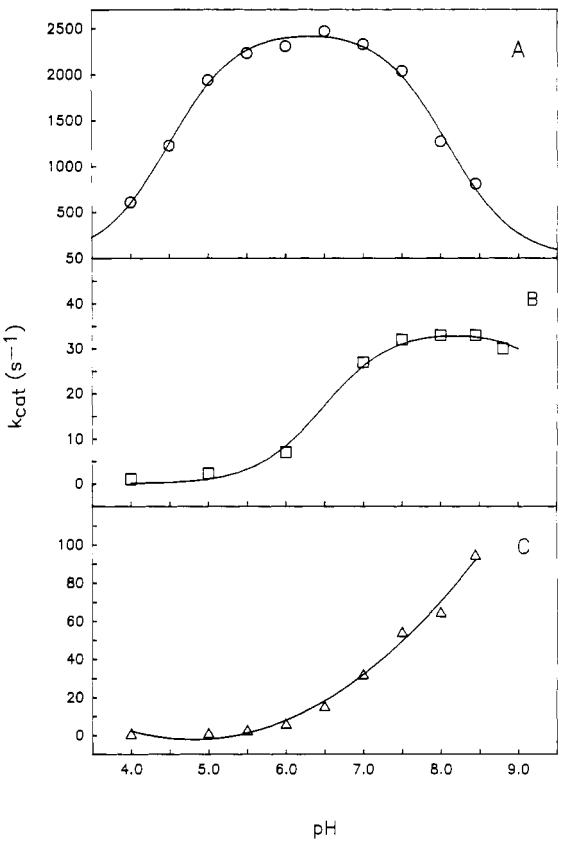


FIGURE 4: Effect of pH on k_{cat} for the β-lactamase-catalyzed hydrolysis of phenoxymethylpenicillin. Wild-type, K234E, and K234A data are shown in panels A–C, respectively. The solid line in panel A represents the curve calculated from eq 1 with values of $pK_1 = 4.5 \pm 0.1$ and $pK_2 = 8.1 \pm 0.1$ for the wild type.

Effects of the K234A and K234E Mutations on the pH Dependence of the Catalytic Parameters. We examined the

Table III: Effect of pH on Kinetic Parameters of the K234E and K234A Mutant β-Lactamases Relative to Wild Type with Phenoxymethylpenicillin[a]

	K234E				K234A			
pH	k_{cat}	K_m	k_{cat}(rel)[b]	K_m(rel)[b]	k_{cat}	K_m	k_{cat}(rel)[b]	K_m(rel)[b]
4.0	1.6	0.12	390	1.6	0.7	0.12	870	1.6
7.0	31	0.8	80	0.13	32	2.8	77	0.035
8.5	33	1.9	24	0.034	95	6.0	8.5	0.009

[a] Units are s^{-1} for k_{cat} and mM for K_m. Conditions were 50 mM buffer, 0.5 M KCl, 30 °C. [b] Ratio of wild-type to mutant enzyme.

Table IV: Effect of Ionic Strength on Kinetic Parameters of Wild-Type β-Lactamase and the K234E and K234A Mutants toward Phenoxymethylpenicillin[a]

	wild type			K234E			K234A		
[KCl]	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
0	1936	0.042	4.6×10^7	29.0	3.0	9.7×10^3			
0.15	2552	0.10	2.5×10^7	31.7	1.5	2.1×10^4			9.3×10^3
0.3				37.3	1.2	3.1×10^4			
0.5	2420	0.10	2.4×10^7	31.0	0.8	3.9×10^4	32.2	2.8	1.2×10^4
1.0	2600	0.10	2.6×10^7	27.8	0.4	6.9×10^4			
1.5	2450	0.10	2.4×10^7	32.0	0.3	1.1×10^5	30.3	1.0	3.0×10^4

[a] Units are s^{-1} for k_{cat}, mM for K_m, and M^{-1} s^{-1} for k_{cat}/K_m. Conditions were pH 7.0, 50 mM sodium phosphate buffer, 30 °C.

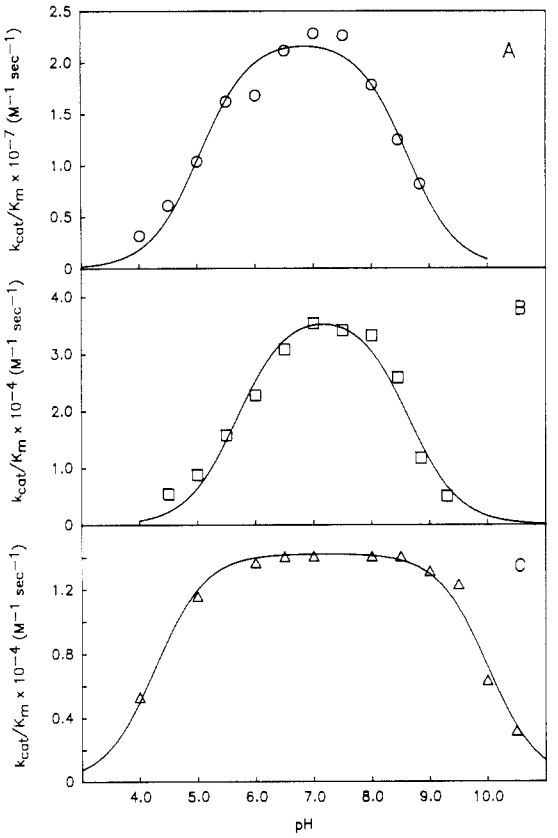


FIGURE 5: Effect of pH on k_{cat}/K_m for the β-lactamase-catalyzed hydrolysis of phenoxymethylpenicillin. Wild-type, K234E, and K234A data are shown in panels A–C, respectively. The solid line in panels A–C represents the curve calculated from eq 1 with values of $pK_1 = 5.0 \pm 0.1$ and $pK_2 = 8.6 \pm 0.1$ for the wild type, $pK_1 = 5.7 \pm 0.1$ and $pK_2 = 8.6 \pm 0.1$ for the K234E mutant, and $pK_1 = 4.3 \pm 0.3$ and $pK_2 = 10.0 \pm 0.3$ for the K234A mutant. Limiting values of k_{cat}/K_m were 2.2×10^7, 3.4×10^4, and 1.4×10^4 M^{-1} s^{-1} for wild-type, K234E, and K234A β-lactamase, respectively.

effect of pH on k_{cat} and K_m for phenoxymethylpenicillin over the pH range 3.5–10.5 using both the wild-type and mutant enzymes (Figures 3–5). The K_m for the wild-type enzyme did not change except for a small increase at low pH (Figure 3). For both the K234E and K234A enzymes, the values of K_m were essentially the same as those for wild type in the low-pH range (pH <6.0), but at higher pH increased substantially relative to the wild type.

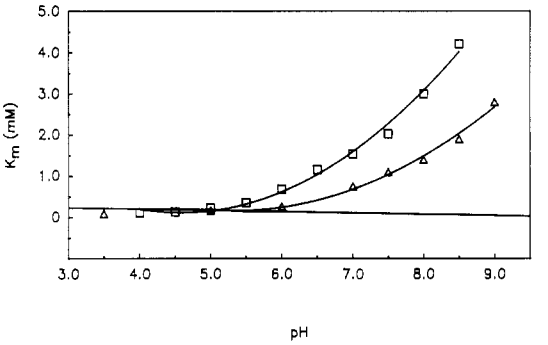


FIGURE 6: Effect of pH on K_m for the K234E β-lactamase-catalyzed hydrolysis of phenoxymethylpenicillin at two ionic strengths; (□) $I \cong 0.20$ M, (△) $I \cong 0.55$ M. The solid line is wild-type enzyme under the same conditions at $I \cong 0.55$ M.

The data from the corresponding k_{cat} vs pH curves (Figure 4) were fit to eq 1 to obtain pK values. It is apparent that the pK_1 of the ionizing group responsible for the acidic limb

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

of the curve is much higher in the mutants; 4.5 for the wild-type, 6.5 for the K234E, and ≥7.4 for K234A enzyme with phenoxymethylpenicillin. The value of pK_2 is also significantly increased in the mutants. For both the K234E and K234A mutant enzymes we estimate $pK_2 \gg 9.5$. As a consequence of the different pKs, the ratios of k_{cat} and K_m for the wild type to the K234E mutant vary with pH (Table III).

Examination of the k_{cat}/K_m vs pH profiles (Figure 5) for the K234E β-lactamase shows the acidic pK_1 is shifted up by 0.7 unit while the alkaline pK_2 remains the same with respect to the wild type. For the K234A β-lactamase, the acidic pK_1 is shifted down by 0.7 unit from the wild type, and the alkaline pK_2 is shifted up by 1.4 pH units.

Effects of Ionic Strength. The effect of increasing ionic strength on the K234E β-lactamase-catalyzed hydrolysis of phenoxymethylpenicillin was investigated (Table IV). Increasing ionic strength at pH 7.0 had little effect on k_{cat} for the wild-type or the mutant enzymes. Increasing ionic strength caused a small increase in K_m for the wild-type enzyme, but resulted in a decrease in K_m for the K234E and K234A mutants. A plot of K_m vs pH (Figure 6) of the K234E β-lacta-

Table V: Effect of pH on Kinetic Parameters of the Wild-Type, K234E, and K234A β-Lactamases with 3α-(Hydroxymethyl)-2,2-dimethyl-6β-(phenoxyacetamido)penam[a]

		substrate			
		phenoxymethylpenicillin		penicillanyl alcohol	
enzyme	pH	k_{cat}	K_m	k_{cat}	K_m
wild type	4.0	1165	0.39		
	5.0	2400	0.32		
	6.0	2700	0.22		
	7.0	2700	0.16		≫2.0
	8.0	1650	0.13	112	0.9
	9.0	415	0.08	65	0.28
K234E	4.0	1.5	0.1	0.5	0.2
	5.0	2.3	0.15	4.1	0.7
	6.0	7.0	0.26		≫1.0
K234A	5.0	1.2	0.13	8.0	0.9

[a] Units are s^{-1} for k_{cat} and mM for K_m. Conditions were 40 mM buffer, 0.4 M KCl, and 20% methanol at 30 °C except for the data given for K234E β-lactamase with phenoxymethylpenicillin (conditions same as given in Table III).

mase at two different ionic strengths shows that K_m decreases for the K234E mutant with increasing ionic strength, and that the values approach those of the wild-type enzyme.

Determination of Rate-Limiting Step: Low-Temperature Acyl-Enzyme Trapping Experiments. Conventional tests for determining the rate-limiting step in β-lactamase-catalyzed hydrolysis by using more potent nucleophiles such as methanol or hydroxylamine have been unsuccessful for class A enzymes such as *Bacillus cereus* β-lactamase, *TEM-2* β-lactamase, and *S. aureus* β-lactamase (Anderson & Pratt, 1981; Bicknell & Waley, 1985). The acyl-enzyme for these enzymes is thought to be shielded from external nucleophiles (Govardhan & Pratt, 1987). As expected, methanol had no effect on the kinetics of hydrolysis of FAP or phenoxymethylpenicillin by wild-type, K234E, or K234A β-lactamase. Interestingly, hydroxylamine was an inhibitor for the K234E enzyme (65% reduced rate at 2.5 mM and 99% reduction in rate at 25 mM hydroxylamine).

Cryoenzymology can be used to trap the acyl-enzyme intermediate of β-lactamase for substrates in which deacylation is rate-limiting (Cartwright et al., 1989). In such experiments, a 1:1 stoichiometry was found between FAP and either wild-type or K234E enzyme, indicating deacylation is rate-limiting. Unlike FAP, less than 5% of the [^{14}C]benzylpenicillin was trapped with either the wild-type or K234E enzyme, indicating acylation is rate-limiting for this substrate.

Kinetic Characterization of Wild-Type, K234E, and K234A β-Lactamases with a Penicillin Derivative Lacking the C3 Carboxylate. The hydrolysis of the penicillanyl alcohol by wild-type and K234E β-lactamases was investigated as a function of pH in the presence of 20% methanol. The K_m for the wild-type enzyme with this substrate is greater than 2 mM at pH 7.0 and decreases substantially at higher pH (Table V). Compared to the hydrolysis of phenoxymethylpenicillin by the wild-type enzyme at pH 7.0, the value of K_m is increased by at least 10-fold (Table V). It should be noted that K_m for wild-type enzyme with phenoxymethylpenicillin is measured in the presence of 20% methanol and the values obtained are higher than those found in a purely aqueous system. The values of K_m for the K234E enzyme with penicillanyl alcohol are slightly higher than those found with phenoxymethylpenicillin (Table V). The k_{cat} value for the wild-type enzyme with penicillanyl alcohol is reduced by 15-fold at pH 8.0 when compared to the value of k_{cat} obtained with phenoxymethylpenicillin. For the K234E enzyme, the value of k_{cat} with the penicillanyl alcohol is slightly higher than found with phenoxymethylpenicillin (Table V). Similar results were obtained for the K234A mutant enzyme. The determination of k_{cat} and K_m as a function of pH (Table V) was limited by the solubility of the alcohol substrate.

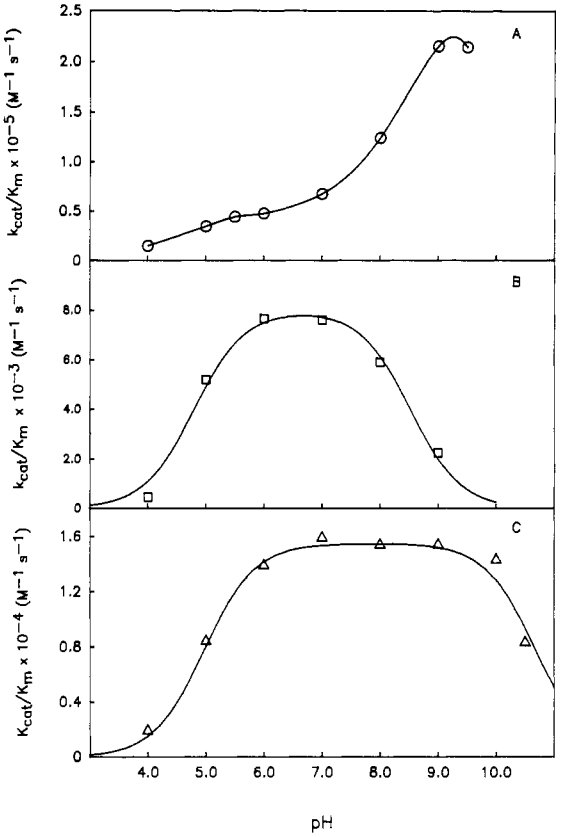


FIGURE 7: Effect of pH on k_{cat}/K_m for the β-lactamase-catalyzed hydrolysis of 3α-(hydroxymethyl)-2,2-dimethyl-6β-(phenoxyacetamido)penam (penicillanyl alcohol). Wild-type, K234E, and K234A data are shown in panels A–C, respectively.

Examination of the k_{cat}/K_m vs pH profiles (Figure 7) for the wild-type enzyme with the penicillanyl alcohol shows two ionizations, both increasing with increasing pH, which are quite distinct when compared to the results obtained with phenoxymethylpenicillin (Figure 5). The same pattern of k_{cat}/K_m vs pH was obtained with low substrate concentrations in the absence of methanol. Thus, the effect was not due to methanol. The k_{cat}/K_m vs pH profile (Figure 7) for the K234E and K234A β-lactamases with penicillanyl alcohol is very similar to that obtained with phenoxymethylpenicillin (Figure 5) except for a 4-fold reduction in the catalytic efficiency.

Discussion

Physical Characterization. The similar circular dichroism, fluorescence spectra, susceptibility to proteolysis, and thermal stability of the wild-type, K234E, and K234A β-lactamases demonstrate that the conversion of Lys-234 into Glu-234 or Ala-234 causes negligible effect on the gross structure of the protein. The observed decreases in the p*I* are expected based on the change from the positively charged ammonium group of Lys-234 to the negatively charged carboxylate of Glu-234 or the neutral residue Ala-234. Therefore, effects on catalysis do not stem from gross changes in the protein's conformation.

The effect of pH on the thermal stability of the K234E enzyme relative to that of the wild-type enzyme can be explained by electrostatic effects. At pH >7.0, the effective conversion of a positive ammonium to a negative carboxylate in the mutant leads to destabilization, possibly due to unfavorable electrostatic interactions with Glu-166. On the other

hand, at low pH the mutant becomes relatively more stable, presumably due to having removed an unfavorable interaction between Lys-234 and Lys-73. The cause of the enhanced stability of the K234A mutant may also reflect the abolition of an unfavorable interaction with Lys-73 in the wild-type enzyme. The different pH dependence of the stability of the wild-type and mutant proteins emphasizes the importance of measuring such properties at more than a single pH, especially when substituting ionizable groups.

Kinetic Characterization and the Role of Lys-234. If the major role of Lys-234 is to facilitate substrate binding (Herzberg & Moult, 1987), then we would expect the effects of these mutations to be most clearly manifested in K_s (the enzyme–substrate binding constant). Values of K_s can be determined from the observed kinetics on the following basis. For a two-step (acyl-enzyme) mechanism, $K_m = k_3K_s/(k_2 + k_3)$, where k_2 and k_3 are the rate constants for acylation and deacylation, respectively (Gutfreund & Sturtevant, 1956). The low-temperature acyl-enzyme trapping experiments demonstrate that deacylation is at least partially rate-limiting for the FAP substrate. Thus, separate determinations of k_2 and K_s are not possible from the FAP experimental data. For benzylpenicillin, acylation is rate-limiting and therefore for this substrate the value of K_m is equal to that of K_s. By analogy this should also be true for phenoxymethylpenicillin because benzylpenicillin and phenoxymethylpenicillin are closely related structurally, and their pH profiles of k_{cat}, K_m, and k_{cat}/K_m are similar (plots for benzylpenicillin not shown). The data for phenoxymethylpenicillin (Figure 3) would indicate that at low pH the mutations at Lys-234 cause a small increase in substrate affinity.

The affinity of the K234E and K234A enzymes for the substrate decreases at pH >6.0. This is probably caused by pH-dependent electrostatic repulsion between mutant enzyme and the C3 carboxylate of the substrate due to the loss of Lys-234. Alternatively, it could involve a small pH-dependent conformational change in the active site that causes poor binding. It seems unlikely that the observed pH dependence of K_m (presumed to equal K_s, Figure 3) at high pH is due to the pK of the C3 carboxylate of phenoxymethylpenicillin [pK_a = 2.7; Buckingham (1982)] for three reasons. First, an increase in pK of at least 5 or 6 units would be required, and second, no such pK is seen for the wild-type enzyme. And finally, the same pH-dependent increase in K_m was observed for the K234E and K234A enzymes with the penicillanyl alcohol substrate. Consequently, if we assume a near-normal pK for the carboxyl at C3 of the substrate, then at pH 5.5, where both wild-type and mutant enzymes have the same K_s, we can rule out direct electrostatic interaction of the Lys-234 as a significant contributor to substrate ground-state binding.

The effects of the mutations on k_{cat} reflect a substantial decrease in transition-state binding. For example, at low pH the values of k_{cat} for the mutants are only 0.2% of wild type. At higher pH, both substrate binding and transition-state binding are affected. This is reflected in the 30–100-fold increase in K_m and 10–20-fold decrease in k_{cat}. Furthermore, the k_{cat} vs pH profile shows a substantial perturbation in the pK values of the E·S complex for the mutants compared to the wild-type enzyme. We conclude that Lys-234 is important in optimizing the electrostatic potential of the transition-state during catalysis.

The contribution of Lys-234 to catalytic efficiency can be calculated from the data in Table III by using eq 2. For

$$\Delta G_{app} = -RT \ln [(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wild\ type}] \quad (2)$$

penicillin and cephalosporin substrates, the K234E mutation increases the free energy barrier in going from the free enzyme and substrate to the transition state by 4.0–4.9 kcal/mol and the K234A mutation results in 4.8–5.8 kcal/mol increases, respectively. These values are consistent with the deletion of a side chain forming a hydrogen bond with a charged group on the substrate (Fersht et al., 1985). It is interesting that transition-state binding energy is weaker for the K234A as compared to the K234E enzyme and may reflect the absence of hydrogen-bonding potential for the alanine substitution.

On the basis of the X-ray structure of *B. licheniformis* β-lactamase (Moews et al., 1990) it is reasonable to assume the group responsible for pK_1 of the enzyme is Glu-166. Our data are consistent with this proposal. The acidic and basic groups responsible for the bell-shaped dependence of k_{cat}/K_m for the wild-type enzyme are still present for the mutant enzymes and therefore Lys-234 is not responsible for these. The pK_1 for the K234E mutant is 0.7 unit higher than the wild-type enzyme. It is reasonable that the reversal in charge at residue 234 should destabilize the negative charge of Glu-166 and therefore cause the observed increase in the pK_1. Contrary to the expected result, the removal of Lys-234 for the K234A mutant enzyme results in a significant decrease in pK_1, attributed to deprotonation of Glu-166, and an increase in pK_2, which may reflect the ionization of Lys-73. This is consistent with exposure of Glu-166 and Lys-73 to a more polar environment. Perhaps replacement of Lys-234 with a rather small amino acid such as alanine creates a water-filled cavity.

Effects of Ionic Strength. If electrostatic interactions are important in substrate binding and catalysis, then the catalytic parameters should be sensitive to the ionic strength of the solution. The data (Table IV) suggest that increasing salt screens a deleterious effect caused by the K234E and K234A mutations. At high ionic strength, the value of K_m for the K234E mutant enzyme with phenoxymethylpenicillin approaches that of the wild type. In contrast to the wild-type enzyme, which shows very small changes in catalytic efficiency as a function of ionic strength at pH 7.0, k_{cat}/K_m for K234E β-lactamase shows a linear increase with ionic strength from 0.20 to 1.55 M. A plot of K_m vs pH (Figure 6) of the K234E β-lactamase at two different ionic strengths shows that the decreasing K_m value for the K234E mutant with increasing ionic strength at pH 7.0 correlates with a shift in pK.

Role of the C3 Carboxylate. In order to further understand the factors involved in the interaction of Lys-234 with the C3 carboxylate of the penicillin substrate, we have replaced this carboxylate with the neutral side-chain hydroxymethyl. As expected, wild-type enzyme shows an increase in K_m and a decrease in k_{cat} (Table V). In contrast to wild-type enzyme with phenoxymethylpenicillin, which shows a bell-shaped dependence for k_{cat}/K_m as a function of pH, the wild-type enzyme with the alcohol substrate shows two ionizations with increasing pH (Figure 7). The dominant effect at higher pH causing this increase is the decreasing K_m at alkaline pH (Table V). The second ascending limb is due to an ionization that could be in either the protein or the substrate. The results suggest that more than one ionization state of the enzyme is capable of catalyzing the hydrolysis reaction. Since this behavior is not observed with the K234E or K234A mutant enzyme with the alcohol substrate (Figure 7), the group responsible may be the Lys-234. Perhaps, at higher pH, binding of the alcohol substrate is improved when Lys-234 becomes neutral. Since plots of k_{cat}/K_m vs pH reflect the pKs of the free enzyme and/or substrate (Fersht, 1985), the data in Figure 7A may reflect some other complex process. It seems unlikely that the pH profile reflects the ionization of the

substrate since the pKs of alcohols are quite high.

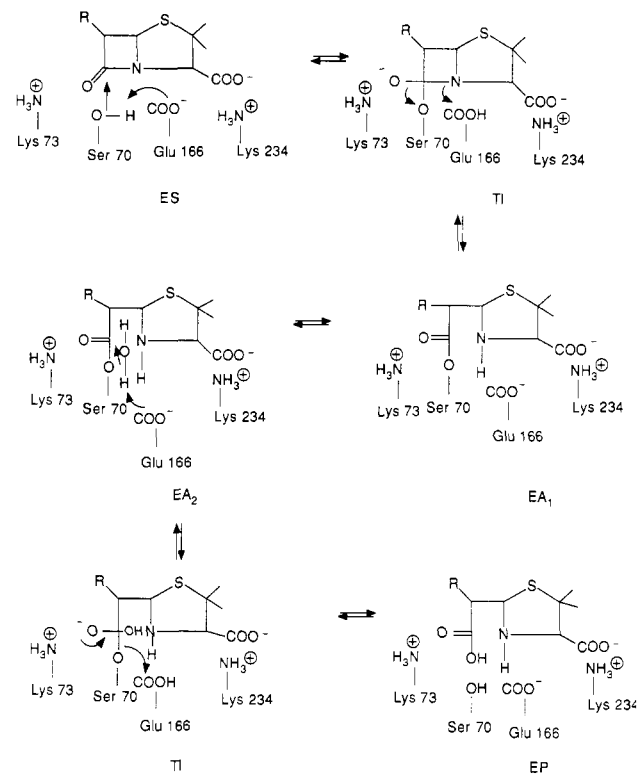
Surprisingly, the K234E and K234A β-lactamases showed no compensatory activity toward the alcohol substrate. The K_m was increased with respect to the carboxyl substrate and the k_{cat} was of similar magnitude (Table V). One possible explanation is that the substrates do not interact with the Glu-234 directly and are bound in an orientation that makes the carboxylate or hydroxyl of the substrate inaccessible to the protein residue at position 234. The pH dependence of K_m for the alcohol substrate is similar to that found for the carboxylate substrate, indicating the increase in K_m is not due to the ionization of the substrate.

Proposed Mechanism. Given the strongly conserved amino acid sequence homology between β-lactamases from *S. aureus* and *B. licheniformis*, and the similarity in three-dimensional structure (Kelly et al., 1986, 1987), it is apparent that the only charged groups likely to be directly involved in catalysis are Lys-73, Lys-234, and Glu-166. We assume the group responsible for pK_1 is Glu-166 (Madgwick & Waley, 1987). Since the pH dependences of k_{cat}/K_m of both wild type and the Lys-234 mutants are fairly similar, we conclude that pK_2 is not due to Lys-234. The data of Carroll and Richards (1990) indicate that the pK_2 for the K73R mutant of RTEM β-lactamase is significantly lower than that of the wild-type enzyme.

Taken together, these studies suggest one of two possibilities. The Lys-73 and Lys-234 both contribute to the observed pK_2 in the wild-type enzyme; thus, when one is removed the effect of the other remains. Alternatively, pK_2 may be due to a residue outside the active site. There are two conserved charged residues that are possible candidates, Tyr-105 or Lys-111 (Joris, et al., 1987). From the crystal structure of *S. aureus* and *B. licheniformis* β-lactamase, Tyr-105 is located near the active-site and has a pK that could reasonably explain the observed ionization around 9 for wild-type enzyme. Nitration of Tyr-105 does not result in loss of catalytic activity at pH 7.0 (Bristow & Virden, 1979). If Tyr-105 is responsible for the observed pK_2, its function in catalysis would most likely involve a conformational change. Such a role could be analogous to that of Tyr-248 in carboxypeptidase, which is postulated to be responsible for pK_2 (Gardell, et al., 1985; Auld, 1987). Replacement of Tyr-248 in carboxypeptidase A by Phe has very little effect on k_{cat} and a small effect on K_m. X-ray diffraction analysis shows carboxypeptidase Tyr-248 undergoes a substrate-induced conformational change resulting in the phenolic hydroxyl moving from the surface of the enzyme to within hydrogen-bonding distance of the cleaved peptide (Rees & Lipscomb, 1981). Site-directed mutagenesis studies currently underway in our laboratory (Y105F) should address this question directly. Lys-111, which is a highly conserved residue of β-lactamase, is located away from the active site in the α-3 helix and therefore seems less likely to be responsible for pK_2.

On the basis of our results, crystallographic studies (Herzberg & Moult, 1987; Moews et al., 1990), and site-directed mutagenesis studies (Carroll & Richards, 1990; Madgwick & Waley, 1987), we postulate the following mechanism for β-lactamase catalysis as a working hypothesis (Scheme I). Initial substrate binding and orientation is facilitated by interactions of the C3 carboxyl with Lys-234, Arg-244, and Thr-235, interactions of the side chain of the penicillin with the protein, and by other electrostatic effects such as the helix dipole moment from the helix containing Ser-70. Glu-166, acting as a general base, facilitates nucleophilic attack by Ser-70 on the β-lactam carbonyl. The

Scheme I



resulting tetrahedral intermediate oxyanion is stabilized by hydrogen bonds to main-chain amides (Herzberg & Moult, 1987). Our results show that Lys-234 must also play a role in the stabilization of this transition state, presumably by contributing to the net electrostatic field. After formation of the acyl-enzyme intermediate, deacylation involves Glu-166 acting as a general base to activate water to attack the acyl carbonyl. Breakdown of the resulting tetrahedral adduct involves Glu-166, acting as a proton donor to Ser-70, and restores the enzyme to its original state. The proposed mechanism is a working model for β-lactamase catalysis; other possibilities exist. For example, the Lys-73 may directly protonate the β-lactam nitrogen. This possibility is unlikely according to the recent crystallographic studies reported by Moews et al. (1990). More likely, Lys-73 may be important in establishing the electrostatic environment necessary for catalysis.

Conclusion

The role of Lys-234 is more complex than originally envisioned. At lower pH, the conversion of Lys-234 to Ala-234 or Glu-234 had no effect on the binding but decreased k_{cat} up to 800-fold. One possible explanation for these results is that at lower pH the substrate is bound in an alternative orientation. This orientation is not optimum for catalysis and thus would explain the low value of k_{cat}. Examination of the X-ray structure of *B. licheniformis* β-lactamase (Moews et al., 1990) suggests that this alternative orientation could involve another charged residue such as Arg-244. This residue is near the thiazolidine ring C3 carboxylate (Moews et al., 1990). The Arg-244 is conserved in all class A β-lactamases except the *Streptomyces albus* G enzyme. At higher pH, the orientation of the substrate is optimum for catalysis (10–20-fold reduction in k_{cat} for the mutants) but for the mutant enzymes binding is poor. Overall, our results indicate Lys-234 establishes an electrostatic environment that is important for substrate binding as well as stabilization of the transition state in β-lactamase catalysis.

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