Lysine-73 Is Involved in the Acylation and Deacylation of β-Lactamase†

Eric J. Lietz, Heather Truher, Debra Kahn, Mark J. Hokenson, and Anthony L. Fink*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064

Received November 22, 1999; Revised Manuscript Received February 15, 2000

ABSTRACT: Lysine 73 is a conserved active-site residue in the class A β-lactamases, as well as other members of the serine penicillin-sensitive enzyme family; its role in catalysis remains controversial and uncertain. Mutation of Lys73 to alanine in the β-lactamase from *Bacillus licheniformis* resulted in a substantial reduction in both turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m), and a very significant shift in pK_1 to higher pH in the bell-shaped pH−rate profiles (k_{cat}/K_m) for several penicillin and cephalosporin substrates. The increase in pK_1 is consistent with the removal of the positive ammonium group of the lysine from the proximity of Glu166, to which the acid limb has been ascribed. The alkaline limb of the k_{cat}/K_m vs profiles is not shifted appreciably, as might have been expected if this limb reflected the ionization of Lys73 in the wild-type enzyme. The k_{cat}/K_m at the pH optimum for the mutant was down about 200-fold for penicillins and around 10^4 for cephalosporins, compared to the wild-type, suggesting significant differences in the mechanisms for catalysis of penicillins compared to cephalosporins. Burst kinetics were observed with several substrates assayed with K73A β-lactamase, indicating an underlying branched-pathway kinetic scheme, and rate-limiting deacylation. FTIR analysis was used to determine whether acylation or deacylation was rate-limiting. In general, acylation was the rate-limiting step for cephalosporin substrates, whereas deacylation was rate-limiting for penicillin substrates. The results indicate that Lys73 plays an important role in both the acylation and deacylation steps of the catalytic mechanism. The effects of this mutation (K73A) indicate that Lys73 does not function as a general base in the catalytic mechanism of β-lactamase. The existence of bell-shaped pH−rate profiles for the K73A variant suggests that Lys73 is not directly responsible for either limb in such plots. It is likely that both Glu166 and Lys73 are important to each other in terms of maintaining the optimum electrostatic environment for fully efficient catalytic activity to occur.

β-Lactamases are the primary means of defense for pathogenic bacteria against β-lactam antibiotic medications. The mechanism of how β-lactamase cleaves the lactam amide bond, destroying the antibiotic's efficacy, has remained "enigmatically" elusive (*1*). Only the active-site Ser70 has had its function firmly established as the nucleophile which attacks the lactam carbonyl, forming an acyl-enzyme intermediate as part of a two-step (acylation, deacylation) mechanism (*2*−*4*). Strong evidence has been presented that Glu166 is a catalytic residue involved in deacylation (*5*−*7*).

Structural data, as well as modeling studies, in conjunction with kinetic results from wild-type and site-specific mutants, have resulted in the proposal of several detailed catalytic mechanisms for the enzymatic hydrolysis of β-lactams. The two major categories of mechanisms for class A β-lactamases are the following: (1) Lys73 acts as a general base, activating Ser70 for attack on the lactam carbonyl by way of a hydrogen bond network involving three protons. A proton is back-delivered to the (leaving group) secondary amine produced upon acylation. Deacylation is effected by activation of a water molecule by Glu166, acting as a general base. The resulting hydroxide attacks the acyl carbonyl, hydrolyzing the acyl-enzyme intermediate (*8*). (2) In an alternative mechanism (*9, 10*), Glu166 acts as the general base, either activating Ser70 by way of a water molecule that is situated between the two residues, or directly (*11*). While the deacylation half of this proposed mechanism is essentially the same in both models, there are major differences in the mechanism of acylation. Additional factors likely to be important in acylation are the presence of an "oxyanion hole" that polarizes the β-lactam carbonyl, rendering this moiety a better acceptor of the Ser70 nucleophile, and the dipole moment resulting from Ser70's position at the N-terminus of the α-helix, which lowers the serine hydroxyl proton's pK_a (*5, 12*). Based on solvent kinetic isotope effects, a mechanism involving asymmetric double-displacement, in which class A β-lactamases use different functional groups for acylation and deacylation, has been suggested (*13*). Of the various mechanisms proposed, the major unresolved question involves the details of the acylation reaction, in which the γ-OH of Ser70 is activated to attack the lactam carbonyl, and the leaving lactam amine group becomes protonated.

Lys73 is a totally conserved residue in the serine penicillin-sensitive enzyme family (which includes β-lactamases, D-Ala-D-Ala-carboxypeptidases, and transpeptidases). Several investigations have suggested that the major active-site residue involved in deacylation for the class A β-lactamases

† This research was supported by a grant from the National Science Foundation.

* Correspondence should be addressed to this author at the Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064. Tel: (831) 459-2744; Fax: (831) 459-2935; E-mail: enzyme@cats.ucsc.edu.

10.1021/bi992681k CCC: $19.00
Published on Web 04/05/2000

is Glu166. As a consequence, it has been assumed that Lys73 is probably most important for the acylation half of the reaction. In terms of the catalytic mechanism of the class A β-lactamases, there are a number of specific questions that relate to Lys73: For example, what is its role in acylation and deacylation; is it responsible for one of the limbs of the bell-shaped pH−rate profile; does it function as an acid or base catalyst?

A particular point of contention is whether the side chain of Lys73 acts as a general base or general acid catalyst. Proposed mechanisms in which it functions as a general base (by analogy to His57 in the serine proteases) require that Lys73 be in a nonprotonated state, and thus have a very low p*K* (around 5) for an ammonium group. It has been suggested that such a dramatic perturbation of the pK_a is due to local electrostatic effects (*8*). NMR and chemical modification experiments to investigate the protonation state for this residue indicate that the pK_a of Lys73 is >10 in the free enzyme, and therefore Lys73 could not act as a general base (*14*). Proponents of Lys73 acting as a general base suggest that the p*K* may be very different in the presence of bound substrate.

In an effort to shed further light on the role of Lys73 in β-lactamase catalysis, we have mutated Lys73 to alanine in the enzyme from *Bacillus licheniformis*. From a detailed investigation of the effects of this substitution, especially on a variety of substrates (a substrate "profile"), and on the pH-dependence of the reaction for both penicillin and cephalosporin substrates, we have discovered a number of very interesting aspects of the catalytic reaction. Particularly notable are the substantial shifts in the bell-shaped plots of k_{cat}/K_m against pH, the small effects on k_{cat}/K_m at the pH optimum for penicillin substrates compared to the wild-type enzyme, and the presence of "bursts" of product in the early stages of the catalytic reaction, consistent with rate-limiting deacylation.

MATERIALS AND METHODS

Materials. Luria−Bertani (LB) medium ingredients were purchased from Difco or Pharmacia. The thermostable polymerase Ultma used for polymerase chain reactions (PCR) was obtained from Perkin-Elmer. Restriction endonucleases, DNA ligase, and DNA molecular weight markers were from Boehringer-Mannheim or New England Biolabs. The 100 bp DNA Ladder was supplied by GIBCO BRL. Oligonucleotide primers were synthesized by facilities within the Department of Biology, UC Santa Cruz, or purchased from Genosys, Inc. Plasmid DNA or gel extraction kits were from Qiagen or Bio101. Sequenase was obtained from United States Biochemicals.

Nitrocefin was purchased from Beckton-Dickenson. Benzylpenicillin, cefoxitin, cefotaxime, phenoxymethylpenicillin, and IPTG[1] were purchased from Sigma. PADAC and *N*-(2-furylacryloyl)penicillin (FAP) were from Calbiochem.

PCR, Mutagenesis, and Subcloning. Genetic manipulations, as well as protein expression, were carried out using the high-expression vector pELB1, a pET9a (Novagen) derivative utilizing the T7 RNA polymerase promoter system. The details of the construction of pELB1 will be reported elsewhere. The overlap−extension PCR method (*15*) was used to produce the site-specific Lys→Ala mutation at position 73. To reduce the possibility of mistranslation, two base pairs comprising the substituted alanine codon were modified. The mutation primers used to produce the K73A mutation were:

"Sense" primer:

5′ TGCGTCGACGATT**GCC**GGCTTTA 3′

"Antisense" primer:

5′ C**GGC**AATCGTCGACGCAAAA 3′

The three-base codon resulting in the substitution of alanine for lysine at position 73 is indicated in boldface. The mutation primers included a *Sal*I unique restriction site (underlined) to facilitate screening of candidate subclones. Overlap−extension PCR product was cut with restriction endonucleases *Xba*I and *Sfu*I, and the resulting DNA gene fragment containing the K73A mutation was ligated into pELB1 (from which the corresponding wild-type gene fragment had been excised using the same restriction enzymes).

Ligation reactions were used to transform *E. coli* DH5α cells made competent by the procedure as described (*16*). Candidate transformant colonies were screened by growth on LB−carbenicillin plates, as well as by isolating plasmid for restriction analysis. Several K73A subclones passing these screens were sequenced by the Sanger dideoxy method (*17*).

Protein Expression, Isolation, and Purification. Four 1 L LB medium (plus 50 μg/mL kanamycin) cultures were inoculated using 6 mL starter cultures. The cultures were incubated at 37 °C for 6 h under moderate aeration (shaking). Cells were harvested by centrifuging the cultures at 4 °C, and the media supernatant discarded. The pelleted cells were washed by resuspending in 400 mL of cold 10 mM Tris-HCl (pH 8.0)/30 mM NaCl buffer. The washed cells were centrifuged 5 min at 7000 rpm, 4 °C, to pellet them, and then resuspended in 200 mL of 20% sucrose, 30 mM Tris-HCl, pH 8.0, 1 mM EDTA. The cells were subsequently centrifuged 5 min at 5000 rpm, 4 °C, and the supernatant was discarded. The cell pellets were osmotically shocked by gently resuspending in 200 mL of deionized water. The cells were on ice during this resuspension (which took approximately 20 min, due to the fact that the sucrose-treated cells were sticky, and resuspended with some difficulty). The resuspended cells (which at this point are assumed to be spheroblasts) were centrifuged 4 min at 7000 rpm, 4 °C, and the osmotic shock supernatant was collected for subsequent protein purification steps.

The following manipulations were performed at 4 °C. The pH of the osmotic shock supernatant was lowered to 4.8 by the addition of 100 mL of 0.02 M NaOAc (pH 4.8)/0.02 M NaCl. Precipitate was removed by centrifugation at 7000 rpm for 20 min. The supernatant was loaded onto a 3 × 25 cm CM-Sepharose column [previously equilibrated to 0.02 M NaOAc (pH 4.8)/0.02 M NaCl by washing 5 times with 0.2 M NaOAc (pH 4.8)/0.02 M NaCl, and 5 times with 0.02 M NaOAc (pH 4.8)/0.02 M NaCl]. The column was rinsed with 0.02 M NaOAc (pH 4.8)/0.02 M NaCl buffer until the absorbance at 280 nm returned to the preloading base-

[1] Abbreviations: FAP, *N*-(2-furylacryloyl)penicillin; PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo) pyridiniummethyl]-3-cephem-4-carboxylic acid; IPTG, isopropyl thio-β-D-galactoside; WT, wild-type; ND, not determined.

line (approximately 300 mL), and then eluted with a 1 L salt gradient (0.02−0.5 M) with absorbance monitoring at 280 nm, yielding β-lactamase activity at approximately 0.18 M NaCl. Fractions showing β-lactamase activity against PADAC or nitrocefin were pooled and exchanged into 0.05 M KPhos buffer (pH 7.0) via dialysis. The protein was concentrated using Centricon 10 filters and stored at either −20 or 4 °C.

Physical Characterization. Far-UV circular dichroism (CD) measurements were made on an Aviv Circular Dichroism Spectrometer (model 60DS). Thermal denaturation was monitored by tryptophan fluorescence using a Spex (ISA, Inc.) Fluoromax 2. A linear gradient of 0.4 °C/min was used to denature 1.0 mL protein samples of approximately 1 μM concentration. Data were fit to a sigmoidal two-state folding mechanism according to eq 1:

$$f_U = (y_F - y)/(y_F - y_U) \quad (1)$$

where f_U represents the unfolded fraction, y_F and y_U represent the folded and unfolded states, respectively, and y is measured fluorescence (*18*). The T_m's reflect the temperature at which 50% unfolding had occurred.

Enzyme Kinetics. β-Lactamase activity toward various penicillin and cephalosporin substrates was determined spectrophotometrically using Hewlett-Packard 8452A or Cary 118 spectrophotometers. Path lengths employed were 1.0, 0.4, or 0.1 cm. Data analyses were performed using a least-squares algorithm (Kaleidagraph, Abelbeck Software, Inc.). The hydrolysis of the amide bond in the lactam ring was followed at the following wavelengths: benzylpenicillin (240 nm), $\epsilon = 820$ M^{-1} cm^{-1}, $\Delta\epsilon = 570$ M^{-1} cm^{-1}; cephaloridine (295 nm), $\epsilon = 1560$ M^{-1} cm^{-1}, $\Delta\epsilon = 1210$ M^{-1} cm^{-1}; cefotaxime (282 nm), $\epsilon = 4630$ M^{-1} cm^{-1}, $\Delta\epsilon = 880$ M^{-1} cm^{-1}; FAP (340 nm), $\epsilon = 3460$ M^{-1} cm^{-1}, $\Delta\epsilon = 1905$ M^{-1} cm^{-1}; nitrocefin (482 nm), $\epsilon = 1405$ M^{-1} cm^{-1}, $\Delta\epsilon = 17\,400$ M^{-1} cm^{-1}; PADAC (466 nm), $\epsilon = 9450$ M^{-1} cm^{-1}, $\Delta\epsilon = 9590$ M^{-1} cm^{-1}; phenoxymethylpenicillin (240 nm), $\epsilon = 845$ M^{-1} cm^{-1}, $\Delta\epsilon = 520$ M^{-1} cm^{-1}. Final enzyme concentrations were usually 3.0 nM for wild-type, 5−10 μM for K73A, and 75 μM for K73A/K234A.

The kinetic parameters k_{cat} and K_m (and/or k_{cat}/K_m) were determined by one or more of the following methods: (a) progress curve data were analyzed by fitting the differential form of the Michaelis−Menten equation to the plotted first-derivative (rate) vs [S] data; (b) initial rates vs [S] were plotted and fit to the differential form of the Michaelis−Menten equation; (c) under first-order conditions (where $K_m \gg$ [S]), absorbance vs time data were fit to a single exponential to obtain k_{obs} from which k_{cat}/K_m was obtained; (d) absorbance vs time data were fit to the integrated form of the Michaelis−Menten equation.

Substrate Specificity Profile. For several penicillin and cephalosporin substrates (Figure 1), β-lactam hydrolysis was monitored at 30 °C, pH 8.0, in 0.05 M sodium pyrophosphate or sodium phosphate buffer, 0.5 M KCl.

pH Profiles. The hydrolytic activity of K73A β-lactamase against nitrocefin, FAP, and benzylpenicillin was assayed over a range of pHs. Each data point represents an average of at least three assays at a given pH. All assays were run at 30 °C. Reactions were buffered by sodium acetate (pH 4.0−5.5), sodium phosphate (pH 6.0−8.0), sodium pyrophosphate (pH 8.0−9.0), CHES (pH 9.5−10.0), and CAPS (pH 10.5). Buffer concentrations for all kinetic assays were 50 mM, in 0.5 M KCl. Ionization constants were determined by fitting the (k_{cat}, K_m, and k_{cat}/K_m) pH profiles to the expression in eq 2.

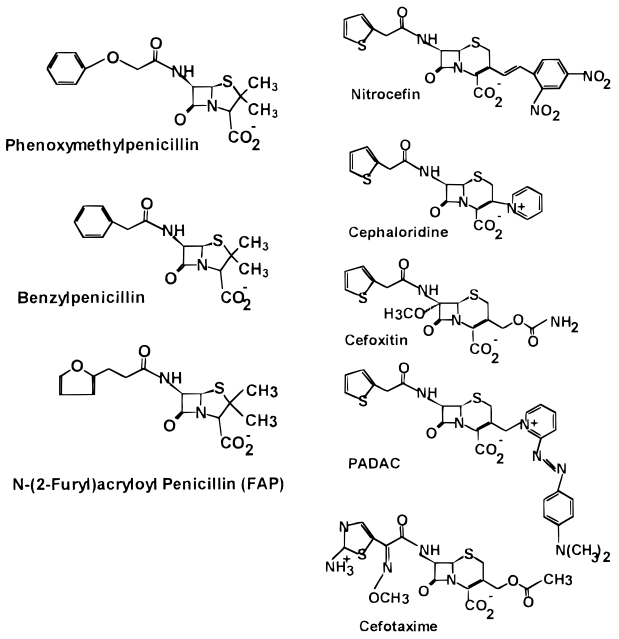


FIGURE 1: Structures of penicillin and cephalosporin substrates used in the present study.

$$k_{obs} = k_{lim}/[1 + [H^+]/K_1 + K_2/[H^+] + K_2/K_1] \quad (2)$$

Burst Kinetics. Burst magnitudes for PADAC, nitrocefin, cephaloridine, cefoxitin, and FAP were measured in 0.05 M sodium pyrophosphate (or sodium phosphate), 0.5 M KCl buffer (at pH 8.0, 15 °C unless otherwise indicated). Burst magnitude measurements were made by extrapolating the linear zero-order fit back to the point of enzyme addition. Burst measurements for all substrates were made under a variety of combinations of [S], [E_0], and temperature in order to detect initial vs steady-state rate changes in the progress curves as well as burst magnitude variations. For FAP, the profile of burst magnitude vs pH was determined by averaging at least three burst measurements at each pH. Reactions were buffered as previously indicated for pH−activity profiles.

The numbering scheme developed by Ambler et al. (*19*) is used throughout this paper. Reactions between a given enzyme and substrate are indicated by 'enzyme/substrate' nomenclature (e.g., K73A/nitrocefin).

RESULTS

Structural Characterization of K73A. Far-UV circular dichroism (CD) was used to compare K73A protein secondary structure to that of wild-type at 25 °C and pH 7.0. The K73A and wild-type spectra were superimposable within experimental error (Figure 2A), indicating the absence of any gross secondary structural modification caused by the K73A mutation. The stabilities of wild-type and K73A mutant β-lactamase toward thermal denaturation at pH 4.0, 7.0, and 10.0 were obtained by monitoring tryptophan fluorescence emission (Figure 2B). The thermal stabilities,

Table 1: Specificity Profile: Comparison of Wild-Type and K73A β-Lactamase Kinetic Parameters for Different Penicillin and Cephalosporin Substrates[a]

	wild-type			K73A			
substrate	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}/K_m
furylacryloylpenicillin	540	4.9×10^{-5}	1.3×10^{7}	0.11	3.0×10^{-6}	4.2×10^{4}	2.3×10^{-3}
benzylpenicillin	2650	1.2×10^{-4}	2.2×10^{7}	0.33	6.0×10^{-6}	5.5×10^{4}	2.5×10^{-3}
phenoxymethylpenicillin	2550	1.0×10^{-4}	2.5×10^{7}	0.33	1.5×10^{-6}	2.2×10^{5}	8.9×10^{-3}
nitrocefin	500	6.0×10^{-5}	8.0×10^{6}	0.28	2.3×10^{-4}	1.2×10^{3}	1.5×10^{-4}
PADAC	667	1.4×10^{-4}	4.6×10^{6}	1.4×10^{-3}	2.1×10^{-6}	673	1.5×10^{-4}
cefotaxime	63	1.6×10^{-3}	3.6×10^{4}	1.6×10^{-2}	8.1×10^{-3}	19.3	5.4×10^{-4}

[a] Conditions were 30 °C, pH 8.0, 0.5 M KCl.

Table 2: Kinetic Parameters at pH 7.0 for Wild-Type vs K73A β-Lactamase[a]

	wild-type			K73A		
	FAP	nitrocefin	benzylpenicillin	FAP	nitrocefin	benzylpenicillin
k_{cat} (s^{-1})	1060	1390	2650	0.017	ND	0.052
K_m (μM)	56	100	120	15	ND	3.8
k_{cat}/K_m (s^{-1} M^{-1})	1.6×10^{7}	1.84×10^{7}	2.15×10^{7}	11700	425	14000

[a] Conditions were 30 °C, 0.5 M KCl/0.05 M sodium phosphate buffer. For nitrocefin, the high value of K_m precluded accurate measurement of the individual values of k_{cat} and K_m.

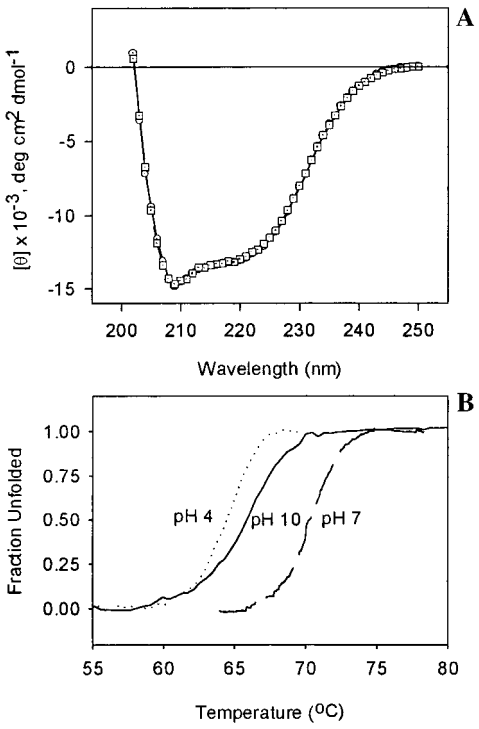


FIGURE 2: Effects of the K73A mutation on the structural properties of β-lactamase. Top panel: comparison of the far-UV circular dichroism spectra of wild-type (squares) and K73A (circles), pH 7.0, 30 °C. Lower panel: effect of pH on the thermal stability of K73A β-lactamase. The thermal unfolding was monitored by tryptophan fluorescence at pH 7.0, 30 °C, and converted to fraction unfolded (F_U). The observed T_ms are comparable with those of the wild-type enzyme. Dotted line, pH 4.0; solid line, pH 10.0; dashed line, pH 7.0.

as represented by T_ms, suggest negligible effects of the mutation. At pH 7.0 and 10.0, the T_ms for wild-type and mutant protein were the same within experimental error (69 and 57 °C, respectively). At pH 4.0, the wild-type protein T_m (45.6 °C) was 10 °C lower than that for K73A, indicating a substantial stabilizing effect of the Lys → Ala substitution at low pH. At all pH values, the K73A protein denatured in a more cooperative fashion. The far-UV CD spectra of native and refolded thermally denatured K73A protein were compared and found to be superimposable, indicating complete reversibility of the process (data not shown). The thermal stability of the K73A β-lactamase at pH 7.0 was also monitored by CD, and a T_m of 70.0 °C was obtained, confirming the T_m determined by Trp fluorescence. Taken together, the data indicate that the substitution of Ala for Lys at position 73 does not result in significant structural perturbation.

Kinetic Characterization of K73A: Substrate Specificity Profile. Previous investigations of the effects of mutations of class A β-lactamases on the catalytic properties of the enzyme have shown that some mutations result in significant differences in the specificity of the enzyme toward penicillins compared to cephalosporins. Consequently, we chose to investigate the effects of the K73A substitution on the catalytic properties of the enzyme on a number of penicillin and cephalosporin substrates: benzylpenicillin, *N*-(2-furylacryloyl)penicillin (FAP), phenoxymethylpenicillin, nitrocefin, PADAC, cefotaxime, cephaloridine, and cefoxitin (for structures, see Figure 1). The assays were done at pH 8.0 because the pH-optima for the K73A mutant were shifted to the vicinity of pH 8.0−8.5 (see below). For the cephalosporin substrate cefoxitin, no reaction with the K73A β-lactamase could be detected. For cephaloridine, complex burst kinetics were observed. For the remaining six substrates (three penicillins, three cephalosporins), the values for k_{cat}, K_m, and k_{cat}/K_m for wild-type and mutant are summarized in Table 1. For three of the substrates, we also measured the catalytic activity as a function of pH. For these substrates, the kinetic parameters for wild-type and mutant at pH 7.0 (the pH optimum for the wild-type enzyme) are summarized in Table 2. The k_{cat} and k_{cat}/K_m values at the pH-optima for both wild-type and mutant are indicated in Table 3.

Examination of the k_{cat}/K_m data in Table 1, which is assumed to be a good measure of the overall effect of the mutation on the catalytic properties of the enzyme, reveals that at pH 8.0 the major effect of the mutation on the

Table 3: Kinetic Parameters at the pH-Optimum (Shown in Parentheses) for Wild-Type and K73A β-Lactamase[a]

	wild-type			K73A		
	FAP	nitrocefin	benzylpenicillin	FAP	nitrocefin	benzylpenicillin
k_{cat} (s^{-1})	1160 (pH 6.5)	1560 (pH 6.3)	3100 (pH 6.3)	0.47 (pH 9.3)	ND	1.2 (pH 10.0)
k_{cat}/K_m (s^{-1} M^{-1})	1.7×10^7 (pH 6.8)	1.2×10^7 (pH 7.3)	1.7×10^7 (pH 6.8)	1.4×10^5 (pH 9.2)	1180 (pH 8.3)	1.1×10^5 (pH 8.5)

[a] Conditions were 30 °C, 0.5 M KCl/0.05 M buffer. See text for buffers used.

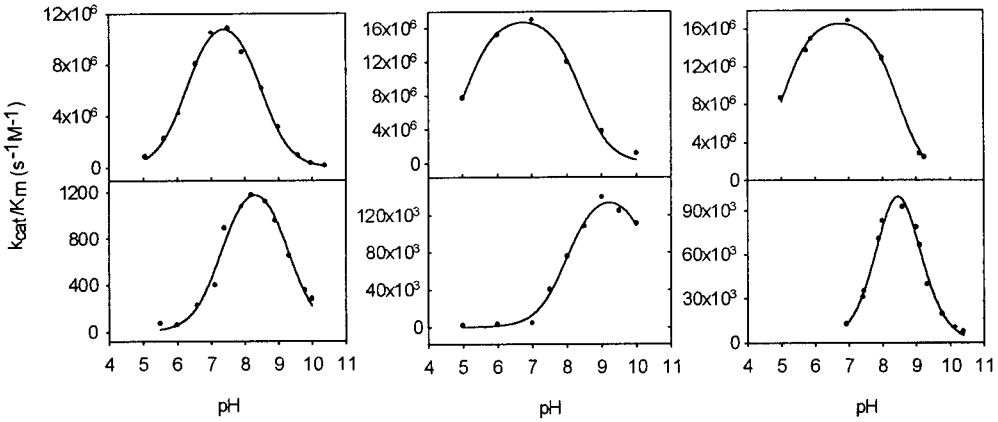


FIGURE 3: pH-dependence of k_{cat}/K_m of wild-type (top panels) and K73A (lower panels) β-lactamase at 30 °C, pH 7.0. Substrates are nitrocefin (left), FAP (center), and benzylpenicillin (right). The corresponding pK values are given in Table 4. Note the different ordinate scales for the wild-type and mutant enzymes.

enzyme's specificity is a ~10-fold greater decrease in activity toward cephalosporin substrates compared to penicillin substrates. This is mostly due to effects on K_m. The substitution of alanine for lysine resulted in a drop in K_m of 1−2 orders of magnitude for the penicillins and PADAC, and an *increase* in K_m for the other cephalosporin substrates. With the exception of PADAC, the k_{cat} values for all the substrates were decreased about the same amount in the mutant, 4000−8000-fold compared to the wild-type β-lactamase. It is important to note that these comparisons, at pH 8.0, are not comparisons at the pH-optima. If the comparisons are made at pH 7.0, the pH-optimum for the wild-type enzyme, the differences between the penicillins and cephalosporins become more prominent (Table 2). In fact, the large values of K_m prevented determination of the individual values of k_{cat} and K_m due to limited substrate solubility. However, using first-order conditions it was possible to measure the value of k_{cat}/K_m for nitrocefin. Whereas this parameter is decreased 1400−1500-fold for the penicillin substrates, it is decreased by more than 43 000-fold for nitrocefin at pH 7.0!

When comparing the kinetic parameters at the pH-optima, however, a different picture emerges, but again the effects are much more significant on the cephalosporin substrates compared to the penicillins. The pH-optima for the wild-type enzyme are in the pH 6.5−7 range, whereas for the K73A β-lactamase the optima are much higher (mostly pH 9−10). If the reductions in catalytic efficiency (k_{cat}/K_m) are compared at the pH-optima for each enzyme/substrate pair (Table 3), then for benzylpenicillin and furylacryloylpenicillin the decrease in the mutant is less than 200-fold, whereas it is 10 000-fold for the cephalosporin.

Kinetic Characterization of K73A: pH Profiles. The pH-dependence of k_{cat}/K_m for two penicillins and one cephalosporin for K73A β-lactamase is compared to that for the wild-type enzyme in Figure 3. In each case, a bell-shaped pH profile was observed. However, the K73A mutation resulted in a substantial shift of the acid limb of the curve to higher pH values for FAP and benzylpenicillin, with shifts of ≥3 pH units in pK_1. The pK_a values are listed in Table 4. Significant increases in pK_2 were also observed for the mutant compared to wild-type. The values of pK_2 for K73A β-lactamase were very consistent with a lysine residue being responsible for the ionizations reflected in these pK_as. The pH-dependence of k_{cat}/K_m reflects the pK_as of ionizable groups in the free enzyme and free substrate. Since the substrates do not have ionizable groups in the pH range where pK_1 and pK_2 are observed, these ionizations must reflect groups in the enzyme. Thus, the pK_a values should be independent of the substrates; however, this is not the case (Table 4), indicating that additional complexities may be involved. It is reasonable to assume that pK_1 for the free enzyme is approximately 8.0 for K73A β-lactamase, and that pK_2 is around 9.0. The significantly higher value for FAP could be a reflection of the potentially different mechanism at high pH (see below). The effect of varying the ionic strength on the reaction of K73A β-lactamase with FAP at pH 7.0 was investigated: no effect was observed.

Table 4: Effect of the K73A Mutation on the pK_as of Ionizing Groups Responsible for the Bell-Shaped k_{cat}/K_m vs pH Plots[a]

	wild-type		K73A	
substrate	pK_1	pK_2	pK_1	pK_2
benzylpenicillin	5.0	8.5	8.0	8.9
FAP	5.1	8.4	8.1	10.4
nitrocefin	6.2	8.5	7.3	9.3

[a] Wild-type values are from (*32*).

The pH-dependence of k_{cat} for FAP and K73A β-lactamase is compared to that for the wild-type enzyme in Figure 4; similar-shaped data were obtained with benzylpenicillin. For both wild-type and mutant enzyme, bell-shaped plots are observed. Again, very dramatic shifts to higher values in the

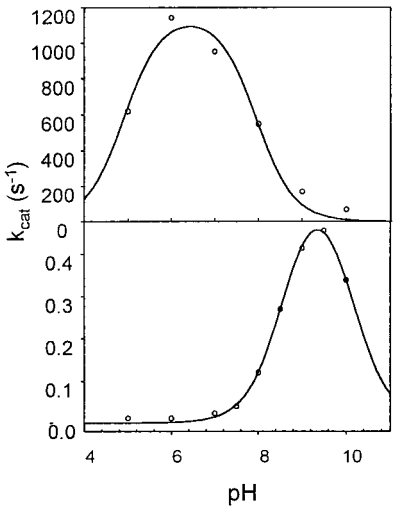


FIGURE 4: pH-dependence of k_{cat} for wild-type (top panel) and K73A (lower panel) β-lactamase with FAP at 30 °C, pH 7.0.

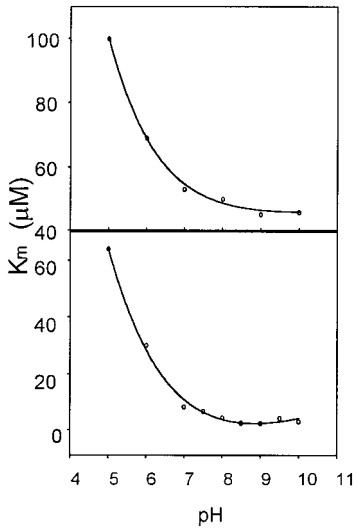


FIGURE 5: pH-dependence of K_m for wild-type (top panel) and K73A (lower panel) β-lactamase with FAP at 30 °C, pH 7.0.

pK_as are seen for the mutant. For both penicillin substrates, the value of k_{cat} for the mutant is decreased by at least 2000-fold compared to the wild-type at their respective pH-optima. Comparable data for the pH-dependence of K_m are shown in Figure 5. Interestingly, in these experiments, CHES buffer was found to inhibit K73A catalytic activity against benzylpenicillin at pH's below the pK_a of CHES.

Burst Measurements and Analyses. β-Lactamase is known to show "burst" kinetics when reacting with substrates with large $\Delta\epsilon$'s under conditions where the acylation reaction is fast and deacylation is slow, and also under conditions where a branched-pathway mechanism (Scheme 2) operates and an alternate conformation of the acyl-enzyme accumulates (*20*−*25*). In the former situation, the magnitude of the burst will be stoichiometric with the enzyme concentration (or less). On the other hand, with a branched-pathway mechanism, the magnitude of the burst may correspond to thousands of turnovers (*20, 22, 26*). Although the presence of a burst suggests that the rate-limiting step is deacylation and not acylation, for the branched-pathway mechanism, burst kinetics may also be seen under certain conditions when k_2 (acylation) is rate-limiting (*20*). Branched-pathway kinetics

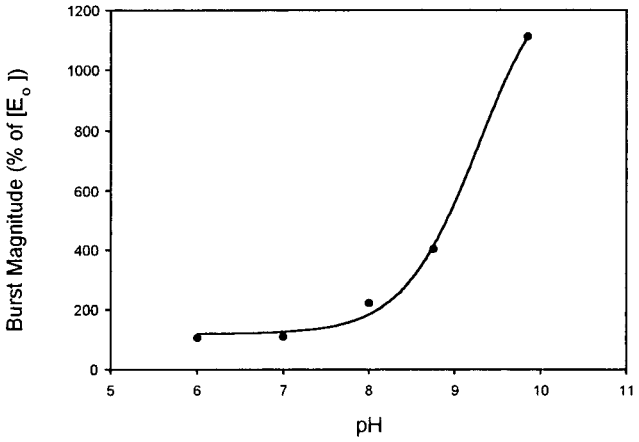
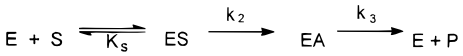
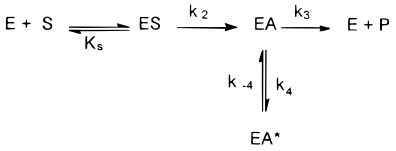


FIGURE 6: pH-dependence of the magnitude of the initial burst in the reaction of K73A β-lactamase with FAP at 30 °C. The magnitude of the burst is shown as a percentage of the enzyme concentration.

Scheme 1



Scheme 2



are manifested as an initial rate, v_i, with progressive inhibition to a steady-state reaction rate value, v_s, and may be modeled by eq 3 (*20*):

$$P = v_s t - (v_s - v_i)(1 - e^{-kt})/k \quad (3)$$

in which P is the concentration of product at time t and k is the rate constant representing the change from v_i to v_s. These parameters may be related to the underlying rate constants in Scheme 2 using the formalism developed by Waley (*20*).

Despite the large $\Delta\epsilon$ for nitrocefin, and the previous observations of bursts with other β-lactamase mutants, and with wild-type β-lactamase at subzero temperatures (*3, 24*), we were unable to detect any burst when this substrate was reacted with K73A. Progress curves consistent with normal Michaelis−Menten kinetics occurred under conditions that were varied to reveal any differences in transient and steady-state rates characteristic of branched-pathway kinetics (*20*). Thus, for the K73A/nitrocefin reaction, acylation is rate-limiting, and branched pathway kinetics are negligible.

Burst kinetics were observed, however, for the reaction between K73A and the penicillin substrate FAP. The bursts were fast, occurring within the manual mixing time of the reaction, and the burst magnitudes were determined to be stoichiometric with enzyme concentration at pH $\leq$ 7 and greater than the stoichiometric equivalent of enzyme at pH $>$ 7 (Figure 6). At higher pH, the burst magnitude increased to $>10\times$ the enzyme concentration, indicative of a branched pathway (*20, 25*) as outlined in Scheme 2. A similar result occurred with the cephalosporin substrate PADAC; the magnitude of the burst at pH 8.0 was 200 $\pm$ 10% of the stoichiometric equivalent of enzyme present. The atypical

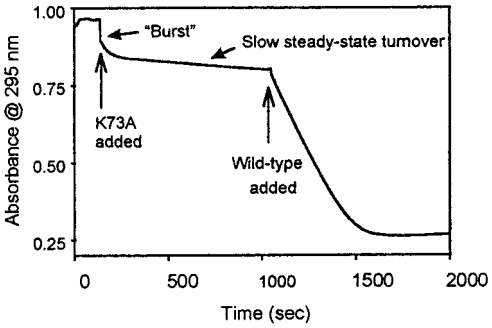


FIGURE 7: Reaction of K73A β-lactamase (10.0 μM) with cephaloridine (0.5 mM) at pH 7.0, 30 °C, showing the initial burst reaction. Wild-type β-lactamase (11.0 nM) was added at 1000 s.

kinetics of K73A with PADAC (see also Table 1) undoubtedly reflect additional complexities due to loss of the leaving group attached to C3 on formation of the acyl-enzyme (*27, 28*).

A burst with an extrapolated magnitude much larger than a stoichiometric equivalent of [E_0] also occurred with cephaloridine as substrate. A typical kinetic trace is depicted in Figure 7: this figure also shows the effect of adding wild-type β-lactamase during the steady-state part of the K73A-cephaloridine reaction, which serves as a graphic demonstration of the effect of the K73A mutation. For the wild-type β-lactamase reaction with cephaloridine, normal Michaelis−Menten kinetics are observed with no detectable burst (data not shown) (Scheme 1). Interestingly, the wild-type *Staphylococcus aureus* PC1 class A β-lactamase exhibits biphasic kinetics with cephaloridine (*27*).

With the cephalosporin substrate cefoxitin, reaction with *wild-type* β-lactamase resulted in a burst with a magnitude that was much larger than the total enzyme concentration, again consistent with a branched-pathway mechanism. However, neither a burst nor any steady-state activity could be detected with K73A β-lactamase against cefoxitin, indicating rate-limiting acylation with this cephalosporin substrate.

Burst kinetics for reactions between K73A β-lactamase and the substrates benzylpenicillin, cefotaxime, and phenoxymethylpenicillin could not be detected due to these substrates' small $\Delta\epsilon$ and the low mutant enzyme activity. Thus, to determine whether acylation or deacylation was the rate-limiting step, we turned to an alternative approach, namely, FTIR.

FTIR Analysis of the Rate-Limiting Step. The reaction of K73A β-lactamase with several substrates was monitored using attenuated total reflectance (ATR) FTIR spectroscopy. In work to be described elsewhere, we have shown that the position of the absorbance band corresponding to the carbonyl stretch frequency for noncovalent ES complexes of β-lactamase and penicillin or cephalosporin substrates is at 1755 ± 2 cm^{-1}, and that the corresponding frequencies for the acyl-enzyme are typically in the vicinity of 1740 cm^{-1} and below. Thus, it is possible, for a given combination of enzyme and substrate, to determine if acylation or deacylation is rate-limiting, from the buildup of either the ES complex (for rate-limiting acylation) or the acyl-enzyme (for rate-limiting deacylation). Some typical data for K73A β-lactamase with FAP, benzylpenicillin, cephalosporin C, and cefotaxime are shown in Figure 8. The reaction of K73A β-lactamase with the penicillins showed bands at 1740 cm^{-1}, consistent with rate-limiting deacylation. In contrast, the

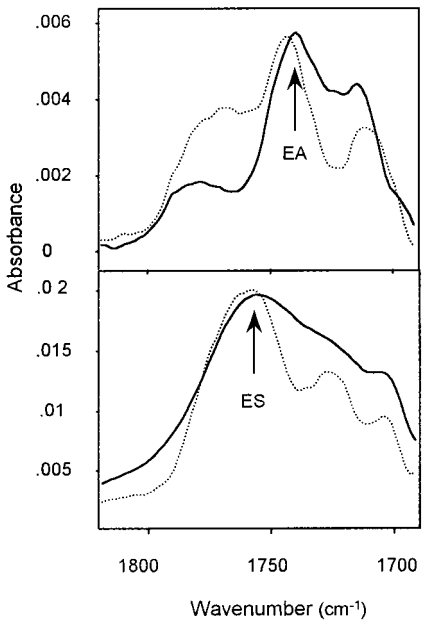


FIGURE 8: FTIR difference spectra of K73A β-lactamase with penicillins and cephalosporins. Top panel: benzylpenicillin (solid line) and FAP (dotted line). Bottom panel: cephalosporin C (solid line) and cefotaxime (dotted line). These difference spectra were obtained by taking the spectrum of the mixture of enzyme (45 μM) plus substrate (450 μM) 2 min after mixing at pH 6.0, and subtracting the spectrum of the enzyme and substrate alone under identical conditions.

difference spectra for the cephalosporin substrates showed substantial absorbance at 1755 cm^{-1}, consistent with rate-limiting acylation. This relationship was also observed for a number of other penicillin and cephalosporin substrates examined. The FTIR data thus confirm the kinetic results suggesting that acylation is the rate-limiting step for penicillin substrates with K73A β-lactamase, whereas deacylation is the rate-limiting step for cephalosporins.

DISCUSSION

Since Lys73 is such a highly conserved residue in the class A β-lactamases and related penicillin-sensitive enzymes, one would expect it to play a very critical role in the reaction of these enzymes with β-lactams. Thus, there are two very unexpected findings in our results: the values of k_{cat}/K_m for penicillins at the pH-optima for the K73A mutant are only decreased by 100−200-fold over the values for the wild-type β-lactamase, and the plots of k_{cat}/K_m for K73A β-lactamase are bell-shaped, as they are with the wild-type, although there are significant shifts in the corresponding pK_as. In particular, pK_1 is shifted from a value of ~5.0 in the wild-type to ~8.0 in the K73A mutant. Substantial increases in pK_2 were also observed, which has shifted from 8.5 with the wild-type to the 9−10 region for K73A β-lactamase. Our data also indicate significant differences in the effects of this mutation on the catalyzed hydrolysis of penicillins compared to cephalosporins, in particular, that the rate-limiting step is deacylation for penicillin substrates and acylation for cephalosporins. The remaining key observation is the presence of greater than stoichiometric bursts with several of the substrates, indicating an underlying branched-pathway kinetic scheme with the K73A mutant. The implications of these findings on the catalytic mechanism of the class A β-lactamases are discussed in detail below.

The mutation-induced shifts in the pH-dependence of the catalytic parameters, which result in significant shifts in the pH-optima, and large differences in catalytic activity between pH 7 and pH 8 demonstrate that kinetic characterization at a variety of pH values is essential in analyzing the effects of mutations on enzyme function.

Different Rate-Limiting Steps for Penicillin and Cephalosporin Substrates with K73A β-Lactamase. Several observations suggest that for K73A β-lactamase the rate-limiting step is acylation with cephalosporin substrates and deacylation with penicillin substrates. In contrast, both acylation and deacylation rates are similar in the wild-type (*30*, *31*). The lack of an observed burst in the reaction of K73A β-lactamase with nitrocefin is strong evidence for rate-limiting acylation, as is the lack of reaction of K73A with cefoxitin. The clearest evidence for rate-limiting acylation for cephalosporins and rate-limiting deacylation for penicillins comes from the FTIR data. The effects of the K73A mutation on K_m are also most readily explained by rate-limiting acylation for cephalosporins and rate-limiting deacylation for penicillins. For example, if the rate-limiting step for cephalosporins is acylation, then $K_m = K_s$, and if the rate-limiting step for penicillins is deacylation, then $K_m = (k_3/k_2)K_s$. Thus, the much smaller values of K_m for the penicillin substrates with K73A β-lactamase reflect the fact that K_m is decreased by the k_3/k_2 ratio, a value $\ll 1$ by definition, if deacylation is rate-limiting. The observed K_m for the cephalosporin substrates represents the substrate dissociation constant, K_s, which is clearly in the millimolar range. One possible molecular basis for a decrease in the rate of deacylation with the mutant is that the deacylating water molecule is not correctly positioned, due to changes in the position of Glu166 due to the absence of Lys73 (in the wild-type enzyme these two residues are within H-bonding distance).

A complication arises in the cases of the cephalosporins PADAC and cephaloridine, which have a potential leaving group at position C3, since these may form longer-lived acyl-enzyme intermediates following loss of the C3 leaving group, leading to apparent burst reactions, which are also observed with the wild-type enzyme (*27*). This change in mechanism, due to the facile loss of the C3 group from the initially formed acyl-enzyme to yield a long-lived modified acyl-enzyme, also accounts for the uniquely large decrease in k_{cat} for PADAC.

Origins of the pK_as in K73A β-Lactamase Catalysis. The most notable aspect of the k_{cat}/K_m vs pH curves for the K73A mutant compared to the wild-type enzyme, with penicillin substrates in particular, is that they are so similar except for the 3 unit shift to higher values for pK_1. Since Lys73 is absent in the K73A mutant, these bell-shaped transitions cannot reflect the ionization of Lys73. The variation in pK_a observed for k_{cat}/K_m vs pH in the K73A mutant may reflect changes in the catalytic mechanism, for example, from the simple acyl-enzyme scheme to a branched-pathway scheme (Schemes 1 and 2 respectively). As will be discussed in more detail below, we attribute pK_1 in the k_{cat}/K_m vs pH plots for K73A (and wild-type) to Glu166. Thus, the major effect of the mutation at residue 73 is on the pK_a of Glu166.

The fact that the plots of k_{cat}/K_m vs pH show an alkaline limb similar to that for the wild-type enzyme, except for a somewhat higher pK_2, indicates that Lys73 cannot be (solely) responsible for the observed pK_2s in the wild-type enzyme. Thus, pK_2 must be due to some group other than Lys73—perhaps Lys234, since clearly the ionization reflects a group with a high pK_a. Alternatively, the observed pK_2 for K73A could reflect a different group from that in wild-type β-lactamase catalysis. Thus, there are four possibilities for the group(s) responsible for pK_2 in the wild-type: it could be due to Lys73, Lys234, both Lys73 and Lys234, or another residue such as Ser130 or a water molecule. The results of the present investigation, especially with benzyl- and furyl-acryloyl-penicillin, are inconsistent with the first possibility. Since we have previously shown that the K234A (and K234E) mutants show similar catalytic properties to the K73A mutant, with the exception of a very broad pH profile for k_{cat}/K_m (because pK_1 is shifted to lower values than in the wild-type, and pK_2 is marginally increased), the second possibility can be eliminated (*32*). Thus, we are left with two possibilities: either the alkaline limb for the wild-type enzyme reflects the ionization of both Lys73 and Lys234 or it represents the ionization of another group. In the former case, we are presented with two options: in the wild-type enzyme, either both lysine residues participate in catalysis, or only one does, but it can be replaced by the other. Since plots of k_{cat}/K_m vs pH indicate only a single ionizing group is responsible for pK_2, it would seem that either lysine can replace the other if one is missing. We have shown previously (*3*) that the heat of ionization of the group responsible for pK_2 in the wild-type enzyme is large (10 kcal/mol), and indicative of a lysine residue. Finally, the results with the K234H mutant in *S. albus* G β-lactamase suggest that pK_2 may reflect the pK_a of residue 234 (*33*) [although this is not true for the *B. licheniformis* enzyme, in which conversion of Lys234 to either Ala or Glu had little effect on pK_2 (*32*)].

In the case that pK_2 is not due to Lys73 or Lys234, the most likely candidates for the group responsible for pK_2 are Ser130 or a water molecule. Both would normally have pK_a values much higher than the pK_2 observed for K73A; however, it is possible that the active-site environment perturbs them substantially, but it is highly unlikely that they would be shifted as low as 8.0 as in the wild-type. Further, mutations of Ser130 have established that this residue is not as essential as might be expected if it were the source of pK_2 (*10*, *34*). In addition, the pH-dependence of the group responsible for pK_2 is active in its acidic state, which makes it most unlikely that it would be either water or serine.

The interpretation of the pH-dependence of k_{cat} is somewhat complicated since it reflects the pK_as in enzyme—substrate complexes, and will also be affected by any changes in the rate-limiting step, and possibly by other aspects of a complex mechanism. However, in general the effects of the K73A mutation on k_{cat} are similar to those on k_{cat}/K_m, namely, a substantial increase in pK_1.

Thus, we conclude that pK_2 in the K73A mutant reflects the ionization of Lys234. This residue makes several key hydrogen bonds in the active site, including one to Ser130, and is highly conserved in the serine β-lactamases. This analysis then raises the issue of the group responsible for pK_2 in the wild-type enzyme. The replacement of either lysine (73 or 234) by alanine leads to a decrease in k_{cat}/K_m of about 100-fold for good penicillin substrates and major shifts in pK_1 compared to the wild-type enzyme. Replacing

Lys234 with His in *Streptomyces albus* G β-lactamase led to a substantial shift in pK_2 for k_{cat}/K_m from 9.9 with the wild-type to 6.3 with K234H with benzylpenicillin, but only a 10-fold decrease in magnitude at the pH-optimum. This observation suggests that pK_2 reflects K234 in the wild-type enzyme. Consistent with this, replacement of Lys234 by Arg in the TEM β-lactamase was reported to have little effect on the reaction with penicillin substrates (*35*). There are two structural features that Lys73 and Lys234 have in common that are relevant to the catalytic mechanism: they both are hydrogen-bonded to Ser130, and they both have positively charged side chains. Thus, either Lys could, in principle, provide a positively charged electrostatic environment around the region of the substrate undergoing reaction, and both could shuttle a proton to Ser130. The mechanistic implications of this are considered in the following section. It is very likely that the position normally occupied by the ammonium group of Lys73 will be occupied by a water molecule in the K73A mutant. Such a water molecule would be expected to be able to substitute for the lysine side chain if it acted as an acid/base catalysis, whereas if its main role were electrostatic, then the substituting water would be unable to undertake that role. It is clear that electrostatic interactions are an important component of β-lactamase catalysis.

We now turn to consideration of the expected effect of the K73A mutation on the pK_a of Glu166. Assignment of pK_1 to Glu166 means that its pK_a has been increased by ~3 units in the mutant compared to the wild-type. In the catalytic reaction of the wild-type β-lactamase, pK_1 (5.0) is virtually unperturbed from the intrinsic value (e.g., propionate pK_a = 4.9), assuming that it is due to Glu166. Structural analysis indicates that Glu166 is hydrogen-bonded to Lys73 (*12*). The solvent accessibility of Glu166 (and Lys73) is very low; consequently, with the removal of the neighboring ammonium group from Lys73 in the K73A mutant, the environment around the Glu166 carboxyl will become significantly more nonpolar, consistent with a substantial increase in its pK_a. Thus, the anticipated environmental effects on Glu166 due to the elimination of the Lys73 ϵ-amino group are as follows: (i) removal of the proximal ammonium group, with the possible introduction of a water molecule to take its place, resulting in an alkaline shift in the pK_a, as observed; (ii) possible reorientation of Glu166 to closer association with Asn170, stabilizing charge distribution, and possibly dislocating the putative deacylating water molecule, creating a less favorable orientation for deacylation; (iii) Asn132 has been implicated in ground-state recognition as well as indirect involvement in the catalytic mechanism (*36, 37*). Since Lys73 is also hydrogen-bonded to Asn132 (*12*), elimination of the ϵ-amino group might allow Asn132 to reorient itself to interact with Glu166, possibly contributing to the increase in its pK_a. The lack of hydrogen bonding between Asn132 and Ala73 in the K73A mutant may allow it to reorient to form a stronger bond with substrate. Given the preceding factors, the observed increase in the pK_a assigned to Glu166 seems reasonable. Attributing pK_1 to Glu166 means the side-chain carboxyl has a pK_a of 8.5 in K73A—this is very high for a glutamate but not without precedent (*38*).

Branched-Pathway Kinetics. The observed magnitude of the bursts for the K73A/FAP reaction is equal to the stoichiometric equivalent of enzyme concentration up to pH 7.0, and then increases exponentially to pH 9.8 (Figure 6). The simplest interpretation is that $k_3 \ll k_2$ at all pHs. The pH-dependence of both k_{cat} and the burst magnitude reflects the pH-dependence of k_2 and/or k_3, and presumably the ionization of Glu166. We have previously shown with the E166C mutant that branched-pathway kinetics are observed with a magnitude equivalent to the enzyme concentration (*25*): thus, in the present case, it is likely that the branched-pathway scheme also operates at pH <7, but at higher pH values the k_3/k_4 ratio (Scheme 2) increases, due to the pH-dependence of k_3.

The question arises as to why branched-pathway kinetics are observed, e.g., with FAP/K73A. As discussed in the context of the E166C mutant (*25*), we believe that that in any enzyme/substrate combination in which the deacylation reaction (k_3) is sufficiently slow, the acyl-enzyme will isomerize into the alternate more stable conformation (EA* in Scheme 2). Thus, the observation of the branched pathway in the case of K73A β-lactamase is consistent with rate-limiting deacylation.

Models for the β-Lactamase Mechanism. Any reasonable mechanistic proposal for the catalytic mechanism of the class A β-lactamases must account for the conserved nature of Lys73 in all penicillin-sensitive enzymes. This evolutionary conservation suggests a role either in acylation and/or in binding, but does not necessarily preclude an evolutionarily subsequent role in deacylation as well in the class A enzymes. Although it has generally been assumed that the role of Lys73 is mainly in the acylation half of the catalytic reaction, our data suggest that it also may play a very substantial role in deacylation. In previous investigations into the catalytic mechanism, two types of roles have been ascribed to Lys73: as a general acid, participating in a proton shuttle to protonate the lactam nitrogen during formation of the acyl enzyme (*39*); or as a general base, activating Ser70 for nucleophilic attack on the β-lactam carbonyl (*8*).

Unlike the serine proteases, which catalyze a very similar reaction to β-lactamase, there is no clear-cut acid/base catalyst in β-lactamase equivalent to His57 in the serine proteases. In the serine proteases, His57 initially activates the serine O-γ in the nucleophilic attack on the carbonyl and then also supplies the proton to the leaving nitrogen. In the β-lactamases, there are two significantly different factors involved in the acylation reaction: the substrate amide is in the four-membered lactam ring which is more strained than a typical peptide bond, and the attacking serine/substrate carbonyls are in the positive electrostatic field of the dipole of helix-2, both of which would be expected to make the acylation reaction significantly more facile in the β-lactamases. However, Page and co-workers have provided considerable data to indicate that the nucleophilic attack by a protonated nucleophile, such as Ser70, would definitely require the assistance of (general) base catalysis (*40*).

For the reasons elaborated below, we believe that the main role of Lys73 is in facilitating the protonation of the lactam leaving group in acylation and of the Ser70 O-γ leaving group in deacylation. This is most likely effected through electrostatic effects on other residues, rather than direct participation by the lysine. For example, the substantial activity of K73R β-lactamase (*6*) implies that the positive

charge is important either directly or less likely as a general acid.

It has been suggested that Lys73 has an unusually low pK_a and that it acts as a general base catalyst in the catalysis; i.e., it is responsible for pK_1 with the wild-type enzyme (*8*, *41*). However, several observations indicate that Lys73 is unlikely to act as a general base in activating Ser70 for nucleophilic attack, for example, the limited impact on catalysis of the substitution by Arg, which cannot act as a general base catalyst (*6*), the observed effects on pK_1 (which would reflect the pK_a of Lys73 were it to act as a general base) for mutations at Lys234 (*32*), and a pK_a of Lys73 in the free enzyme of >10 by direct measurement (*14*). In addition, calculations suggest that Lys73 will have a p$K_a > 10$ in both free enzyme and the ES complex (*42*, *43*).

The most likely base catalyst in activating Ser70 is Glu166, either directly or via a bridging water molecule. In the free enzyme, the distance between Glu166 and Ser70 O-γ is too far for direct proton transfer; however, there is a conserved water molecule in hydrogen bonding distance of Glu166 and Ser70. The flexibility of the omega loop could potentially allow Glu166 to act as the general base in acylation; it is also possible that binding of the substrate results in displacement of the water bound to Glu166 and Ser70 in the free enzyme (*11*). Mutations at Glu166 have been shown to result in a greater effect on deacylation than acylation, but the rate of acylation is also decreased significantly in Glu166 mutants. The simplest mechanism would involve the bridging water as a transient proton acceptor to form the tetrahedral transition state/intermediate. The partitioning of this species is determined by the relative rates of C−O and C−N cleavage, the latter leading to the acyl-enzyme, the former returning to the ES complex. Partitioning toward products will be facilitated by proton donation to the lactam nitrogen. Page (*40*) has shown that nucleophilic attack on the β-lactam carbonyl occurs from the exo face, and thus the β-lactam nitrogen lone pair is syn to the incoming nucleophile in the tetrahedral intermediate/transition state. This would favor the same residue acting as the general base to extract the serine O-γ and as the general acid to donate the proton to the nitrogen. Although it has been suggested that Glu166 is also responsible for this proton donation (*40*), this seems unlikely due to the long distance between the carboxyl and the nitrogen. However, it is possible that the bridging water could act as a proton shuttle between Glu166 and the lactam N.

The side chain of Glu166 in the acyl-enzyme is well-positioned to act as a general base in activating the deacylating water molecule for its attack on the acyl group. Mutagenesis experiments on Glu166 have generally shown smaller effects on acylation and major effects on deacylation rates (*5*−*7*, *44*−*46*). The fact that deacylation is rate-limiting in the reaction of penicillins with K73A β-lactamase most likely reflects the fact that the role of Lys73 as a proton donor is more critical in deacylation than in acylation. From the TEM β-lactamase acyl-enzyme structure (*8*), Lys73 is within hydrogen bonding distance of Ser70 O-γ, and could thus directly donate a proton during collapse of the tetrahedral transition state in deacylation (Lys234 is too far away to do this). However, Ser130 is also within H-bonding distance of the Ser70 O-γ, so a proton shuttle could also operate in deacylation, in which case Lys234 might be able to substitute for the missing Lys73 in the K73A mutant.

From examination of the acyl-enzyme structure of the class A TEM β-lactamase (*8*), the ϵ-amino nitrogen atom of Lys73 is 3.2 Å from the Ser-O-γ, an eminently reasonable distance for the ammonium group to donate a proton the leaving serine group. Lys234 is located 4.6 Å from the Ser70 O-γ in this structure, and thus not in a good position to act as the proton donor. However, in the K73A mutant it is possible that Lys234 moves closer and acts as a surrogate proton donor for Lys73. As has been noted before, Lys73 is very close to the side chain of Ser130 and is well-positioned to participate in a proton shuttle in acylation in which a proton that is transferred from Ser130 to the lactam nitrogen is replenished by one from Lys73, which is in turn replenished by a proton from solvent. Interestingly, Lys234 is situated about the same distance from Ser130 O-γ as Lys73, and thus could take the place of Lys73 in the K73A mutant in this proton shuttle. It is also possible that Ser130 could be the ultimate proton donor to the O-γ of Ser70 in the deacylation step.

Conclusions. Although the results of the present investigation are very interesting, they do little to dispel the "enigmatic" nature of the mechanism of β-lactamase catalysis (*1*). Our results indicate that there are significant differences in the K73A β-lactamase-catalyzed hydrolysis of different β-lactam antibiotics. In particular, the rate-limiting step is acylation for cephalosporins and deacylation for penicillins. We believe the major role of Lys73 is in the protonation steps in both the acylation and deacylation reactions, namely, in donating a proton to the lactam N in the acylation reaction and to the O-γ of Ser70 in the acyl-enzyme in the deacylation reaction. Our data indicate that Lys73 and Glu166 work in concert, modulating each other in both acylation and deacylation. In the absence of the ϵ-amino group of Lys73, a water molecule may serve as a less efficient substitute in the proton shuttle, but cannot substitute for the loss of the positive charge of Lys73.

ACKNOWLEDGMENT

We thank V. Uversky for assistance with the circular dichroism experiments, Joshua Nadherny for the ionic strength effect experiments, and Michael Page for useful discussions.

REFERENCES

1. Galleni, M., Lamotte-Brasseur, J., Raquet, X., Dubus, A., Monnaie, D., Knox, and Frere (1995) *Biochem. Pharmacol. 49*, 1171−1178.
2. Virden, R., Tan, A. K., and Fink, A. L. (1990) *Biochemistry 29*, 145−153.
3. Cartwright, S. J., Tan, A. K., and Fink, A. L. (1989) *Biochem. J. 263*, 905−912.
4. Murphy, B. P., and Pratt, R. F. (1988) *Biochem. J. 256*, 669−672.
5. Escobar, W. A., Tan, A. K., and Fink, A. L. (1991) *Biochemistry 30*, 10783−10787.
6. Gibson, R. M., Christensen, H., and Waley, S. G. (1990) *Biochem. J. 272*, 613−619.
7. Adachi, H., Ohta, T., and Matsuzawa, H. (1991) *J. Biol. Chem. 266*, 3186−3191.
8. Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., and James, M. N. G. (1992) *Nature 359*, 700−705.

9. Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frere, J. M., and Ghuysen, J. M. (1991) *Biochem. J. 279 (Pt 1)*, 213-221.
10. Juteau, Billings, E., Knox, and Levesque (1992) *Protein Eng. 5*, 693−701.
11. Vijayakumar, S., Ravishanker, G., Pratt, R. F., and Beveridge, D. L. (1995) *J. Am. Chem. Soc. 117*, 1722−1730.
12. Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., and Frere, J. M. (1990) *Protein: Struct., Funct., Genet. 7*, 156−171.
13. Adediran, Deraniyagala, Xu, Y., and Pratt (1996) *Biochemistry 35*, 3604−3613.
14. Damblon, C., Raquet, X., Lian, Lamotte-Brasseur, J., Fonze, E., Charlier, P., Roberts, and Frere (1996) *Proc. Natl. Acad. Sci. U.S.A 93*, 1747−1752.
15. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
16. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) *Proc. Natl. Acad. Sci. U.S.A 86*, 2172−2175.
17. Sanger, F. (1981) *Science 214*, 1205−1210.
18. Schmid, F.-X. (1989) in *Protein Structure: A Practical Approach* (Creighton, T. E., Ed.) p 320.
19. Ambler, R. P., Coulson, A. F., Frere, J. M., Ghuysen, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., and Waley, S. G. (1991) *Biochem. J. 276*(Pt 1), 269−270.
20. Waley, S. G. (1991) *Biochem. J. 279*(Pt 1), 87−94.
21. Kiener, P. A., Knott-Hunziker, V., Petursson, S., and Waley, S. G. (1980) *Eur. J. Biochem. 109*, 575−580.
22. Citri, N., Samuni, A., and Zyk, N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1048−1052.
23. Fink, A. L., Behner, K. M., and Tan, A. K. (1987) *Biochemistry 26*, 4248−4258.
24. Virden, R., Tan, A. K., and Fink, A. L. (1990) *Biochemistry 29*, 145−153.
25. Persaud, K. C., Pain, R. H., and Virden, R. (1986) *Biochem. J. 237*, 723−740.
26. Escobar, W. A., Tan, A. K., Lewis, E. R., and Fink, A. L. (1994) *Biochemistry 33*, 7619−7626.
27. Faraci, W. S., and Pratt, R. F. (1985) *Biochemistry 24*, 903−910.
28. Faraci, W. S., and Pratt, R. F. (1986) *Biochemistry 25*, 2934−2941.
29. Banerjee, S., Pieper, U., Kapadia, G., Pannell, L. K., and Herzberg, O. (1998) *Biochemistry 37*, 3286−3296.
30. Christensen, H., Martin, M. T., and Waley, S. G. (1990) *Biochem. J. 266*, 853−861.
31. Matagne, A., Dubus, A., Galleni, M., and Frere, J.-M. (1999) *Nat. Prod. Rep. 16*, 1−19.
32. Ellerby, L. M., Escobar, W. A., Fink, A. L., Mitchinson, C., and Wells, J. A. (1990) *Biochemistry 29*, 5797−5806.
33. Brannigan, J., Matagne, A., Jacob, F., Damblon, C., Joris, B., Klein, D., Spratt, B. G., and Frere, J. M. (1991) *Biochem. J. 278*, 673−678.
34. Jacob, F., Joris, B., Lepage, S., Dusart, J., and Frere, J. M. (1990) *Biochem. J. 271*, 399−406.
35. Lenfant, F., Labia, R., and Masson, J. M. (1991) *J. Biol. Chem. 266*, 17187−17194.
36. Jacob, F., Joris, B., Dideberg, O., Dusart, J., Ghuysen, J. M., and Frere, J. M. (1990) *Protein Eng. 4*, 79−86.
37. Osuna, J., Viadiu, H., Fink, A. L., and Soberon, X. (1995) *J. Biol. Chem. 270*, 775−780.
38. Wilson, N. A., Barbar, E., Fuchs, J. A., and Woodward, C. (1995) *Biochemistry 34*, 8931−8938.
39. Lamotte-Brasseur, J., Dive, G., Dehareng, D., and Ghuysen, J. M. (1990) *J. Theor. Biol. 145*, 183−198.
40. Page, M., and Laws, A. P. (1998) *Chem. Commun.*, 1609−1617.
41. Swaren, P., Maveyraud, L., Guillet, V., Masson, Mourey, L., and Samama (1995) *Structure 3*, 603−613.
42. Raquet, X., Lounnas, V., Lamotte-Brasseur, J., Frère, J.-M., and Wade, R. C. (1997) *Biophys. J. 73*, 2416−2426.
43. Lamotte-Brasseur, J., Lounnas, V., Raquet, X., and Wade, R. C. (1999) *Protein Sci. 8*, 404−409.
44. Delaire, M., Lenfant, F., Labia, R., and Masson, J. M. (1991) *Protein Eng. 4*, 805−810.
45. Leung, Robinson, Aplin, and Waley (1994) *Biochem. J. 299 (Pt 3)*, 671−678.
46. Guillaume, G., Vanhove, M., Lamotte-Brasseur, J., Ledent, P., Jamin, M., Joris, B., and Frere, J. M. (1997) *J. Biol. Chem. 272*, 5438−5444.

BI992681K