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Dasantila Golemi-Kotra
University of Notre Dame

Samy O. Meroueh
University of Notre Dame

Choonkeun Kim
University of Notre Dame

Sergei B. Vakulenko
University of Notre Dame

Alexey Bulychev
University of Notre Dame

See next page for additional authors

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Dasantila Golemi-Kotra,† Samy O. Meroueh,† Choonkeun Kim,† Sergei B. Vakulenko,† Alexey Bulychev,† Ann J. Stemmler,† Timothy L. Stemmler,‡* and Shahriar Mobashery†*

†Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA ‡Department of Biochemistry and Molecular Biology, Wayne State University, Detroit, MI 48202, USA

*Corresponding Author: Tel. (574) 631-2933, Fax: (574) 631-6652, E-mail: mobashery@nd.edu

Abstract

β-Lactamases and penicillin-binding proteins are bacterial enzymes involved in antibiotic resistance to β-lactam antibiotics and biosynthetic assembly of cell wall, respectively. Members of these large families of enzymes all experience acylation by their respective substrates at an active-site serine as the first step in their catalytic activities. A Ser-X-X-Lys sequence motif is seen in all these proteins and crystal structures demonstrate that the side chain functions of the serine and lysine are in contact with one another. Three independent methods were used in this report to address the question of the protonation state of this important lysine (Lys73) in the TEM-1 β-lactamase from *Escherichia coli*. These techniques included perturbation of the pK_a of Lys73 by the study of the γ-thialysine-73 variant and the attendant kinetic analyses, investigation of the protonation state by titration of specifically labeled proteins by nuclear magnetic resonance and by computational treatment using the thermodynamic integration method. All three methods indicated that the pK_a of Lys73 of this enzyme is attenuated to 8.0-8.5. It is argued herein that the unique ground-state ion pair of Glu166 and Lys73 of class A β-lactamases has actually raised the pK_a of the active site lysine to 8.0-8.5 from that of the parental penicillin-binding protein. Whereas we cannot definitively rule out that Glu166 activates the active site water, which in turn promotes Ser70 for the acylation event, such as proposed earlier, we would like to propose as a plausible alternative for the acylation step the possibility that the ion pair would reconfigure to the protonated Glu166 and unprotonated Lys73. As such, unprotonated Lys73 could promote serine for acylation, a process that should be shared among all active-site-serine β-lactamases and penicillin-binding proteins.

Introduction

A number of enzymes have evolved a catalytic strategy that depends on a transient acylation of an active site serine. The catalytic serine residue in these enzymes is followed by a lysine three residues toward the C-termini of the proteins (i.e., …Ser-X-X-Lys…). This sequence motif is seen in serine-dependent β-lactamases and penicillin-binding proteins (PBPs),[1] of which several hundred members are known. The catalytic implication of this Ser-X-X-Lys sequence motif for β-lactamases is debated in the literature, but the role of these residues in catalysis is likely to be general for the large group of proteins that share this sequence.

β-Lactamases are bacterial resistance enzymes to β-lactam antibiotics, which include penicillins and cephalosporins. Members of the class A β-lactamases are the most common among pathogenic bacteria. These enzymes undergo acylation and deacylation at Ser70 during substrate turnover ([i],[ii]). The process of deacylation of the acyl-enzyme intermediate is best understood. Glu166 appears to be the active-site general base that promotes a water molecule in the deacylation step ([iii],[iv],[v]). On the other hand, how the active-site serine experiences acylation is an issue of much debate (iii,iv,[vi],[vii],[viii],[ix],[x],[xi],[xii],[xiii]).

The contention stems largely from a lack of clear knowledge of the titration states of the active-site residues involved in catalysis. The active-site moieties that could undergo titration are Glu166, Lys73, and Lys234. Two groups have suggested that Lys73 is not protonated and can serve as the general base in activation of Ser70 during its acylation (iv,vi). Others believe that a positively charged Lys73 would necessitate other catalytic strategies by these enzymes (viii,ix,x,xi,xii). We present herein experimental evidence that indicates that the active site lysine in the class A TEM-1 β-lactamase from *Escherichia coli* has a lower pK_a than a typical lysine. The pK_a of Lys73 was also determined computationally using the molecular dynamics–based thermodynamic integration method. The results were in good agreement with experiments. Further calculations were carried out on the Glu166Ala mutant to determine the influence of Glu166 on the pK_a of Lys73. It was found that the pK_a of Lys73 in the Glu166Ala mutant was reduced to 6.0, arguing that the proximity of Glu166 to Lys73 has raised its pK_a to 8.0-8.5 in class A β-lactamases. Based on these findings, we propose as a plausible alternative mechanism the possibility of a protonated Glu166 and an unprotonated Lys73. The unprotonated Lys73 would promote the active site serine for acylation by the substrate. This notion unifies the mechanism(s) for serine acylation by substrates for all active-site serine β-lactamases and PBPs.

Experimental Procedures

Materials—Penicillin G was purchased from Sigma. Mezlocillin was the generous gift of Dr. Robert Bonomo. The growth medium was purchased either from Difco Laboratories (Detroit, Michigan) or Fisher Scientific. The chromatography media were from Bio-Rad Laboratories. Isolation and purification of all enzymes (wild-type and mutants) were carried out as described earlier (xiv). The 99% $^{15}NH_4Cl$ was purchased from Isotec Inc., and [6-^{13}C] L-lysine (99%) was from Cambridge Isotope Laboratories.

Generation of the mutant TEM-1 β-lactamases—Mutagenesis of Lys73 of the TEM-1 β-lactamase to Ala was accomplished by using QuikChange Site-Directed Mutagenesis Kit (Stratagene®), in accordance with manufacturer's recommendations. The TEM-1 β-lactamase gene that was previously subcloned into an expression vector pET24a(+) was mutated using two primers: forward, 5'-CAATGCTGCGCACTTTTGCAGTTCTG-CTATGTGGC-3', and reverse, 5'-GCCACATAGCAGAACTGCAAAAGTGCTCATCATTG-3' (the codon for alanine is underlined) . Mutated DNA was used to transform *E. coli* JM83 and selection of transformants was performed on agar supplemented with kanamycin A (30 μg/ml). DNA from several transformants were initially screened by digestion with the restriction endonuclease *Dra*I (this site was removed by mutation). Subsequently the nucleotide sequence of the entire gene was verified.

The double mutant Cys77Ser/Cys123Ala and the triple mutant Lys73Cys/Cys77Ser/Cys123Ala of the TEM β-lactamase were generated using the pTZ19-4 vector essentially as described earlier (xv). First, Cys123Ala substitution was generated, utilizing primer TEM123 (5'-GGTTATGGCAGCACTGGCTAATTCTCTTACTG-3'; mutated codon underlined). Subsequently Cys77Ser substitution was introduced using primer TEM77 (5'-AATACCGCGCCAGATAGCAGAACTTTAAAAG-3'; mutated codon underlined) to produce the double-mutant enzyme. Finally, Lys73 was mutated to cysteine with primer TEM73 (5'-CGCCAGATAGCAGAACACAAAAAGTGCTCATC-3'; mutated codon underlined). Both DNA strands of the mutant genes were sequenced.

Cloning of mutant TEM b-lactamase genes into expression vector and purification of enzymes—To facilitate b-lactamase secretion into the growth medium, we fused the PCR-generated genes for the Cys77Ser/Cys123Ala and Lys73Cys/Cys77Ser/Cys123Ala mutant b-lactamases with the leader sequence of the OmpA protein in the pSV106 vector, as described earlier (xiv). After resequencing of the entire genes, they were recloned into the *Nde*I-*Hind*III sites of the pET24 expression vector. Growth conditions for protein induction and purification were essentially the same as described recently (xiv).

Labeling (^{15}N and ^{13}C) of enzymes and protein preparation—To prepare the proteins uniformly labeled with ^{15}N, we utilized *E. coli* BL21DE3 and *E. coli* BL21DE3 pLysS strains, carrying vector pET24a(+) with the TEM-1 β-lactamase and the Lys73Ala mutant β-lactamase genes, respectively. Strains were incubated at 37 ºC in a minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source ([xvi]), 400 mM D-sorbitol, 2.5 mM betaine, and 20 μg/ml of kanamycin A. When the optical density at 600 nm for bacterial growth (A_{600}) reached 0.3, expression of proteins was induced by the addition of IPTG (0.4 mM final concentration), and bacteria were grown for another 20 h at room temperature. The proteins were isolated both from the growth medium and from the periplasm by osmotic shock.

For production of proteins selectively labeled with $^{13}C(\varepsilon)$-lysine, each strain was incubated at 37 ºC in minimal medium containing 100 μg/ml of each amino acid (excluding lysine), 150 μg/ml of ^{13}C-labeled lysine ([xvii],[xviii]), 400 mM D-sorbitol, 2.5 mM betaine, and 20 μg/ml of kanamycin A to A_{600} of 0.3. An additional portion of ^{13}C-labeled lysine (150 μg/ml) was added just before induction. Induction and isolation of the selectively labeled proteins was performed essentially as described above.

The desired proteins were purified from the growth medium to homogeneity in one step by DEAE-Sepharose ion-exchange chromatography (2.5 × 20 cm) using a linear gradient of 10-100 mM Tris (1.0 liter), pH 7.0.

Modification of the Lys73Cys/Cys77Ser/Cys123Ala mutant protein—A total of 5 mg of the triple mutant protein was denatured in 8 M urea, 200 mM AMPSO, 15 mM EDTA, pH 8.5, in a final volume of 1.0 ml. The exposure of cysteine to solution was assessed by its titration (10-μl aliquot of the denaturation mixture) with 5,5'-dithio-bis-2-nitrobenzoic acid (DNTB), per method of Ellman ([xix]). The mixture was kept at 25 °C with gentle mixing under an atmosphere of argon for 15 min. Upon full denaturation, the protein was incubated with a freshly prepared solution of 2-bromoethylamine in the denaturing buffer that provided a final concentration of 40 mM. The mixture was then gently mixed for 20 h at 25 ºC, at which point the cysteine was fully modified.

Refolding of the enzyme was carried out according to a modified literature method ([xx]). The enzyme was diluted into 100 mM sodium phosphate, 600 mM ammonium sulfate, 20% glycerol, pH 7.0 (refolding buffer; total volume of 50 ml) and was kept gently mixing for 1 h at 4 ºC. It was necessary to maintain a protein concentration of ≤ 0.1 mg/ml after the dilution. Removal of urea and ammonium sulfate was accomplished by extensive stepwise dialysis against 100 mM sodium phosphate buffer, pH 7.0, containing 20% glycerol and ammonium sulfate (0.3 M, 0.1 M and 0 M, in

three steps). The protein was concentrated to a maximum of 0.3 mg/ml. In excess of this concentration the protein precipitated.

The pH dependence of catalysis by the TEM-1 β-lactamase and of its modified Lys73Cys/Cys77Ser/Cys123Ala mutant variant—Mezlocillin was used as the substrate for these experiments. Buffers for the pH range of 5.0 to 9.5 were 50 mM sodium acetate (pH 5-6), sodium phosphate (pH 6-7.5) and Tris (pH 8-9), supplemented with 150 mM sodium chloride to keep the ionic strength constant. Analyses of the data were carried out according to the methods of Lineweaver-Burk and Hanes-Wolf.

NMR Spectrometry—Protein samples were dialyzed against 25 mM sodium phosphate buffer, pH 6.5 and 10% D_2O. Final concentrations for all samples were 1.0 mM protein, with initial sample volumes of 550 µl. Samples were adjusted for a range of pH values (6.00, 6.50, 7.06, 7.50, 8.06, 8.77, 9.30, 9.33 and 11.06) by adding small (microliter) aliquots of 10 mM HCl or 100 mM NaOH directly into the 5 mm NMR tubes, which contained the protein solution. Samples were then gently mixed and the final pH was measured in the NMR tube using a Mettler Toledo Analytical NMR tube pH electrode and a Corning pH meter.

All NMR spectra were collected on the Varian Unity Plus 720 MHz NMR spectrometer with a Varian (^1H, ^{13}C, ^{15}N) triple resonance probe equipped with z-axis pulsed-field gradients. The instrument was operating at the unique proton frequency of 719.86 MHz, with carbon and nitrogen frequencies of 181.02 and 72.95 MHz, respectively. NMR spectra were collected at 293 K. Transmitter and decoupler offsets were referenced with sodium 3-(trimethylsilyl)-1-propanesulfonate as an external standard dissolved in 25 mM phosphate buffer and 10% D_2O.

Carbon filtered proton resonances from the $^{13}C(\varepsilon)$-Lys labeled samples were observed using the ^{13}C HSQC experiment ([xxi]). Transmitter and decoupler offsets and spectral widths for the ^1H and ^{13}C dimension were set at 2.59 ppm/2000 Hz and 42.61 ppm/1000 Hz, respectively. Data were collected with 128 transients at 2048 complex points in the ^1H and 64 increments in the ^{13}C dimension (linear prediction to 96 points and zero-filling to 128 points were applied during the Fourier transformation process), which resulted in a total data collection time of 4.5 hours per pH point. Data were analyzed using Felix 2000 ([xxii]). Felix matrices were generated for each spectrum in the identical fashion (i.e., matrix size, linear prediction in the carbon dimension, phasing parameters in both dimensions, referencing) to ensure continuity of signals.

Quality control spectra to monitor the protein integrity at all pH values were measured on globally ^{15}N labeled protein using the ^{15}N HSQC experiment ([xxiii]). Transmitter and decoupler offsets and spectral widths for the ^1H and ^{15}N dimension were set at 4.73 ppm/12001.20 Hz and 83.57

ppm/7199.42 Hz, respectively. ^{15}N HSQC spectra were measured over the pH range of 6.0 to 11.5. Data were collected with 32 transients at 2048 complex points in the ^{1}H and 256 increments in the ^{15}N dimension (linear predicted to 384 points and zero-filled to 512 applied during the Fourier transformation process) for a total data collection time of 30 minutes per pH point.

Computational Methods—Thermodynamic integration method was used to determine the free energy change for the protonation of Lys73 and Lys146 (a surface lysine with normal pK_a) in the wild-type and in the Glu166Ala mutant enzymes. The protonation of each residue constitutes one half of the full thermodynamics cycle used to determine the pK_a of Lys73 (see below). The protein was fully solvated and the particle mesh Ewald method (PME; [xxiv]) was used to treat long-range electrostatics as implemented in the Sander module of the AMBER 7 package.

Calculation of the pK_a of Lys73, pK_a(BH$^+$), is based on the following equation ([xxv]):

$$2.3RT[pK_a(BH^+) - pK_a(AH^+)] = \Delta\Delta G_{aq}\,(B - BH^+) - \Delta\Delta G_{aq}\,(A - AH^+) \qquad (1)$$

where pK_a(AH$^+$) corresponds to that of Lys146, expected to have a typical pK_a value of 10.8, and pK_a(BH$^+$) is the pK_a of Lys73. Free energy simulations using the thermodynamic integration technique were used to determine $\Delta\Delta G_{aq}$ (B – BH$^+$) and $\Delta\Delta G_{aq}$ (A – AH$^+$), the free energy changes for the deprotonation of Lys73 and Lys146, respectively. Along with the known pK_a of Lys146, the computed values of $\Delta\Delta G_{aq}$ (B – BH$^+$) and $\Delta\Delta G_{aq}$ (A – AH$^+$) were used in equation 1 to determine the pK_a of Lys73.

The thermodynamic integration method can be used to evaluate the free energy differences between the two states X and Y:

$$G_X - G_Y = \Delta G = \int_0^1 \left\langle \frac{\partial V(\lambda)}{\partial \lambda} \right\rangle_\lambda d\lambda \qquad (2)$$

where λ is a coupling parameter, G_X and G_Y and the free energies of states X and Y, and V(λ) is the potential energy. The integrand is determined by carrying out molecular dynamics simulations at discrete values of λ ranging from 0 to 1.

The initial coordinates for the wild-type TEM-1 β-lactamase were taken from the Research Collaboratory for Structural Bioinformatics (www.rcsb.org/pdb/index.html) Protein Databank (accession number 1BTL). The Glu166Ala mutant enzyme was generated by mutating Glu166 to alanine *in silico*. The enzyme was protonated and counter ions were added to the enzyme using the "protonate" and "addions" programs, respectively, which are parts of the AMBER 7 package ([xxvi]).

Crystallographic water molecules were retained and the system was fully solvated in a box of TIP3P [xxvii] waters, such that no atom in the enzyme was less than 10 Å from any edge of the box. The total number of atoms in the fully solvated system was 37012 for the wild-type enzyme and 37015 for the mutant protein. Before starting the simulations for the free energy calculations, the system was equilibrated based on a combination of energy minimizations, slow heating and 300 K molecular dynamics simulation, as described in a previous study [xxviii]. All bonds involving hydrogen atoms in the system were constrained using the SHAKE algorithm. Particle mesh Ewald (PME) was used to treat long-range electrostatics. All simulations were carried out in parallel using the Sander program of the AMBER 7 package on 8 processors of a Linux cluster.

The integral in equation 2 was estimated numerically using a 12-point Gaussian quadrature. A total of 40 ps of simulation was carried out at each λ value, which consists of 10 ps of equilibration and 30 ps of data collection.

Results and discussion

Determination of the protonation state of Lys73 would clarify its mechanistic role in the catalytic turnover of substrates by class A β-lactamases, and the elucidation of the mechanism of these enzymes would have implications for those of the related enzymes. We have approached this task for the mechanism of the TEM-1 β-lactamase from *E. coli*, as a representative member of these families of enzymes, by three complementary methods in this report. First, we have generated a lysine analogue, γ-thialysine, by combining site-directed mutagenesis and chemical modification of the cysteine [xxix], introduced in place of Lys73 to perturb its pK_a in comparison with the wild-type enzyme. Second, we have evaluated the pK_a value of Lys73 by NMR experiments. Finally, we have evaluated the pK_a for Lys73 by state-of-the-art computational treatment using the molecular dynamics-based thermodynamic integration method.

It has been reported by Hermann and Lemke that the side chain of *N*-acetyl-γ-thialysine titrates at a pK_a value of 9.39 [xxx]. We verified this observation by preparing *N*-acetyl-*S*-ethyl(^{15}N)amine-cysteine (i.e., *N*-acetyl-γ-thialysine with the side chain amine labeled), which gave a pK_a of 9.52 ± 0.01 in our hands, in good agreement with the earlier report. The presence of the sulfide moiety in γ-thialysine attenuates the pK_a of the side chain by approximately one pK unit, compared to *N*-acetyllysine. This pK_a attenuation gives an experimental handle for the evaluation of the influence of residue 73 in the course of enzymic catalysis. γ-Thialysine has been used previously to elucidate the role of lysine residues in the active sites of several proteins such as aspartate aminotransferase [xxxi], ribonuclease A [xxxii], acetoacetate decarboxylase [xxxiii] and leader peptidase [xxxiv].

We set out to generate a γ-thialysine at position 73 of the TEM-1 β-lactamase. These experiments turned out substantially more complicated than anticipated. For example, to generate γ-thialysine according to earlier methodology ([xxxv]), one generates a cysteine at the position, and then the cysteine thiolate is modified by bromoethylamine to give γ-thialysine. This seemingly straightforward process did not work in our hands with the TEM β-lactamase. After considerable troubleshooting, we concluded that a disulfide exchange reaction was taking place between the disulfide bond linking residues Cys77 and Cys123 and the introduced cysteine at position 73. Hence, the resultant protein was misfolded. We took out the disulfide bond in the enzyme by generating the double mutant Cys77Ser/Cys123Ala. The choice of the amino acid substitutions was based on earlier precedents (xx,[xxxvi],[xxxvii]). This enzyme showed properties very much similar to those of the wild-type enzyme (e.g., a mere 2- to 3-fold effect on the steady-state kinetic parameters for substrate turnover). These results agreed well with previous studies on the role and effect of the disulfide-bridge in the TEM-1 β-lactamase (xx,xxxvi,xxxvii). Hence, the loss of the disulfide bond was not detrimental from a mechanistic point of view. Having determined this, the triple mutant Lys73Cys/Cys77Ser/Cys123Ala was prepared. We observed that this protein would not express well (2 mg per liter of growth). Despite this challenge, we were able to purify to homogeneity sufficient amounts of this protein for our experiments. The protein was devoid of activity (0.04% activity compared to the wild-type), as the critical Lys73 was mutated. Subsequently, it became clear that modification of Cys73 in the triple mutant protein (both by the Ellman reagent and by 2-bromoethylamine) was not taking place in attempts at generating γ-thialysine. According to the X-ray structure, Lys73 in the wild-type enzyme is solvent inaccessible, so we reasoned the same may be true for the mutant protein. Therefore, the protein was denatured for modification by 2-bromoethylamine, and then the reconstituted enzyme was refolded. Several different methods for denaturation and folding were tried before we settled on the method that worked best in our hands (see the Experimental Procedures). The yield of the "modified triple-mutant enzyme" (possessing the γ-thialysine) after renaturation was quantitative. The reconstituted modified triple-mutant protein was an active enzyme, with roughly 50% of the wild-type activity (see below).

One of the goals of preparing the γ-thialysine-73 variant of the TEM-1 β-lactamase was our expectation to study the γ-thialysine moiety suitably labeled for titration by NMR experiments. For example, $^{15}N_{\varepsilon}$□ or $^{13}C_{\delta}$-labeled γ-thialysine would be useful to study the protonation state of the side chain of residue 73, since NMR signals are sensitive to the protonation states. In essence, we would be able to determine the pK_a of a single residue in the fully constituted enzyme. Unfortunately, the high concentration of the γ-thialysine variant of the TEM-1 β-lactamase that was necessary to

attempt the NMR experiments could not be attained, as the protein precipitated at such concentrations, precluding this experiment. This is in contrast to the wild-type enzyme, which is stable at high protein concentrations.

The pH dependence of k_{cat}/K_m of the wild-type enzyme with mezlocillin (a penicillin), an indicative of the state of the free enzyme or the substrate, revealed two titratable residues with pK_a values of 5.0 ± 0.2 and 8.0 ± 0.1 (Fig. 1). For the γ-thialysine-73 variant, the pK_a for the basic limb decreased to 7.5 ± 0.1, and the pK_a for the acidic limb was 4.6 ± 0.4, which is virtually unchanged within the error. The optimum pH was also shifted slightly to lower pH values for the γ-thialysine mutant (Fig. 1). The pH dependence of k_{cat}, an indicator of the complex of enzyme and the substrate, for the wild-type TEM-1 enzyme with mezlocillin was bell-shaped with pK_1 = 4.5 ± 0.6 and pK_2 = 8.5 ± 0.1, and also for γ-thialysine we measured pK_1 = 4.9 ± 0.1 and pK_2 = 8.4 ± 0.1. The pH dependence profile for K_m was essentially the same for the wild-type and the mutant (Fig. 1C).

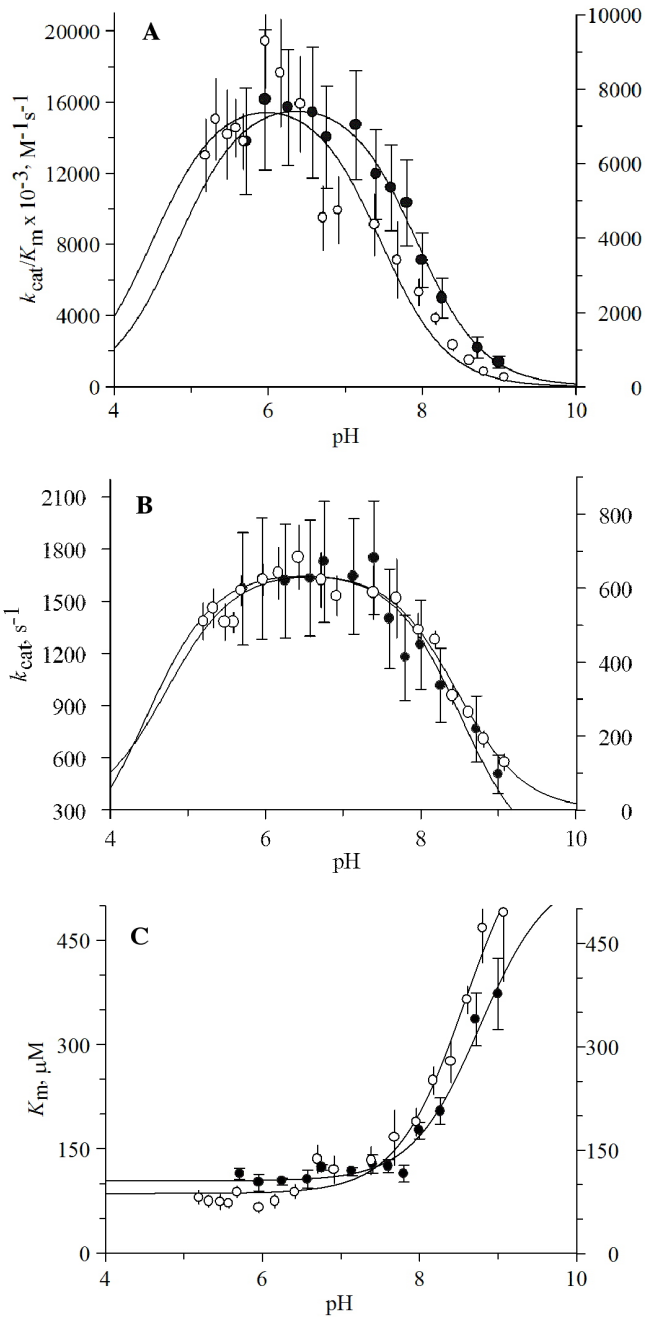


Figure 1. The pH dependence of k_{cat}/K_m (A), k_{cat} (B), K_m (C) of the wild-type (closed circles; left y-axis) and of γ-thialysine (open circles; right y-axis) TEM-1 β-lactamases with mezlocillin.

These findings indicate that the pK_a for the basic limb of the k_{cat}/K_m versus pH profile was attenuated in the case of the γ-thialysine-73 variant enzyme, hence, the basic limb is due to titration of Lys73. Therefore, the implication is that at the *free state of the enzyme (uncomplexed by the substrate)*, Lys73 is protonated. In light of a lack of observation of any difference for the basic limb of the plots for the two enzymes with reference to the pH-dependence for k_{cat} (Fig. 1B), we surmise

that the basic limb for k_{cat} is not due to Lys73. Since the pH dependence for k_{cat} (Fig. 1B) is an indicator of the complex of enzyme and the substrate, we cannot conclude anything about the protonation state of Lys73 within the complex. Indeed, the titratable residues that define the pH dependence of k_{cat}/K_m and of k_{cat} need not be the same.

To follow up on these measurements using an additional technique, we utilized NMR spectrometry to explore the titration states of the 11 lysines in the TEM-1 β-lactamase. Proton and carbon chemical shift changes at the lysine ε position were used to determine a pK_a profile of all 11 lysines. Aliphatic $H_2C(\varepsilon)$ resonances are unaffected by solvent exchange broadening and their chemical shifts serve as useful markers in characterizing the chemical environment of the N(ζ) group directly attached to them. By selectively labeling only the C(ε) positions in all lysines in the TEM-1 β-lactamase, we were able to remove ambiguity of signal overlap with additional aliphatic resonances from other residues and focus directly on the pK_a values of the 11 lysines.

The pH profile for the 11 lysine $H_2C(\varepsilon)$ resonances was determined by measuring ^{13}C HSQC spectra over the pH range of 6.0 to 11.1. The overlap of signals in a complimentary set of ^{15}N HSQC spectra collected on uniformly ^{15}N-labeled TEM-1 β-lactamase samples between pH 4 and 11.7 were used to monitor the integrity of the protein. Samples above pH 11.1 showed precipitation in the NMR tube and shifts in the amide backbone chemical shifts to the unfolded region in the ^{15}N filtered HSQC spectra (spectra not shown). The data from the globally ^{15}N-labeled TEM-1 β-lactamase indicated that the enzyme was stable in the pH range of 5-11 (data not shown).

Carbon filtered HSQC spectra were collected on both $^{13}C(\varepsilon)$ labeled wild-type and Lys73Ala mutant TEM-1 β-lactamases to determine which resonance(s) in the wild-type spectra were due to the active site Lys73. The wild-type and mutant $^{13}C(\varepsilon)$ HSQC spectra are shown in Fig. 2 at two different pH values. Figure 2A shows the ^{13}C HSQC spectrum of the 11 lysine $H_2C(\varepsilon)$ resonances in the TEM-1 β-lactamase at pH 6.5. Eleven distinct lysine signals were observed and these were labeled 1 to 11 for clarity, with peaks correlated to the same lysine labeled with the a/b label extensions. At pH 6.5, all peaks seen in the wild-type sample (Fig. 2A) appeared to be present also in the Lys73Ala mutant spectrum (Fig. 2B). However, when the pH of these samples was raised to 9.3, differences in these spectra were observed (Figs. 2C and 2D). Peak 4 in the wild-type protein spectrum is the feature that is absent when compared to the mutant at high pH spectrum. Peak 7 in the mutant spectra still followed the chemical shift vs. pH profile seen in the wild-type spectra, while peak 1b in the mutant protein resisted the behavior of merging with peak 1a, which was seen in the wild-type spectra. As in the spectrum for the mutant protein, the remaining peaks were unaffected by the elevated pH up to a value of 9.3. Because peak 4 was the absent peak in the Lys73Ala mutant

spectrum at high pH when compared to the wild-type spectrum, we attribute this peak to the $H_2C(\varepsilon)$ resonance of Lys73 in the TEM-1 β-lactamase.

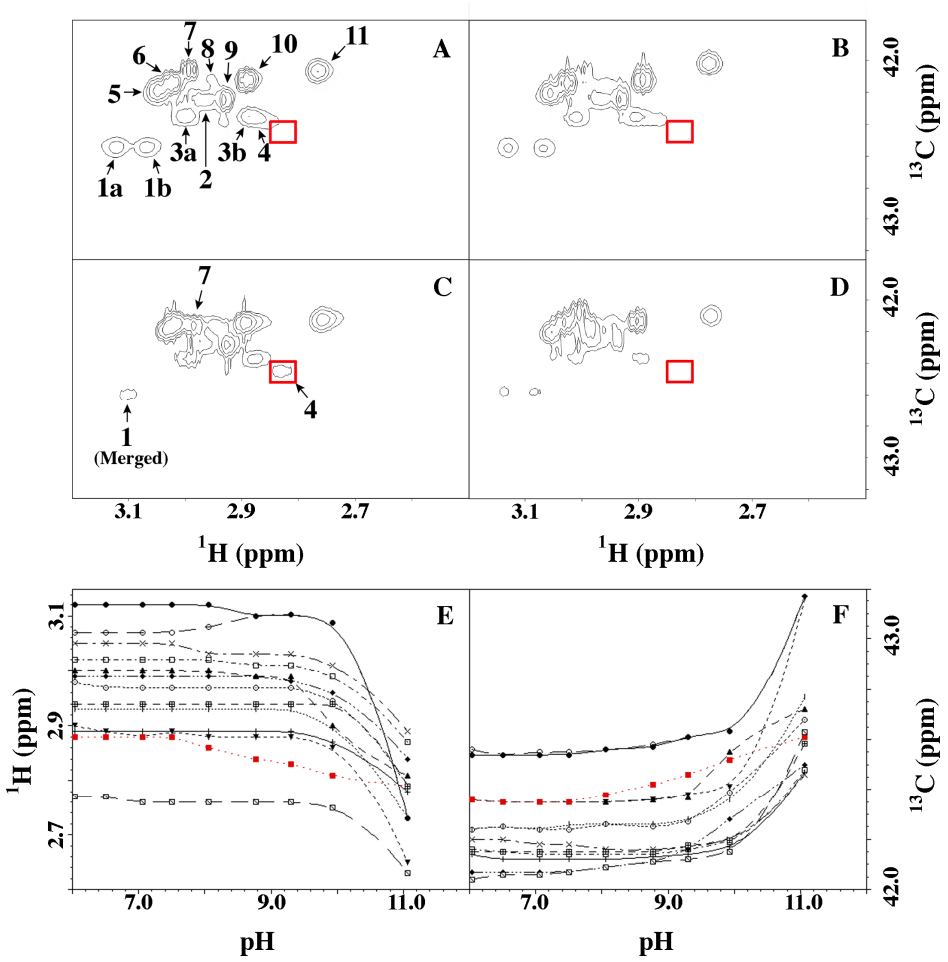


Figure 2. ^{13}C HSQC spectra for wild-type and Lys73Ala mutant TEM-1 β-lactamase, specifically ^{13}C labeled at the C(ε) position, and the corresponding ^{1}H-^{13}C(ε) chemical shifts from all lysines for the wild-type protein, obtained from the pH titration. Spectral features are labeled in panel A. The wild-type spectra at pH 6.0 (panel A) and pH 9.3 (panel C), and those of the Lys73Ala mutant protein at pH 6.0 (panel B) and pH 9.3 (panel D) are depicted. Red boxed areas designate the position (or lack thereof) for peak 4, attributed to the Lys73 signal. Chemical shifts of proton (panel E) and carbon (panel F) as a function of pH for all lysine $H_2C(\varepsilon)$ resonances. The profile for peak 4 is shown in red. Peak labels are as follows: Peaks 1a (●), 1b (❍), 2 ([+]), 3a (▲), 3b (▼), 4 (■), 5 (X), 6 (□), 7 (◆), 8 (◎), 9 (|), 10 (+), 11 ([\]).

Panels E and F of Fig. 2 show the ^{1}H(ε) and ^{13}C(ε) chemical shift profiles of all lysines from the ^{13}C HSQC spectral titration at all pH values for the wild-type protein. At pH 7.5, peak 4 begins to separate from its adjacent peak, with which it overlaps (called 3b, as it is correlated with 3a in higher pH values). Peak 4 repositioning is monitored in the red box (see Fig. 2A and 2C) and shows

chemical shift changes in the upfield direction in the proton and downfield direction in the carbon dimensions. In addition, peak 1b begins to merge with peak 1a in the wild-type enzyme. By pH 8.77, peak 4 has emerged from the new peak 3b, and peaks 1a and 1b have merged, while peak 7 has begun to undergo a carbon chemical shift change to the downfield position. However, the nine remaining signals (including peaks 1 and 3) are unchanged in their chemical shifts up to pH 8.77. The results of Fig. 2 indicate that all lysine side chains have normal pK_a values of 10-11, with the sole exception of that for Lys73, which starts titrating at pH 7.5. Since the enzyme is virtually inactive at pH above 9.0 (consistent with kinetics), we used the NMR titration data for pH values of 6.0 to 9.3 for the titration of Lys73. The fitting of these data to a titration transition revealed a pK_a of 8.4 ± 0.1 and 8.7 ± 0.2, whether the ^{13}C or ^{1}H chemical shifts were used for analyses, respectively. As a cautionary note, the trends for the chemical shifts of the side chain of Lys73 cannot be attributed to different "conformational" states, rather than the two titration states, since integration of the signal after its separated from peak 3b did not change to the end of the titration.

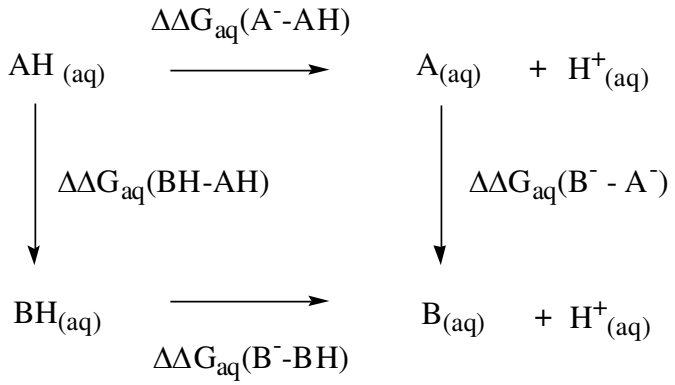
The results from the NMR experiments are inconsistent with similar experiments performed by Damblon et al. earlier (xii). ^{13}C HSQC spectra for the wild-type $^{13}C(\varepsilon)$ labeled TEM-1 have been previously published at similar spectral resolution as our data, only at a lower spectrometer frequency, with fewer ^{1}H transients and at fewer pH points in the low pH range. Despite the differences in data collection conditions, except for the absence of the low intensity peaks 2 and 8 in the data of Damblon et al., our spectra and those of Damblon et al. look very much similar. The fact that we see these less intense peaks (2 and 8) when the previously published work did not, is most likely a reflection of the higher sensitivity for our instrument (a 720 MHz spectrometer, compared to a 600 MHz instrument for the earlier study). The earlier work had not studied a mutant at position 73 to delineate which NMR signal corresponded to that for the desired residue. As a consequence, the NMR signal that was attributed to Lys73 was inadvertently incorrect. The experiments performed herein unequivocally identified the NMR signal due to Lys73 and monitored its titration as a function of changing pH. As evidenced by Fig. 2, Lys73 is indeed unique among the 11 lysines in the TEM-1 β-lactamase in that it starts titrating at pH values above 7.5.

We decided to complement the experimental results with state-of-the-art computational treatment of the TEM-1 β-lactamase. Computational analyses of the pK_a values of the active site residues in the TEM-1 β-lactamase have been reported previously. These studies have used the continuum electrostatic methods to compute pK_a of ionizable groups in the native TEM-1 β-lactamase (vi,[xxxviii],[xxxix]) or in complex with substrates (vi,ix). The first continuum electrostatics calculation of the pK_a of Lys73 was carried out by Swarén et al. (vi) who evaluated its pK_a at 8.0 in the substrate-

free enzyme. This proposal was subsequently explored further with additional similar calculations by another group, but no decrease in the pK_a of Lys73 was found (xxxviii). Subsequent studies, also using continuum electrostatics calculations, have estimated the pK_a of Lys73 at around 10 (xxxix).

Whereas the continuum electrostatics calculations have been useful in studies of enzymes, the results with β–lactamases have not clarified the picture. It would be desirable to use sophisticated methods that would provide a more definitive and realistic representation of the system. Free-energy perturbation and thermodynamic integration methods have been shown to determine free energy change within 1 kcal/mol of the experimental values, hence they afford high accuracy (xl). The thermodynamic integration approach takes into consideration several factors that are important for the accurate calculations of the pK_a of ionizable groups. These factors include protein flexibility, explicit treatment of water and counter ions and a robust and extensively used AMBER force field for the treatment of electrostatics and van der Waals interactions surrounding the ionizable residues. For these studies, we have used the fully solvated structure of the TEM-1 β-lactamase with PME for treatment of long-range electrostatics, which, needless to say, preserves the unique environment of Lys73.

The pK_a of an ionizable residue is related to the free energy difference between the protonated and the unprotonated states. The following free energy cycle, which forms the basis of equation 1, was used to calculate the pK_a of Lys73:



where AH is the protonated Lys73 and BH is the protonated Lys146. Since the pK_a of Lys146 is expected to be that of a typical lysine (as per NMR experiments), equation 1 can be used to determine the pK_a of Lys73, given that $\Delta\Delta G_{aq}$ (B – BH$^+$) and $\Delta\Delta G_{aq}$ (A – AH$^+$) are known. These free energy terms are computed using the thermodynamic integration free energy method. In general, the free energy difference between two states X and Y can be determined, if an ensemble of structures is available for both states. However, the difference in energy between states X and Y can be large and might lead to inaccuracies in the free energy. This problem is solved with the introduction of a series of non-physical intermediate states joining the two physical states X and Y.

In practice, these intermediate states are defined by introducing a coupling parameter λ and the potential energy of these states is defined by

$$V(\lambda) = (1- \lambda)\ V_A + \lambda V_B \qquad (3)$$

Calculation of the free energy difference between the protonated and unprotonated states of Lys73 and Lys146 using the thermodynamic integration methods thus consists of collecting an ensemble of structures using molecular dynamics simulations at each λ value and using a 12 point Gaussian quadrature to determine the integral in equation 2. The resulting difference between $\Delta\Delta G_{aq}$ (B – BH^+) and $\Delta\Delta G_{aq}$ (A – AH^+) was found to be –3.8 kcal/mol. Using Equation 1, along with the known pK_a of 10.8 for a typical lysine assigned to Lys-146, the computed pK_a of Lys73 was found to be 8.0.

As determined by kinetic experiments with the γ-thialysine mutant and with the wild-type β-lactamase, the pK_a of Lys73 is around 8.0-8.5 for the native enzyme without a ligand in the active site. The NMR experiment and computations also indicated that Lys73 of the native TEM-1 β-lactamase has a reduced pK_a, with a value of the same range. Swarén et al. had computed a pK_a value of 8.0 for Lys73 based on electrostatic considerations of the X-ray structure of the enzyme (vi). The more sophisticated computational analysis reported herein are supportive of this earlier determination (vi) and contradicts other computations (xxxviii,xxxix).

The protonation state of this lysine residue has been debated in the literature. There are proponents for both a protonated (iii,vii,viii,ix,x,xi) and unprotonated (iv,vi) Lys73, each favoring a different mechanism for the turnover chemistry by class A β-lactamases. The proponents of a free-base Lys73 argue that the side-chain amine of this residue is the basic entity that activates Ser70 for the acylation event by the substrate. Those favoring a protonated Lys73 have proposed that Glu166, via an intervening water molecule, activates Ser70 for acylation. The fact that Glu166 functions as the general base for promotion of a water molecule for the deacylation step is undisputed by the two camps.

The sequence analysis of over 140 penicillin-binding proteins and β-lactamases that undergo transient acylation in the active-site serine revealed that the only motif that is conserved in all these proteins is the Ser-X-X-Lys sequence in the active site ([xli]). In essence, this is a minimal requirement shared by all these enzymes that transiently undergo acylation at the active site serine in the course of catalysis. There are several representative X-ray structures for both PBPs and β-lactamases in the literature by now (i). It is revealing that practically in all the cases among members of both families of enzymes—class A β-lactamases being the exception—there is no other basic residue other than the lysine three residues downstream of the active site serine to carry out

the activation step. Acylation of the active site by the respective substrates clearly happens in these enzymes, so it would appear that there should be no choice other than relying on the active site lysine to facilitate it. Insofar as β-lactamases are widely accepted to have descended from PBPs (ii,xli,[xlii]) it would appear that the mechanism of acylation is handed down from the parental PBPs to various β-lactamases, as suggested earlier (xli). As such, the side chain of an unprotonated lysine interacting with that of the active-site serine in the preacylation complex would be an intuitive mechanism for its activation. It is important to note that there are no natural variants of any of these proteins that have experienced substitutions at either active-site serine or lysine, so both residues are critical for the activities of these enzymes.

As we proposed earlier (xli), the evolution of a nascent β-lactamase from a PBP would require the advent of a catalytic step for the deacylation event. We conjectured that as class A β-lactamases evolved, Glu166 was introduced within the active site to activate a water molecule for deacylation of the acyl-enzyme species (Fig. 3). We acknowledge that introduction of a polar amino acid such as Glu166 within the active site for evolution of the deacylation step could also influence the acylation event. A number of reports on properties of mutant variants of β-lactamases at positions 73 and 166 have appeared (xi,[xliii],[xliv],[xlv],[xlvi],[xlvii],[xlviii]). Mutations at position 166 have revealed to have effects on both the rates of acylation and deacylation of class A β-lactamases (xlvi,xlvii). However, the effect is substantially more for the deacylation step (six orders of magnitude), such that a mutant variant at position 166 has been useful in determination of an X-ray structure for the acyl-enzyme species with a preferred substrate, namely a penicillin (iv). This X-ray structure argues that the glutamate carboxylate at position 166 was dispensable for the acylation event, although one would not argue against it having some influence on the microscopic rate constant for the acylation step (xlvi,xlvii), since the residue is within the active site. In essence, in the absence of the Glu166 carboxylate in the mutant variant, acylation of the active site Ser70 by the β-lactam substrate takes place, although with a somewhat attenuated microscopic rate constant.

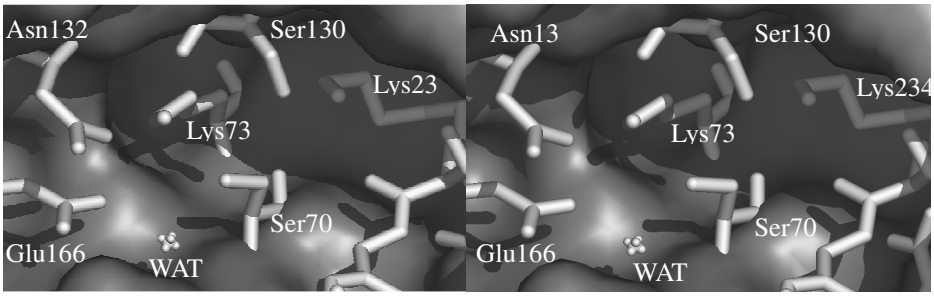


Figure 3. Stereo view of the active site of the TEM-1 β-lactamase from the X-ray structure depicted as a Connolly surface.

We have documented in this report that clearly Lys73 is different than a typical lysine in a protein by having its side chain pK_a attenuated to 8.0-8.5. Attenuation of the pK_a of Lys73 is due to the influence of Lys234 (4.7 Å away), also an active site residue. This type of electrostatic suppression of the pK_a of the side chain of lysine was first reported by Schmidt and Westheimer [xlix] and it is precedented in several enzymes (xxxiii,[l,li]). While Lys73 of class A β-lactamases has an attenuated pK_a compared to a typical lysine, the results of Fig. 1 indicate that it is protonated in the native state in the absence of substrate in the active site. This result is a direct consequence of the electrostatic effect of the side chain of Glu166, which is within 3.5 Å of the Lys73 side chain nitrogen. The proximity of the carboxylate of Glu166 indeed would elevate the pK_a of Lys73.

To explore this possibility, further calculations of the pK_a of Lys73 were carried out with the Glu166Ala mutant enzyme to determine the pK_a of Lys73 in the absence of the glutamate carboxylate. The free energy change for protonation of Lys73 and Lys146 were determined using the same procedure that was used for the wild-type enzyme. Two distinct molecular dynamics simulation, one for Lys73 and the other for Lys146, were carried out at various intermediate states, as the lysine residues were converted from the deprotonated to their protonated forms. The results were intriguing. Mutation of Glu166 to alanine resulted in a pK_a of 6.0 for Lys73. *In essence, the advent of Glu166 in evolution of class A β-lactamases created a unique electrostatic balance that settled at a pK_a of 8.0-8.5 for the side chain of Lys73 in the native state.*

Therefore, the question remains how acylation of the active site serine in class A β-lactamases, and in other β-lactamases and PBPs, takes place. We provide an alternative mechanistic possibility in this report. We speculate here that introduction of Glu166 has not altered the overall mechanism for acylation of class A β-lactamases, compared to PBPs, their kin enzymes. It is conceivable that an unprotonated lysine is involved in activation of the active site serine. In the native state, as evidenced by our experiments, Glu166 and Lys73 of class A β-lactamases form an ion pair (i.e., a protonated Lys73). *In the course of catalysis, Glu166 becomes protonated, resulting in an unprotonated Lys73 that activates Ser70*. The mechanism for acylation for all known active-site-serine β-lactamases and PBP would appear to be the same and class A β-lactamases are not exceptions. We underscore that a deprotonated Glu166 and a free-base Lys73 would be unsuitable for the enzyme, as the lone electron pairs in the two side chains would repel each other, probably causing local detrimental conformational changes within the active site, consistent with the enzyme losing activity above pH of 9. This is supported by previous molecular dynamics simulations of TEM-1 with deprotonated Glu166 and free-base Lys73 that resulted in unstable trajectories [lii].

We acknowledge that the catalytic event that precipitates active site acylation might benefit from protonation of the β-lactam nitrogen in the substrate by Ser130, facilitated by the carboxylate of the substrate itself, such as proposed for the first time by Makinen and colleagues (ix).

It is interesting that class D β-lactamases ([liii],[liv]) and a penicillin-binding protein from *Staphylococcus aureus*, the BlaR protein ([lv]), have a carboxylated version of the active site lysine. The side chain amines of these enzymes—as free bases—undergo modification by carbon dioxide to give carbamates. The carbamate has been shown to serve as the active site base for serine acylation in both the OXA-10 class D β-lactamase (liii) and in BlaR from *S. aureus* (lv). Therefore, despite this alteration of the structure of the active site lysine in these proteins, the function remains the same, namely as a base in promotion of serine acylation.

Two recent ultrahigh resolution x-ray structures of class A β-lactamases have appeared in the literature that are worthy of comment (x,xiii). Because of the resolutions of better than 1 Å in these structures, many of the hydrogens in the two proteins were visualized. Whereas, neither structure could settle the number of hydrogens that reside on the side chain nitrogen of Lys73, the protonation state of this residue can be inferred from the immediate environment around it. One structure, that of the native SHV-2 β-lactamase, shows that the proton on Ser70 is pointing toward the active site water molecule, and not at Lys73 (xiii). This is to be expected as proposed herein, since Lys73 is protonated in the native state in the absence of any ligand (substrate) bound to the active site. The other is an x-ray structure of the TEM-1 β-lactamase with a boronate inhibitor bound to the active site serine. This structure shows that Glu166 is protonated (x). Whereas the authors surmised that the protonated nature of Glu166 argues for it abstracting a proton from the active site water, which in turn would activate Ser70 for acylation, it is also consistent with our proposal that the ion pair of Glu166 and Lys73 would reconfigure to a protonated Glu166 and free-base Lys73 for active site acylation. In essence, in our view what the structure of Minasov et al. reveals is that the free-base lysine has activated Ser70 for modification by the boronate inhibitor, as a consequence of which both Glu166 and Lys73 would be trapped in their protonated forms; the protonation state of the former residue could not be visualized in the X-ray structure.

Concluding Remarks.

The mechanism(s) of active site acylation of penicillin-binding proteins and β-lactamases by their respective substrates is likely to be the same. As for class A β-lactamases, introduction of Glu166 into the active site has perturbed the pK_a of the active site Lys73, but its role for promotion of serine for acylation is to be preserved. The equilibrium for the existence of the ion pair between Glu166

and Lys73 and their respective uncharged versions (protronated Glu166 and free-base Lys73) should be important in the reaction of the enzyme, consistent with the ultrahigh resolution structures for these enzymes reported recently.

Acknowledgements.

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Abbreviations

[1]EDTA, ethylenediaminetetraacetic acid; BEA, (2-bromoethyl)amine; AMPSO, 3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid; IPTG, isopropylthio-β-D-galactoside; PBP, penicillin-binding protein.

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