Inactivation of Class A β-Lactamases by Clavulanic Acid: The Role of Arginine-244 in a Proposed Nonconcerted Sequence of Events

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Abstract: From the refined 2 Å crystal structure of the *Bacillus licheniformis* 749/C β-lactamase, energy-minimized models for active-site binding of the precatalytic ("Michaelis") complex with the clinically utilized inactivator, clavulanic acid, for the acyl enzyme intermediate, and for the ultimate acylated acyclic species that leads to inactivation of class A β-lactamases by clavulanate have been generated. On the basis of these models, the details of the chemistry of inactivation of clavulanate are reassessed. A nonconcerted process for the inactivation chemistry of class A β-lactamases by clavulanate is proposed. These models reveal that the Arg-244 side chain and the Val-216 carbonyl anchor a structurally conserved water molecule, W673, which serves as the most likely source of a critical proton in a stepwise sequence of events. Disruption of this "electrostatic anchor" for W673 by mutational replacement of Arg-244 with Ser in the TEM β-lactamase would account for the resulting observed severe impairment of the efficiency of inactivation of the mutant enzyme by clavulanate. The kinetic impact of the Arg-244-Ser mutation on interaction with clavulanate is reflected by resistance to ampicillin plus clavulanate of a strain of *E. coli* bearing the mutant enzyme. Molecular dynamics computations on the acylated acyclic intermediate—the putative inactivating species—indicated that irreversible inactivation of the β-lactamase may not occur as a consequence of a transimination reaction, in contrast to previous suggestions. The most likely scenario for irreversible inactivation involves the capture of the β-hydroxyl of conserved Ser-130 by the iminium moiety of the acylated acyclic intermediate, followed by a deprotonation at C_6 of clavulanate. The deprotonation is likely to be carried out by the conserved Glu-166 via the intervening crystallographic water W712. Deprotonation prior to nucleophile capture is proposed as the mechanism of generation of the so-called transiently inhibited enamine species. For the wild-type TEM-1 β-lactamase, both irreversible inactivation and the formation of the transiently inhibited species proceed with comparable rates. In addition, a new function for the Ser-130 in the formation of the acyl–enzyme intermediate with both clavulanate and typical β-lactamase substrates is proposed. It is suggested that the β-hydroxyl of Ser-130 stabilizes the transition state for the expulsion of the incipient amine from the high-energy tetrahedral species by hydrogen bonding to the oxazolidine amine in the course of Ser-70 acylation.

Hydrolytic cleavage of the lactam bond of β-lactam antibiotics (*e.g.*, penicillins and cephalosporins) is the principal bacterial activity that confers high-level resistance to these antibacterial agents. As a consequence of extensive therapeutic use of these antibiotics over the past four decades, such resistance to β-lactams has become common in clinical pathogens. Two strategies have been employed to counter this resistance problem. First, new β-lactam drugs have been synthesized which are inherently more resistant to the action of β-lactamases.[1] A second approach utilizing combinations of a mechanism-based inactivator for β-lactamases [e.g., clavulanate (compund **1**) and sulbactam] and a penicillin has been introduced into clinical use.[2] The rationale for such combined therapy is based on a synergistic effect of two molecules: the inactivator would abolish the β-lactamase activity, and the active penicillin would thereby be protected from inactivation.

The mechanism of action of clavulanate, as a representative β-lactamase inactivator, has been examined in considerable detail.[3] Nonetheless, a full analysis of interactions between the inactivator and the enzyme active sites was not possible until the recent availability of high-resolution crystal structures for the class A β-lactamases of *Staphylococcus aureus* PC1,[4] *Bacillus licheniformis* 749/C,[5,6] and *Streptomyces albus* G.[7] From the analysis of these crystal structures, it was suggested that Arg-244 may be involved in interactions with mechanism-based inactivators such as clavulanate.[5] Furthermore, we have shown recently that Arg-244[8] of the TEM-1 β-lactamase contributes to the binding of both the transition state and ground state for turnover of penicillins and cephalosporins[9] and that this residue, or a water molecule coordinated to its side chain, facilitates the $\Delta^2 \rightarrow \Delta^1$ pyrroline tautomerization of carbapenem antibiotics.[10] In addition, we had found that selection for resistance to ampicillin plus

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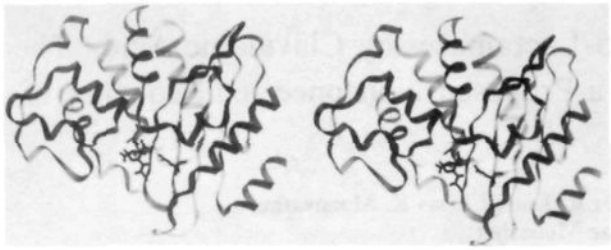

Figure 1. Stereoview of β-lactamase polypeptide chain with clavulanic acid in its minimized position near Ser-70 and Arg-244.

clavulanate in a bacterial strain containing the TEM-1 β-lactamase resulted in a mutant β-lactamase with substitution of Arg-244 by cysteine.[11] We report here on a model for the details of the interactions of clavulanate with class A β-lactamases and examine some mechanistic features of the proposed systems.

Experimental Section

Amino acid substitution at position 244 of the TEM-1 β-lactamase was carried out by site-specific mutagenesis according to the method of Kunkel,[12] and the wild-type and the Arg-244-Ser β-lactamases were purified to homogeneity by a slight modification of a published method,[13] as detailed elsewhere.[9] Benzylpenicillin was purchased from Sigma. Clavulanic acid was obtained from Smith-Kline Beecham. Kinetics measurements were carried out on a Perkin-Elmer Lambda 3B or Hewlett-Packard 452 diodearray instrument. Structures were viewed on an Evans & Sutherland PS330 graphics system.

Structures. The fully refined 2 Å crystal structure of the class A β-lactamase of *Bacillus licheniformis* 749/C[5,6] (4BLM, Protein Data Bank, Brookhaven National Laboratory) was used in the three-dimensional modeling and energy minimization (Figure 1). Hydrogen atoms were added in calculated positions. This β-lactamase shares a high sequence homology with the TEM-1 β-lactamase.[8] However, a few nonconserved residues within the inactivator binding pocket differ between TEM-1 and the *B. licheniformis* enzyme. Therefore, the following three substitutions were made in the crystal structure to mimic more closely the active site of the TEM enzyme: at position 216, from threonine to valine; at 235, from threonine to serine; and at 274, from tyrosine to glutamic acid. The minimization was then continued until complete. A publication on the crystal structure of the TEM enzyme has appeared in the literature recently.[14] Since the crystallographic coordinates have not been deposited in the Protein Data Bank, a direct comparison of our model and the crystal structure in not possible. However, Strynadka *et al.* state that the active-site region of the TEM, *B. licheniformis*, and *S. aureus* β-lactamases are very similar, so that mechanistic deductions from one enzyme should be valid for the others as well.[14] The consensus numbering scheme of Ambler et al. is used throughout this manuscript.[8]

The crystallographic structure of the *p*-nitrophenyl ester of clavulanic acid[15] was used to construct the acid structure. Both *E* and *Z* configurations for clavulanic acid are known. However, as the *Z* configuration is that of the natural product,[15] it was therefore used in our modeling. The inhibitor was initially positioned according to the precatalytic binding scheme proposed for the *B. licheniformis* enzyme complex with penicillin G,[5,6] in which the α-face of the β-lactam is presented on the β-hydroxyl of Ser-70. The carbonyl oxygen atom of the β-lactam moiety is in the "oxyanion hole" formed by the main-chain NH functions of residues 70 and 237, and the carboxylate group is near Ser-130, Lys-234, Ser-235, and Arg-244.

Solvation. The complexes were solvated in a two-step process. About 245 crystallographically determined water molecules were retained for these computations, except for four water molecules which overlapped clavulanic acid atoms by more than 20% of the van der Waals radius. The bound inhibitor was then capped by the addition of 87 Monte Carlo TIP3P water molecules within 15 Å of any clavulanic acid atom.[16] As a result, the surface of both enzyme and bound inhibitor was fully solvated with water molecules for the minimizations which followed.

Energy Minimization. The complexes were energy minimized in two stages with CVFF (version 2.2), an empirical force field which was used in conjunction with Discover version 2.70[17,18] on a Silicon Graphics 4D/70GT and 4D/35S. Initially, all non-hydrogen atoms of the enzyme, clavulanate, and the crystallographic waters were fixed to allow the generated hydrogen atoms and Monte Carlo waters to adopt realistic positions. Hydrogen atom positions were minimized until the change in energy was less than 0.01 kcal/mol/Å. Finally, the crystallographic and Monte Carlo waters, the clavulanate, and all enzyme residues within 10 Å of any atom of the clavulanate were allowed to minimize until the change in energy was less than 0.001 kcal/mol/Å. A constant dielectric of 1.0 was used with a residue-based cutoff distance of 12 Å.

Molecular Dynamics. For the covalently bound species **5** with both rings opened, a molecular dynamics simulation was performed with the CVFF forcefield. This minimized complex was heated to 100 K over 5 ps and then equilibrated for 35 ps at 310 K. A dynamics simulation of 60 ps was performed using a time step of 1 fs. Conformations were sampled every 2.5 ps and were then minimized until the largest change in energy was less than 0.01 kcal/mol/Å.

Assay of Enzyme Activity. A 1.0-mL assay mixture typically consisted of 2 mM benzylpenicillin in 100 mM sodium phosphate, pH 7.0. The hydrolytic reaction was initiated by the addition of β-lactamase (2×10^{-8} M final concentration) to the assay mixture, followed by monitoring of the hydrolysis at 240 nm.

Kinetics Experiments. Inactivation experiments were performed by the addition of an aliquot of a stock solution of clavulanic acid (0.3–3.2 mM final concentration) to the enzyme (2 μM) in 100 mM sodium phosphate, pH 7.0 at room temperature. A 10-μL aliquot was removed at various time intervals and was diluted 100-fold into the assay mixture containing 2 mM benzylpenicillin. The residual enzyme activity was monitored until the substrate had been completely depleted. Typically, a progressive increase in the rate of substrate hydrolysis was observed initially; this was attributed to recovery of enzyme from transiently inhibited species (*vide infra*). The enzyme activity was calculated from the highest linear rate observed during the course of substrate hydrolysis. These experiments were carried out under conditions of excess substrate, as described by Koerber and Fink.[19]

The partition ratios for clavulanate with the wild-type TEM-1 β-lactamase and the mutant enzyme were determined by the titration method.[20] Several buffered mixtures containing various molar ratios of $[I]_0/[E]_0$ ranging from 1 to 200 for the wild-type and 1 to 8000 for the Ser-244 enzyme in 100 mM sodium phosphate, pH 7.0, were incubated at 4 °C overnight (ca. 20 h). Subsequently, the remaining enzyme activity was assayed as described for inactivation experiments. The extent of nonspecific inactivation of each protein was taken into account by measuring the activity of enzyme incubated under the identical set of conditions in the absence of the inactivator.

The dissociation constants (K_i) for clavulanate with both the wild-type and Ser-244 enzymes were calculated by the method of Dixon.[21] Two concentrations of substrate ampicillin ($\Delta\epsilon_{240} = 538\ M^{-1}\ cm^{-1}$, at pH 7.0), 400 and 500 μM, were used. A series of assay mixtures containing both the substrate and various concentrations of clavulanate (0.5–4 μM with the wild-type, and 2.5–50 μM with the Ser-244 enzyme) were prepared in 100 mM sodium phosphate buffer, pH 7.0. A portion of the enzyme was added to afford a final enzyme concentration of 5 nM in a total volume of 1 mL. The enzyme activity was measured immediately. The rates were calculated from the linear portion corresponding to approximately the first 5% of substrate turnover.

Determination of Minimum Inhibitory Concentrations (MICs). The MICs of ampicillin for various bacterial strains with and without clavulanate were determined by the broth microdilution method with inocula of 5×10^5 colony forming units per milliliter in Mueller–Hinton

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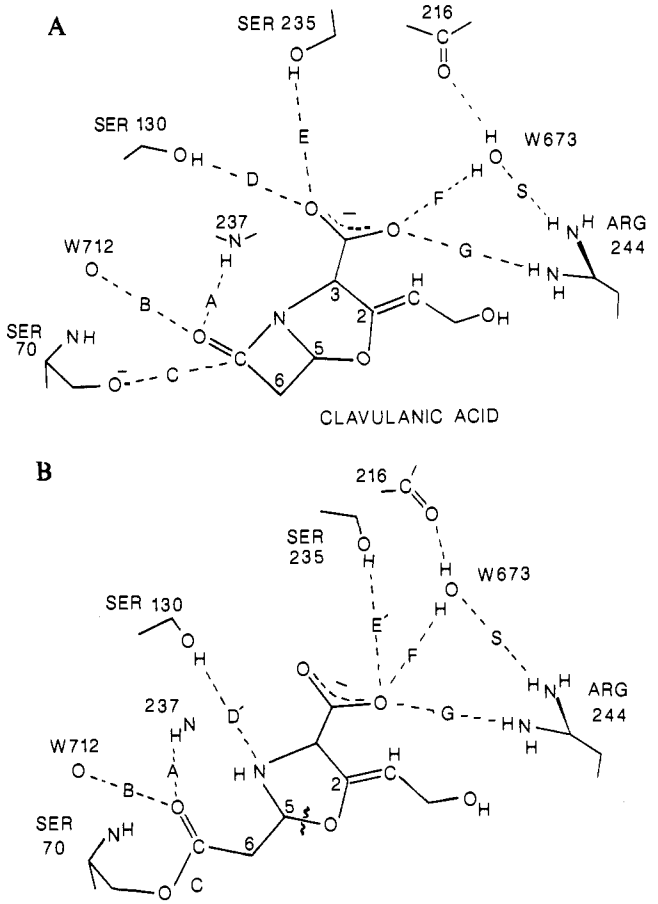

Figure 2. Diagram of the distances in Table I for the (A) preacylation complex and (B) one-ring-opened acylated complex of clavulanate with the β-lactamase.

broth.[22] The MIC was defined as the lowest concentration of antibiotic that prevented the appearance of turbidity after 24 hrs of incubation at 37 °C.

Results

Energy-Minimized Complexes. Results of the minimizations are shown in Table I, which lists calculated distances between two clavulanate species and selected atoms in the binding site (Figure 2). Because earlier modeling studies of β-lactam complexes have shown the importance of the protonation state of the nucleophilic Ser-70,[23] both protonation states of Ser-70 (in the *complexed* enzyme, as opposed to the *substrate-free* enzyme) were examined in the minimizations. The protonation state of another catalytic residue, Glu-166, was made opposite to that of Ser-70 in each complex. In Table I only the distances for the complexes in which Ser-70 is anionic and Glu-166 is protonated are presented, as it is this state that produced the closer and more favorable approach of the Ser-70 oxygen atom to the susceptible carbonyl carbon atom (2.8 vs 3.3 Å). This computational result agrees with the fact that upon close approach of substrate, the serine must at some instant become deprotonated for acylation to occur. Precatalytic deprotonation of the Ser-70 proton is assisted by the larger-than-expected macrodipole-induced positive charge at the N-terminus of the helix[24] on which the serine resides, and by the close proximity of the positively charged invariant Lys-73. We hasten to add that both recent kinetic and

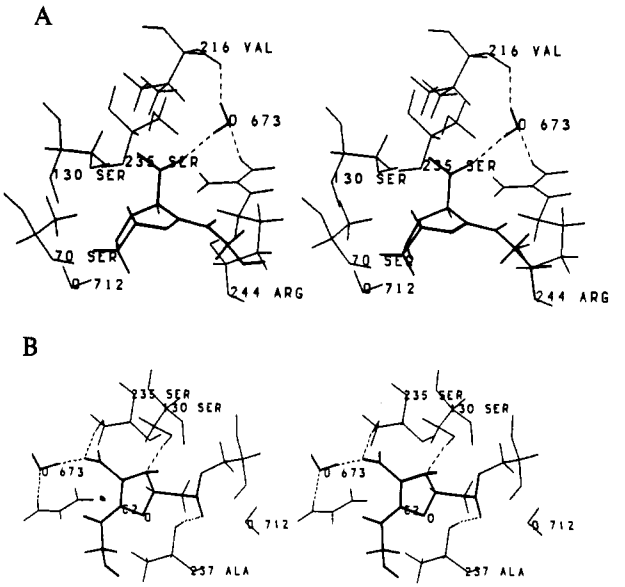

Figure 3. Stereoview of the minimized complexes. Hydrogen bonds are indicated by dashed lines. Crystallographic water molecules W673 and W712 are included: (A) the preacylation complex and (B) the one-ring-opened acylated complex bound to Ser-70.

crystallographic work[25] with the Glu-166-Ala mutant has discounted the contribution of Glu-166 in deprotonating the Ser-70 during acylation.

Interactions in the Energy-Minimized Complexes. (A) Nonacylated Complex. Following energy minimization, intact clavulanate remained near its starting position but with a translation of about 0.75 Å toward the hydroxyl group of Ser-235 (Figure 3A) which hydrogen bonds strongly (2.6 Å) with the carboxylate of clavulanate. This carboxylate also hydrogen bonds to invariant residues Ser-130 and Arg-244 and to crystallographic water W673, so that a full complement of four hydrogen bonds exists around the carboxyl anion. Relative to the position proposed for penicillin substrates anchored in the active site,[5,6] the β-lactam carbonyl in clavulanate is positioned in the oxyanion hole away from the amide of Ser-70 and more toward the amide of residue 237 in the B3 β-strand. The carbonyl now forms an additional hydrogen bond with the presumed hydrolytic water molecule W712. In effect, this water molecule has replaced the Ser-70 amide in polarizing the β-lactam carbonyl prior to nucleophilic attack by Ser-70.

The C_2 substituent of clavulanate lies near Arg-244 (Figure 2A). The sp^2 carbon atom of the substituent is about 3.2 Å from the closest N_η of the guanidinium moiety of Arg-244. Another hydrogen atom near (4.1 Å) the sp^2 carbon atom is that of water W673, which links Arg-244, the clavulanate carboxyl group, and the main-chain carbonyl group of residue 216. This particular water molecule lies directly above the double-bonded carbon atoms. The C_2 substituent exists in the active *Z* configuration; had the clavulanate been in the *E* configuration, the distances between the sp^2 carbon atom and the Arg-244 and W673 would have been much longer. The terminal hydroxyl group of clavulanate is held by a weak hydrogen bond to Glu-274 at 3.3 Å. It is known that this hydroxyl group plays no role in either recognition or the chemistry of inactivation, since its absence does not change the kinetic behavior of 9-deoxyclavulanate compared to clavulanate.[26] It is noteworthy that in this preacylation complex the susceptible carbonyl bond of the β-lactam ring is found to be markedly out of plane by approximately 35°. The distortion is in the direction expected for the formation of the nascent tetrahedral transition-state species, with the incipient

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Table I. Calculated Distances between Non-Hydrogen Atoms in the Minimized Complexes[a]

distances				
Figure 2A	Figure 2B	groups involved	preacylation complex (Å)	acylated complex (Å)
A	A	Ala-237 to lactam CO	2.75	2.98
B	B	W712 to lactam CO	2.63	3.32
C	C	Ser-70 to lactam CO	2.80	1.39
D	D′	Ser-130 to COO^- or lactam N	2.71	2.87
E	E′	Ser-235 to COO^-	2.56	2.52
F	F	W673 to COO^-	2.54	2.61
G	G	Arg-244 to COO^-	2.87	2.84
S	S	Arg-244 to W673	2.97	3.46

[a] The estimated uncertainty in distances is 0.05 Å.

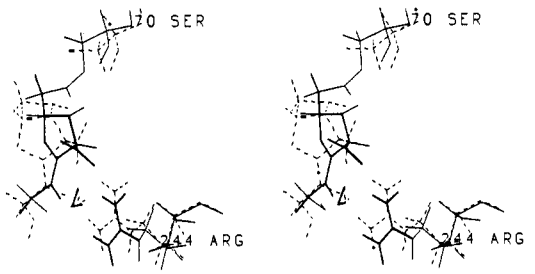


Figure 4. Stereoview of the overlay of the preacylated (dashed) and one-ring-opened acylated species. each in its minimized position near Arg-244.

oxyanion to be held by a hydrogen bond to the backbone amide of residue 237 in the B3 strand.

(B) Acylated Complex. Energy minimization of the β-lactam ring-opened clavulanate species, the product of acylation of Ser-70, produced the structure shown in Figure 2B and Figure 3B. Distances are given in Table I. Though the C_2 substituent remains near Arg-244 and W673, a rotation of approximately 45° about the C_5–C_6 bond has made the plane of the double-bonded carbon atoms almost perpendicular to the plane of the guanidinium group of Arg-244. As a direct consequence of this rotation, Ser-130 no longer hydrogen bonds to the clavulanate carboxylate, but instead donates a hydrogen bond to the lone electron pair on the pyramidal nitrogen atom in the heterocyclic ring. Figure 4 shows an overlay of the preacylated and acylated complexes.

(C) Acylated Linear Complex. To survey the full range of potential movement of the acylated linear complex within the active site, we examined molecular dynamics simulations of the both-rings-opened clavulanate species **5**. Figure 5A is an overlay of six of the 24 minimized conformations sampled during the 20–80-ps period. The composite figure shows that the range of movement of species **5** and important active site residues is rather small and usually less than 1 Å. Average distances between C_5, the sp^2 carbon atom susceptible to nucleophilic attack, and the three potential active site nulceophilic residues Lys-73, Ser-130, and Lys-234, in addition to Arg-244, are listed in Table II. It is seen that the β-hydroxyl atom of Ser-130 approaches C_5 more closely than do the nitrogen atoms of the other residues (Figure 5B).

Kinetic Evaluation of the Function of Arginine-244 in the Chemistry of Inactivation. We had studied earlier the role of Arg-244 in turnover of typical penicillins and cephalosporins in kinetic experiments with the Arg-244-Ser mutant TEM-1 β-lactamase.[9] For these studies the Arg-244-Ser mutant TEM β-lactamase was designed by computer modeling, which indicated the possibility of two new hydrogen bonds between the Ser-244 β-hydroxyl and the main-chain carbonyl of Gly-242 and the Ser-265 side-chain hydroxyl. This arrangement placed the Ser-244 hydroxyl at approximately 9–10 Å from the substrate carboxylate. Furthermore, circular dichroic measurements indicated that no

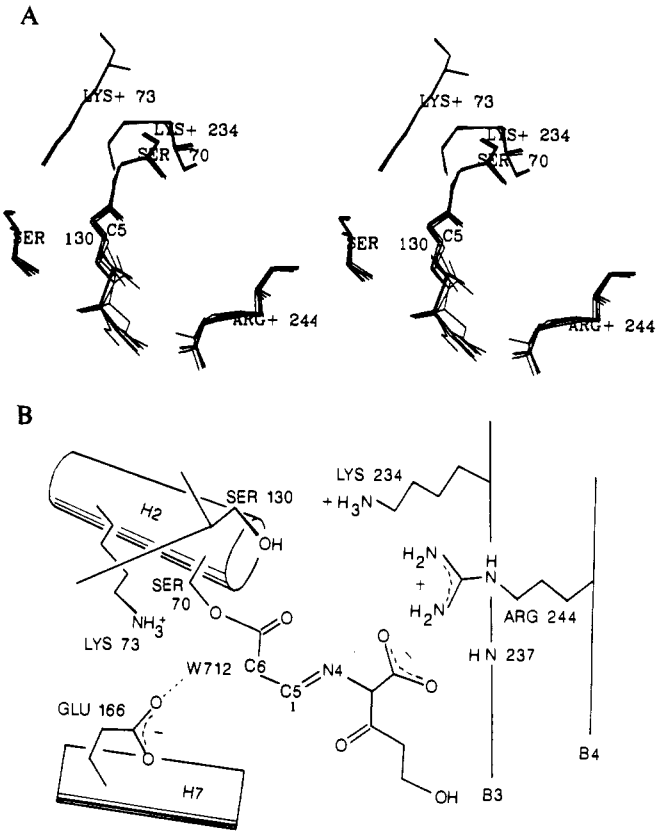


Figure 5. (A) Overlay of six of the 24-minimized complexes of acyclic acylated species **5** sampled during the dynamics simulation period from 20 to 80 ps. Hydrogen atoms are not shown. Distances are given in Table II. (B) Schematic showing disposition of potential nucleophiles around species **5**. The actual protonation states of residues 73. 166. and 234 are not known.

Table II. Calculated Distances in the Complex of the Acyclic Species **5** After the Dynamics Simulation

	distance (Å)	
interaction	range	av
Lys-73 N^{ϵ} to C_5	4.8–5.8	5.3
Ser-130 O^{β} to C_5	3.6–5.4	4.6
Lys-234 N^{ϵ} to C_5	4.2–5.8	4.9
Arg-244 $N^{\eta 1}$ to C_5	6.0–8.2	7.2
Arg-244 $N^{\eta 2}$ to C_5	7.6–9.0	8.4

perturbation of the secondary structural elements of the enzyme resulted as a consequence of the mutation within the pH range of 4–9.[9] This same mutation at residue 244 is useful in exploring the roles of Arg-244 and W673 in the chemistry of clavulanate. The Ser-244 mutant enzyme not only would lack the guanidinium function of arginine near the active site-bound clavulanate but also should not retain water W673 because of lack of coordination by the arginine side chain. The Arg-244-Cys mutant that had been selected by us for resistance to the ampicillin/clavulanate combination[11] would be expected to have the same properties as the Ser-244 enzyme; however, we chose not to work with the Arg-244-Cys mutant enzyme because of potential problems associated with the oxidation and titration states of the cysteine in the protein in solution. Figure 6 shows the results of inactivation of both the wild-type TEM-1 and the Arg-244-Ser mutant enzyme with clavulanate. Inactivation of the wild-type enzyme is biphasic; a very rapid phase is followed by a slower phase, and both phases show dependence on the concentration of clavulanate. Although inactivation of the Ser-244 enzyme is also biphasic, the rates of inactivation in both phases are markedly reduced. As shown in Figure 2 and Figure 3, both W673 and the Arg-244 side chain make hydrogen bonds to the clavulanate carboxylate. Therefore,

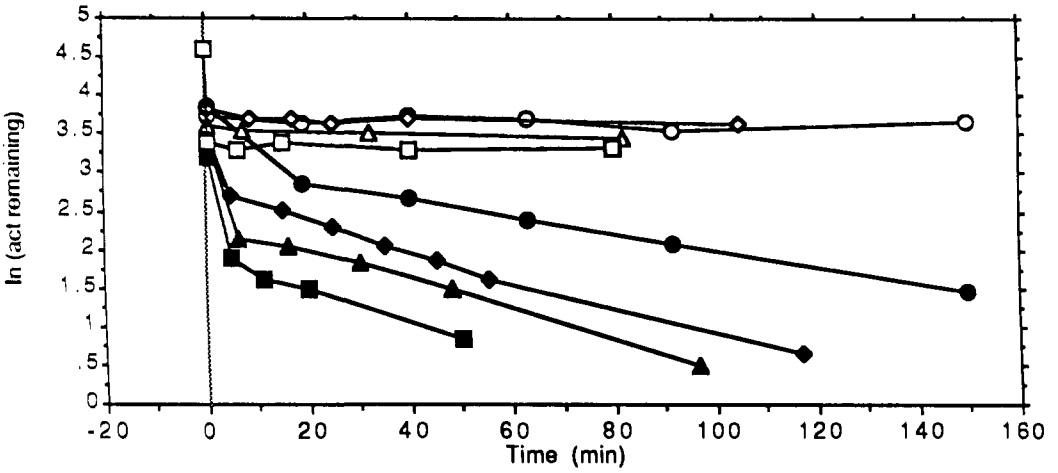


Figure 6. Loss of activity of the wild-type (closed symbols) and the Arg-244-Ser mutant (open symbols) TEM-1 β-lactamase in the presence of 3.2 mM (squares). 1.6 mM (triangles). 0.8 mM (diamonds). and 0.32 mM (circles) clavulanate.

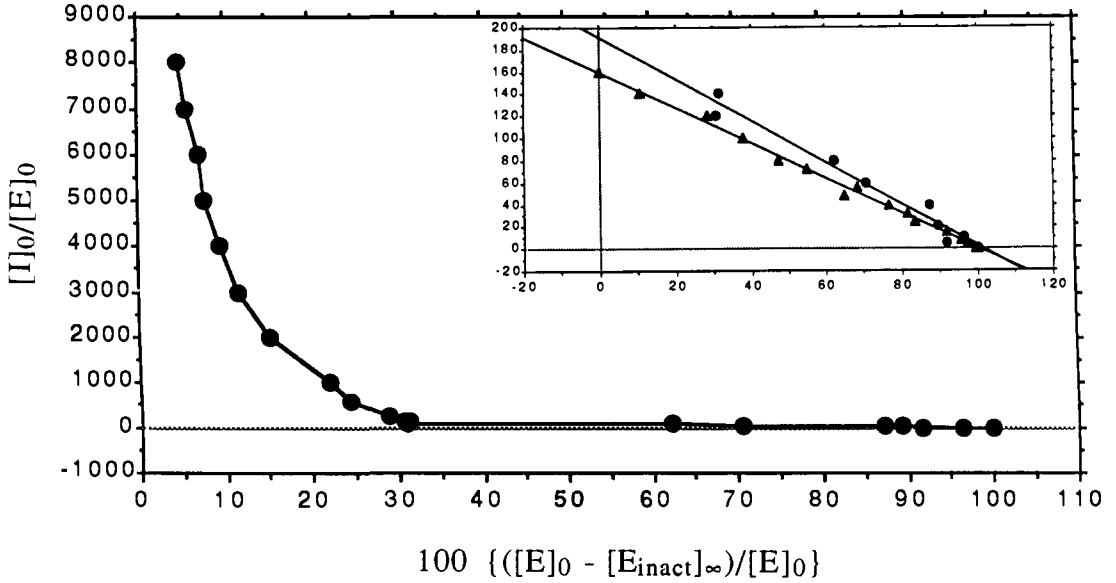


Figure 7. Loss of activity of the wild-type (▲) and Arg-244-Ser (●) TEM-1 β-lactamase in the presence of various molar ratios of clavulanate to enzyme after 20 h of incubation at 4 °C (conditions are given in the text). The inset shows the expansion of the data for $[I]_0/[E]_0$ of less than 200: the wild-type data are shown only in the inset.

the loss of these hydrogen bonds in the Ser-244 enzyme would reduce affinity for clavulanate. The dissociation constants (K_i) for clavulanate were measured at 0.4 and 33 μM for the wild-type and Ser-244 mutant enzymes, respectively (data not shown). We wish to stress that the reduction in rates of inactivation shown in Figure 6 for the Ser-244 enzyme are not due merely to reduced affinity for clavulanate, since each of those experiments was carried out with high clavulanate concentrations that should have saturated the mutant enzyme.[27]

Given "infinite" time, the mutant enzyme is inactivated considerably by clavulanate. Equation 1, which is derived from the integrated form of the ratio of the steady-state rate expressions for turnover of and for inactivation by clavulanate, describes the linear portion of Figure 7. Inactivation of the wild-type enzyme in the presence of varying ratios of the inactivator to the enzyme at the "infinity" is linear for complete inactivation, allowing for the determination of a partition ratio (i.e., k_{cat}/k_{inact}) of 160. The corresponding experiments for the Ser-244 enzyme show somewhat similar results for conditions under which high activity remains (an extrapolation of these data points to a point of zero activity indicates a partition ratio of 190 for this portion of the plot) but subsequently deviate from linearity significantly and approach a hyperbola as the fraction of residual activity declines. Even at a ratio of $[I]_0/[E]_0$ of 8000, complete inactivation is not achieved.

$$\frac{[I]_0}{[E]_0} = \frac{k_{cat}}{k_{inact}}\left(1 - \frac{[E]_0 - [E_{inact}]_\infty}{[E]_0}\right) \qquad (1)$$

Therefore, it is clear that the presence of the Arg-244 guanidinium moiety, and/or the high-affinity chelation to W673 by the enzyme, enhanes the rate of inactivation. This finding is consistent with the bacterial susceptibility results for clavulanate (Table III) that reflect the compromise in the ability of clavulanate to inactivate the Ser-244 enzyme efficiently *in vivo*. The background plasmidless strain lacking β-lactamase activity (*E. coli* MV1190) is susceptible to ampicillin, without significant enhancement of ampicillin inhibitory activity by the presence of clavulanate, which has poor activity by itself. The presence of the wild-type TEM-1 activity. encoded by pTZ18U, confers high-level resistance to ampicillin, which is progressively reversed by the presence of 8 and 16 μL/mL of clavulanate. The ampicillin

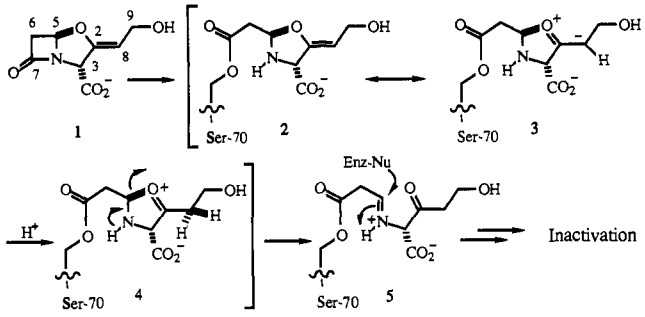
(27) The mere loss of a hydrogen bond with the side chain of Arg-244 was the sole reason for the modest reduction in rates of turnover for substrates.[9] The effect of hydrogen bonding of Arg-244 to substrate carboxylate was at the most 48-fold on k_{cat}/K_m, suggestive of the presence of a long (and weak) hydrogen bond. The effect of mutation at position 244 on the chemistry of clavulanate is substantially larger, indicative of an additional function beyond mere hydrogen bonding.

Table III. Susceptibility[a] of Strains Containing Wild-Type and Mutant TEM β-Lactamases

tested compounds	β-lactamase content of tested strains: none[b]	wild-type TEM-1 (Arg-244)[c]	(Arg-244-Ser)[d]
ampicillin	2	>1024	>1024
ampicillin + clavulanic acid, 8[e]	1	16	>1024
ampicillin + clavulanic acid, 16[f]	1	2	256
clavulanic acid	32	32	32

[a] Minimum inhibitory concentration (MIC) of tested compounds in μg/mL. [b] *E. coli* MV1190. [c] *E. coli* MV1190 (pTZ18U). [d] Mutant of *E. coli* MV1190 (pTZ18U) with Arg-244 of the TEM-1 β-lactamase replaced by Ser. [e] MIC of ampicillin in the presence of clavulanate 8 μg/mL. [f] MIC of ampicillin in the presence of clavulanate 16 μg/mL.

Scheme I



resistance conferred by the Ser-244 enzyme is much less affected by the presence of clavulanate.

Discussion

Clavulanic acid is a mechanism-based inactivator (also known as "suicide substrate" or "k_{cat} inhibitor") for class A β-lactamases, which has been in clinical use for the past few years. As a mechanism-based inactivator, it is recognized as a substrate by the enzyme and is turned over. However, in a deviation from the turnover processes, it leads to irreversible inactivation of β-lactamase. The details of the processes for inactivation were reviewed by Knowles before the tertiary structure of the enzyme was known and are quite complicated.[3] Our discussion here is intended to provide a structure-based analysis of these processes and to reevaluate a number of mechanistic assumptions for the clavulanate action.

Inactivation Chemistry of Clavulanate. We propose that the conversion of clavulanate to the inactivating species **5** (**1** → **5**. Scheme I) needs to be a stepwise process. Crackett and Stoodley had reasoned earlier that since the lactam bond is not in an antiperiplanar orientation with the C_5–O bond, the two bond scissions cannot take place in concert.[28] This reasonable chemical explanation, in conjunction with direct evidence from several laboratories for the existence of acyl–enzyme intermediates for both penicillins and cephalosporins,[29] suggests strongly that an intermediate such as **2** may exist in the course of clavulanate turnover. Furthermore, the C_5–O bond of **2** is nearly orthogonal to the π electrons of the adjacent alkene function. Since this bond cannot hyperconjugate into the π orbitals, the conversion of **2** to **5** should therefore be a stepwise process as well. Conversion of **2** to **5** will be assisted greatly by a protonation event at either the sp^2 carbon β to the ether function (depicted in Scheme I) or at the ether oxygen of **2**. With a pK_a of approximately 19–20 for the incipient enolate at C_8,[30] in the absence of protonation, the expulsion of oxygen from C_5 is a thermodynamically unfavorable reaction. These assertions find precedent in the work by Kresge and others on acid-catalyzed hydrolysis of vinyl ethers which has revealed that lability of the vinyl ether function is directly proportional to the ease with which the initial protonation of the double bond occurs, a process that may be rate-limiting.[31] Similarly, an entropically favorable intramolecular protonation of the double bond accounts for the extraordinary lability of the cyclic vinyl ether of prostacyclin in solution.[32]

The energy-minimized structure for the precatalytic ("Michaelis") complex of clavulanate in the enzyme active site is shown in Figure 2A and Figure 3A. Both the Arg-244 side chain and crystallographuic water W673 come close to the alkene functionality of clavulanate, whereas the ring oxygen is pointing out to the opening of the active site. After acylation of Ser-70 (Figure 2B), despite the rotation of the five-membered ring, the proximity of the C_2 functionality to Arg-244 and W673 remains favorable. We wish to propose as a working hypothesis that the structurally essential clavulanate carboxylate may function as a general base, whereby it facilitates the transfer of a proton from W673 to the carbanion in **3**. via an entropically favored six-membered transition.[33] Transfer of a proton directly from W673 to the carbanion in **3** cannot be ruled out of hand; however, the distance of 3.9 Å between W673 and the carbanion should make this possibility less likely. The source of this essential proton has been presumed previously to be bulk water from the medium. The ideal positioning of W673. chelated by the side chain of Arg-244 and the main-chain carbonyl of Val-216, with respect to the oxazolidine ring of clavulanate, provides an entropic factor in favor of the proton-transfer reaction as proposed here;[34] W673 is in essence an "enzymic residue" in this scheme. This role for W673 finds precedent in our earlier work with carbapenem tautomerization in the β-lactamase active site.[10,33]

To substantiate the proposed role for Arg-244 and W673, we have carried out comparative kinetic experiments for inactivation chemistry of the TEM-1 β-lactamase and the Arg-244-Ser mutant enzyme with clavulanate. Substitution of the wild-type Arg-244 with serine was expected not only to remove the Arg guanidinium moiety from the active site but also to reduce affinity for coordination to W673 by the protein. The results shown in Figure 6 clearly indicate a large attenuation in the rate of β-lactamase inactivation by clavulanate as a consequence of Arg-244 substitution in the mutant enzyme. Yet, inactivation does take place eventually, albeit with severely impaired efficiency (Figure 6 and Figure 7). These *in vitro* effects are reflected in the demonstration of resistance to the synergistic activity of ampicillin plus clavulanate in the strain bearing the mutant enzyme. We find it significant that the same Arg-244-Ser mutant that we designed in the laboratory for our studies has recently been identified in the plasmid of an ampicillin/clavulanate-resistant clinical isolate of *E. coli*.[35] It thus appears that our engineered mutation has

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(32) Cho, M. J.; Allen, M. A. *Prostaglandins* **1978**, *15*, 943.
(33) We have suggested earlier that the Arg-244 side chain or a water molecule coordinated to it may serve as the soruce of an essential proton in turnover of carbapenem.[10] At the time, we did not have access to a crystal structure of β-lactamase which showed the structurally conserved water molecules; however, we had assumed that a water molecule should be coordinated to the Arg-244 guanidinium moiety. It is likely that W673 is the source of the proton in carbapenem turnover as well. Similarly to the clavulanate case, the carboxylate of carbapenem, in principle, may facilitate the transfer of proton from W673 to the five-membered ring in carbapenems.
(34) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678. Page, M. I. *Chem. Soc. Rev.* **1973**, *2*, 295.
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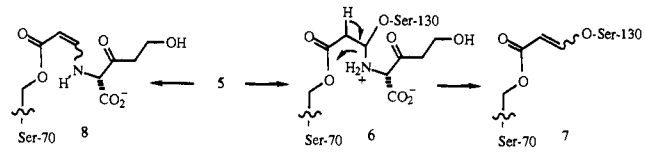
already been selected in nature and thereby compromises the utility of clavulanate in preserving the effectiveness of penicillins in therapy.

A New Proposed Role for the Conserved Ser-130 in the Formation of the Acyl–Enzyme Intermediate. As shown in Figure 2A, Ser-130 contributes to initial binding of clavulanate by coordination to the inactivator carboxylate. After Ser-70 acylation, a rotation of approximately 45° about the C_5–C_6 bond brings the lone electron pair on the oxazolidine nitrogen to the coordination sphere (2.87 Å) of the Ser-130 β-hydroxyl (Figure 2 and Figure 3). Indeed, we speculate that the driving force for the ring rotation may be this new interaction with Ser-130.

The hydroxyl of Ser-70 attacks the β-lactam carbonyl to generate the high-energy tetrahedral species en route to the acyl–enzyme intermediate. The collapse of the tetrahedral species leads to the expulsion of the nascent amine. We propose here that Ser-130 facilitates the transition state for departure of the incipient amine by donation of a hydrogen bond.[36] Fersht showed for the first time that enzymes utilize hydrogen bonds to lower energy barriers, rather than merely to improve affinity for substrates.[37] The role offered here for Ser-130 in the acylation of Ser-70 by the β-lactam moiety is consistent with this thesis, and indeed it finds precedent among some proteases. For example, Tyr-248 of carboxypeptidase A forms a hydrogen bond to the amine of the scissile bond in substrates and substrate analogues.[38] The contribution of this hydrogen bond to lowering of the transition-state energy barrier in solution for turnover or improved binding affinity for an inhibitor has been measured at 2–2.5 kcal/mol.[39] Similarly, catalysis by thermolysin benefits from a bifurcated hydrogen bond between the amine moiety of the scissile bond in substrate and the main-chain amide carbonyl of residue 113 and the side-chain function of Asn-112.[40] This mechanistic contribution by Ser-130 should apply to turnover of typical penicillins and cephalosporins as well, a possibility that has not been considered to date. In summary, the conserved Ser-130 plays at least three important functions in class A β-lactamases: (i) it serves a structural role by making a strong (2.7 Å) hydrogen bond to the side chain of Lys-234,[5,6] (ii) it is involved in substrate carboxylate recognition.[6,9,23] and (iii) it lowers the energy barrier for hydrolysis of substrates by hydrogen bonding to the incipient amine in the course of turnover, as reported here. The contribution of Ser-130 to turnover chemistry has been studied earlier by kinetic analyses of mutant enzymes at this position.[23,41] From these experiments, it is apparent that the conserved residue 130 has an important influence in the process of turnover; however, a clear dissection of the attributed functions to this residue is technically not possible, and thus does not allow one to draw a meaningful conclusion for the contribution of individual roles in turnover.

Generation of Transiently Inhibited and Covalently Modified Enzyme Species. The inhibition pattern of clavulanate, as stated earlier, is complicated. At least two species, a transiently inhibited and an irreversibly inactivated enzyme, have been proposed. Knowles and colleagues suggested that a transimination by a lysine side chain with the iminium species in **5** results in irreversible inactivation. The energy-minimized model for **5** in the enzyme active site revealed the presence of Lys-73 and Lys-234 in fair proximity to the iminium carbon (C_5), but in the crystal structure both lysines are calculated to have zero accessibility to a probe the size of a water molecule.[6] In this admittedly static picture, contact between the C_5 of species **5** and the Lys-234 side chain appears, at first glance, not probable for purely steric reasons. However, considerable structural flexibility may exist in the enzyme, and, as a consequence of the cleavage of both rings, in species **5** as well, to allow contact with the lysine side chain(s). Therefore, we carried out molecular dynamics simulations on **5** over 60 ps to inspect the distances of several active site residues to C_5 (Figure 5). These analyses indicated that the most likely candidate for the potential nucleophilic residue is not a lysine, but rather Ser-130, whose β-hydroxyl approaches to within 3.6 Å of C_5 (Table II). Addition of the Ser-130 hydroxyl to the iminium moiety (**5** → **6**) would proceed with ease as neutral to acidic pH and does not require activation, as indicated by model studies (Scheme II).[42] However, the β-hydroxyl of Ser-130 is hydrogen bonded to Lys-234, which indeed may contribute to the activation of the β-hydroxyl function, provided that the Lys-234 side chain is embedded in the protein in its deprotonated form. Notably, Ser-130 is one of two residues in the active site that have a strained α_L conformation.[6] We suggest that such nucleophilic capture is followed by deprotonation at C_6, mediated by Glu-166 via W712 to afford species **7**, which would account for irreversible inactivation of the enzyme. This suggestion is consistent with Knowles' measurements of a primary deuterium isotope effect of 3 for deprotonation at C_6 on the inactivation rate constant for the related compound sulbactam (analogous to species **6** in the inactivation by clavulanate),[43] the generation of a chromophore with a λ_{max} at 280 nm typical of α,β-unsaturated esters (as in species **7**),[43–45] and the demonstrated release into solution of the portion of the molecule from **6** that constitutes the former five-membered ring of clavulanate, yielding species **7**.[46,47] Brenner and Knowles had shown that both hydrogens at C_6 are exchangeable, although the 6β hydrogen was exchanged more readily.[43] These experimental observations are consistent with our dynamics simulations which indicate favorable proximity and orientation for both C_6 hydrogens, but especially so for the 6β hydrogen, with respect to the activated water W712 (Figure 8).[48] It is more difficult to reconcile with the crystallographic information the observation by Knowles and colleagues that three

Scheme II



(36) *The function offered here for the Ser-130 β-hydroxyl is merely donation of a hydrogen bond and not protonation of the amine anion in the course of turnover.* Recent work from the Fink group discounts the possibility that the Glu-166 side-chain carboxylate serves as the functionality that shuttles a proton from Ser-70 to the amine anion.[25] Alternatively, according to Herzberg, Lys-73 may serve as the proton shuttle.[4] Further experimentation is needed to shed definitive light on the nature of the process of activation of Ser-70 hydroxyl and protonation of the amine anion.

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(45) For a survey of chromophores typical for β-substituted-α,β-unsaturated carbonyl compounds, consult: Ostercamp, D. L. *J. Org. Chem.* **1970**, *35*, 1632.

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(48) Delaire *et al.* (Delaire, M.; Lenfant, F.; Labia, R.; Masson, J. M. *Prot. Eng.* **1991**, *4*, 805) showed that substitution of Glu-166 with 18 other amino acids abolished activity totally. One mutant, Glu-166-Tyr showed some activity. Delaire *et al.* indicated that tyrosine can substitute for glutamate in interactions of the enzyme with some substrates and clavulanate. However, these researchers did not attempt to separate the contribution of irreversible inactivation and reversible inhibition of the enzyme by clavulanate in their analysis.

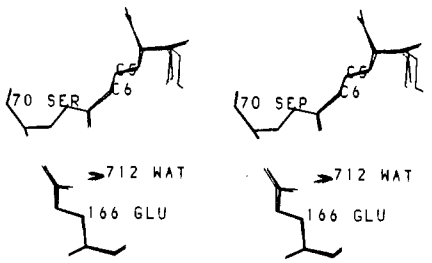

Figure 8. Overlay of three positions of Glu-166. W712. and species **5** from the dynamics simulations. See also Figure 5B.

distinct modified β-lactamases species can be identified by isoelectric focusing after treatment with clavulanate.[26,44,46] It is possible, in principle, that an inactivator-induced conformational change in β-lactamase,[49] not revealed by the dynamics simulations, may bring Lys-73 or Lys-234 side chains close enough to the C_5 of **5** to result in the generation of additional inactivated protein species. However, these three species need not necessarily arise from modification of three different amino acid side chains by **5**. For example, hydrolysis of the Ser-70 ester bond in **7** would yield an inactivated β-lactamase species that forms a distinct band on isoelectric focusing.

The Knowles group described the existence of a so-called transiently inhibited species in the course of the inactivation chemistry by clavulanate, to which they assigned structure **8**.[43,46] This structure arises from the tautomerization of **5** to the more stable enamine **8**, most likely carried out by Glu-166 via the intervening W712. The existence of both *cis* and *trans* forms of **8** have been reported in the literature.[50] The wavy presentation of bonds in structures of species **7** and **8** (Scheme II) is intended to denote the presence of both *cis* and *trans* isomers. The α,β-unsaturated ester in **8** does not serve as a Michael acceptor for an active site nucleophile; however, it affords hydrolytic stability to the ester function. It appears from this discussion that whether the inactivator would be processed into the transiently inhibited or irreversibly inactivated forms is a function of how readily deprotonation of C_6 may take place. If deprotonation occurs before nucleophilic capture by the iminium species, transient inhibition takes place. However, if deprotonation takes place after nucleophilic capture, irreversible inactivation ensues. For the TEM enzyme the two rates are comparable, so both processes take place concurrently.

Acknowledgment. This work was supported in part by grants from Smith-Kline Beecham, Eli Lilly & Co., and the National Institutes of Health Grant AI 33170.

Note Added in Proof: Subsequent to submission of this manuscript, a report by Delaire et al. (Delaire, M.; Labia, R.; Samama, J. P.; Masson, J. M. *J. Biol. Chem.* **1992**, *267*, 20600) supports the kinetic observations reported in this manuscript, albeit the interpretation of the data is different than ours. We note that Lamotte-Brasseur et al. reported a role for Ser-130 in the process of Ser-70 acylation earlier (Lamotte-Brasseur, J.; Dive, G.; Dideberg, O.; Charlier, P.; Frère, J. M.; Ghuysen, J. M. *Biochem. J.* **1991**, *279*, 213. Lamotte-Brasseur, J.; Jacob-Dubuisson, F.; Dive, G.; Frère, J. M.; Ghuysen, J. M. *Biochem. J.* **1992**, *282*, 189). Lamotte-Brasseur et al. suggested that Ser-130 may be a leg in proton relay in the course of Ser-70 acylation, a role which is different than what has been put forth in this manuscript for Ser-130.

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