Class A $\beta$-lactamases are the predominant source of bacterial resistance to the $\beta$-lactam family of antibiotics, such as the penicillins and the cephalosporins. Consequently, these enzymes are targets for anti-resistance drug design and have been extensively studied mechanistically since the seminal studies of Knowles. Among the best studied and most widespread of the class A $\beta$-lactamases is TEM-1, whose catalytic mechanism and potential for inhibition have drawn considerable attention. TEM-1 catalyzes the hydrolysis of $\beta$-lactams near the diffusion limit (10$^8$ M$^{-1}$ sec$^{-1}$) and is considered a “perfect enzyme”. It uses an active site serine nucleophile to cleave the lactam bond of the antibiotics, which inactivates them, in a mechanism that loosely resembles that of serine proteases. The reaction begins with the formation of a pre-covalent encounter complex (Figure 1A), and moves through a high-energy acylation tetrahedral intermediate (Figure 1B) to form a transiently stable acyl-enzyme intermediate, forming an ester through the catalytic residue Ser70 (Figure 1C). Subsequently, the acyl-enzyme is attacked by a hydrolytic water (Figure 1D) to form a high-energy deacylation intermediate (Figure 1E), which collapses to form the hydrolyzed product (Figure 1F). The product is then expelled, regenerating free enzyme. As in serine proteases, this mechanism requires a catalytic base to activate the serine nucleophile to attack the amide bond of the substrate and, following formation of the acyl-enzyme, to activate the hydrolytic water for attack on the ester center of the adduct (Figure 1).

Whereas there is a consensus on the deacylation portion of the mechanism, the acylation part of the reaction has remained controversial. The key residue implicated in deacylation is believed to be the conserved Glu166, which is located on the catalytically critical Omega loop. Previous crystallographic studies suggested that the deacylation water is activated by the catalytic water to activate Ser70 for nucleophilic attack on the $\beta$-lactam ring of the substrate. The hydrolytic mechanism of class A $\beta$-lactamases, such as TEM-1, appears to be symmetrical, as are the serine proteases. From its mechanistic implications, this atomic resolution structure affords an unusually detailed view of the structure, dynamics, and hydrogen-bonding networks of TEM-1, which may be useful for the design of inhibitors against this key antibiotic resistance target.
The Lys73 hypothesis (Figure 2C) is supported by site-directed mutagenesis and structural studies. When class A β-lactamases are substituted at this residue, which is completely conserved, catalytic activity drops by 10^3-fold or more against penicillins. This suggests that Lys73 plays a direct role in acylation and deacylation. In several class A β-lactamase structures, the NZ atom of Lys73 appears to hydrogen bond to Ser70 Oy, consistent with a role as the general base activating Ser70 for nucleophilic attack. Although substitutions at the completely conserved Glu166 also resulted in 10^2 to 10^3-fold reduction in activity, two lines of evidence seem to favor the Lys73 hypothesis: (1) the acylation rate was less affected than the deacylation rate by substitutions at Glu166; and (2) it was possible to trap acyl-enzyme intermediates of β-lactams bound to these mutants. The simplest explanation of these data was that Lys73 acts as the general base in acylation and Glu166 acts in deacylation, although alternative explanations have been proposed (where Lys73 is only important in proton shuttling in both states).

On the other hand, the Glu166 hypothesis (Figure 2B) is supported by activity studies of the pH dependence for wild type (WT), Lys73 mutants and Glu166 mutants. In WT enzyme, the pH dependence of the acylation rate constant is a bell-shaped, with a pK_a1 of 5.0 and pK_a2 of 8.5. The acidic limb pK_a is widely thought to represent the protonation state of Glu166. The origin of the basic limb pK_a remains controversial. Whereas the mutant K73A activity retained bell-shaped pH dependence, with higher pK_a1 8.0 and pK_a2 8.9, the charge-neutral mutant enzymes E166Y and E166C had sigmoidal pH-dependence curves with a single pK_a above 8.0.6,5 In addition, Waley and colleagues showed that acylation and deacylation constants have the same pH profile. These data suggest that Glu166 plays a role as a general base in acylation. Additionally, for Lys73 to act as a catalytic base, it would have to be deprotonated at physiological pH (Figure 2). However, NMR evidence suggests that the pK_a of this residue is greater than 10, which seems inconsistent with the Lys73 hypothesis. Finally, X-ray crystallographic evidence, which has been used to support a role for Lys73 as the catalytic base, is also consistent with a role for Glu166, which is observed to hydrogen bond to the catalytic water (Wat1004 in this study) that, in turn, is observed to hydrogen bond with the nucleophilic Ser70.

Figure 1. The reaction cycle of TEM-1 β-lactamase. The two states in braces represent the acylation and deacylation tetrahedral high-energy intermediates.

Figure 2. Two candidate mechanisms for acylation of TEM-1 β-lactamase. (A) Nucleophilic attack of Ser70 Oy on the lactam carbonyl carbon. (B) Proposed role of Glu166 as the catalytic base in acylation. (C) Proposed role of Lys73 as the catalytic base in acylation. carboxylate of Glu166, which plays the role of the catalytic base in deacylation. In one mechanism, the catalytically key residue Lys73 is proposed to be the catalytic base in its deprotonated state, activating Ser70 for attack on the lactam carbonyl carbon (Figure 2C). This would result in an asymmetrical hydrolytic mechanism. In the second mechanism, Glu166 would play the role of the catalytic base in acylation, activating Ser70 mediated through a bound water (Figure 2B). Despite extensive site-directed mutagenesis studies and structural investigations by both X-ray crystallography and NMR, no consensus has emerged that favors one mechanism over the other.

ultrahigh-resolution structure allows us to observe the protonation state of Lys73, Glu166, and Wat1004, and to determine alternative conformations may help with inhibitor design studies against this key antibiotic resistance target.

Materials and Methods

Preparation of TEM-1 Mutant M182T. This work was conducted with a stabilized mutant TEM-1, M182T.\textsuperscript{31} The substitution Met182 → Thr stabilizes the mutant enzyme by 2.6 kcal/mol relative to wild type (WT),\textsuperscript{32} but has very little effect on catalytic activity;\textsuperscript{31} the substitution is 1 Å from the catalytic serine, far from the active site. The mutant was prepared by a two-step PCR protocol.\textsuperscript{33,34} In brief, two PCR reactions were performed: one involved a mutagenesis anti-sense primer and a sense primer that covered the 5′ site that flanks the NdeI site and another involved a mutagenesis sense primer and a sense primer covering the EcoRI site that flanks the 3′ end of the gene. The reaction volume of 100 μL included 2.5 units of Pfu DNA polymerase (Stratagene), reaction buffer provided by Stratagene, 100 ng of pAlter Ex II TEM-1, 250 ng of flanking primer, 20% (w/v) sucrose at room temperature for 10 min. Cells were then collected and resuspended in ice-cold 5 mM MgCl\textsubscript{2} for 10 min. The supernatant was saved as the periplasmic contents, concentrated to about 100 mL, and dialyzed against 5 mM Tris/HCl, pH 8.0. The crude extract was applied to a Q-Sepharose FF column (Pharmacia, Uppsala, Sweden) equilibrated with 5 mM Tris/HCl, pH 8.0. The column was then washed extensively with 5 mM Tris/HCl, pH 8.0. The enzyme was eluted by 5 mM Tris/HCl, pH 8.0, containing 100 mM NaCl. The active fractions were pooled and dialyzed against 100 mM sodium phosphate, pH 8.0. The enzyme solution was concentrated to 6 mg/mL and stored in 200 mM potassium phosphate, pH 7.0, 50% glycerol at −20°C.

Crystalization and Data Collection. Crystals of the complex of TEM M182T with compound I, ([2-amino-α-(1-carboxyl-1-methyl-ethoxylimino)-4-thiazoleacetyl]amino)methaneboronic acid, which is a boronic acid transition state analogue inhibitor,\textsuperscript{36} were grown by seedling techniques. An 8 μL droplet containing 5 mM of the enzyme and 2.5 mM compound I in 0.65 M sodium–potassium phosphate buffer, pH 8.0, were seeded with microcrystals of apo M182T\textsuperscript{32} and placed over 1.4 M sodium–potassium phosphate well buffer, pH 8.0. Single crystals appeared in 7 days and grew to a maximum size of 0.4 × 0.4 × 0.6 mm\textsuperscript{3} in two weeks. Crystals were soaked in cryo-protectant (25% sucrose in 1.6 M phosphate buffer, pH 8.0) for 60 s and then frozen in liquid nitrogen. Diffraction data were collected from a single crystal on the 5D beamline of the DND-CAT at Advanced Photon Source (Argonne, IL). Two sets of data, at high and low resolution, were collected at a wavelength of 0.7999 Å (λ = 15.5 keV) using a MARCCD detector. A total of 484 frames were integrated, and 1 143 489 reflections were scaled and merged using DENZO/SCALEPACK package.\textsuperscript{27} The data are 100% complete to 0.85 Å resolution (200 302 unique reflections, Table 1). Crystals belong to the space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}2, with unit cell parameters of 41.31, 61.64, and 89.25 Å, which are similar to the unit cell parameters of the M182T apo-enzyme crystals\textsuperscript{33} and to those of WT.\textsuperscript{27}

Refinement. Standard conjugate gradient refinement (CGLS) was carried out with SHELEX\textsuperscript{36} using the apo-M182T\textsuperscript{32} as an initial model. Three percent of the data (6015 reflections) was set aside randomly as the test set. The resolution was increased stepwise from 2.0 to 0.85 Å (Table 2). The model was manually corrected using 2Fo – Fc and Fo – Fc, oS\textsuperscript{36} maps displayed with TURBO\textsuperscript{39} on a Silicon Graphics computer. Compound I was fit into unambiguous difference density. At this stage, about 60% of the residues was modeled in multiple conformations to interpret features of an F(–)Fc electron density map (Table 2). The model was then refined using ADPs (anisotropic displacement parameters) with standard DELU (rigid-bond restraint), SIMU (restraint for spatially adjacent atoms), and ISOR (restraint for isolated atoms to be approximately isotropic) restraints, and converged at R-factor/R\textsubscript{free} 10.9/13.0%.

Table 1. Data and Refinement Statistics

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</table>

Values in parentheses are for the highest resolution shell.


\textsuperscript{40} Merritt, E. A. Acta Crystallogr., Sect. D 1999, 55, 1109–1117.
Here we report the crystal structure of a boronic acid complex with M182T (Table 3) complex of the TEM-1 M182T mutant (Table 3 and Figure 3A). Thus, for Asp273 the electron density at the 2.0 Å level for O2 is discrete, while the density for O1 is closer to and continuous between Cγ, which would be consistent with a single (longer) bond between O2 and Cγ and a double bond between O1 and Cγ (Figure 3A). For the sulfur atoms of Met69, density is consistent with the location of lone pair electrons, which are observed as positive peaks in Fo − Fe map at 1.5σ (Figure 3B). Finally, difference density for more than 70% of all hydrogen atoms (1683/2420) was observed as peaks at the 1.5σ level. We chose this level as a cutoff for modeling a hydrogen atom into density, reasoning that a level of 3.0σ is typically used to assign heavy atom positioning into Fo − Fe maps in lower resolution structures, thus making a 1.5σ cutoff for a hydrogen with only two electrons in its bonding orbital reasonable. The quality of the data allowed us to refine positions of the hydrogen atoms in methyl and hydroxyl groups using torsion angle refinement and to use the “riding” model refinement for the rest of the hydrogen atoms in SHELXL. Figure 3C shows the electron density maps for the Thr180–Thr181–Thr182 region of the structure. Well-defined positive peaks at 2.0σ level in the Fo − Fe density map are located near predicted hydrogen atoms. Peaks were found for all main-chain hydrogen atoms, except for several regions adopting multiple conformations, and for most side-chain hydrogen atoms. Surprisingly, it was even possible to assign proton positions to several water molecules that had unambiguous positive difference peaks (Figure 3D).

**Hydrogen Atoms in the Active Site.** The ultrahigh-resolution structure of TEM-1 β-lactamase provides a detailed picture of the hydrogen atoms in the active site. Unambiguous electron density in both Fo − Fe and 2Fo − Fe maps suggested that the boronic acid transition-state analogue I (Table 3 and Figure 4A and D) is covalently bound to Oγ of Ser70, as expected. Both maps indicated that the side chain of compound I, which mimics the R1-sided chain of cefazidime, adopts two alternative conformations, involving a rotation around the Cβ–Oγ–B–C7 torsion angle (Figure 4A). One hydrogen atom is visible in Fo − Fe density map at 1.4σ level, on the O2 atom of the boronic acid (Figure 4B). We note that the shape of the 2Fo − Fe density map suggested that the proton on the O1 oxygen of the boronic acid, corresponding to the position of the “oxyan-
ion" or "hydroxyl", is pointed toward the carbonyl oxygen of Ala237, consistent with earlier predictions. Nevertheless, we felt that the evidence for these proton positions was not strong enough to include those in the final model. In addition, hydrogen atoms were visible on C7, N10, and C15 of compound 1. No hydrogen atoms were modeled for N18, C19, and C20 that are located at the end of compound 1, due to the high B-factors of these atoms.

Most hydrogen atoms on the side-chain termini of Lys234, Ser130, Lys73, Asn170, Ala 237, and Asn132 are clearly visible at 1.5σ level (see Figure 4B for representative density). The OD1 and OD2 from Glu166 were unambiguously differentiated by their densities and had different bond lengths, consistent with one atom being predominantly double bonded and the other being predominantly single bonded in the asymmetric environment of the protein (Figure 4C). Unexpectedly, even at a pH 8.0 where the crystal was grown, strong positive density at OD2 was present to indicate that Glu166 was protonated (Figure 4C and see below for detailed discussion). Peaks of positive difference density on the proposed deacylation water (WAT1004) pointed toward OD1 of Asn170 (1.6 σ) and toward Oγ of Ser70 (1.4 σ), indicating unambiguous hydrogen atoms on this water (Figure 4C).

Discussion

Perhaps the most interesting result to emerge from this structure is the protonation state of Glu166 and the subsequent hydrogen-bonding network in which it participates in a complex that mimics the high-energy acylation intermediate.

In this complex, Glu166 appears to be protonated on the basis of strong difference density features (Figure 4C). This glutamic acid proton is donated to Wat1004 to form a hydrogen bond. This water, in turn, donates one of its protons to the catalytic Ser70, which, because it is forming an acyl-adduct with the transition-state analogue, can only accept a proton. That Glu166 should be protonated in this structure, even at pH 8.0, and that the key conserved Wat1004 should be positioned to accept this proton and donate its own proton to Ser70, seems compelling to us. An appealing interpretation of these observations is that Glu166, acting as the ultimate catalytic base for acylation, activates Wat1004, which, in turn, activates Ser70. This activation leads to a proton shuttle from the serine to the water to the glutamic acid.

We believe that the boronic acid mimics the acylation transition state on the basis of the geometry it adopts in the site, which closely resembles that of a phosphonate inhibitor bound to the analogous class A β-lactamase, PC1.49,50 This phosphonate structure was thought to represent the acylation high-energy intermediate geometry.49 Correspondingly, the position of the boronic acid group differs considerably from that adopted by another boronic acid bound to TEM-1, which was thought to represent the structure of the deacylation high-energy intermediate.2,3 In the geometry observed here, the O1 oxygen of the boronic acid corresponds to the position of the former lactam carbonyl oxygen in the “oxyanion”47 or “electrophilic”48 hole. The hydrogen bond that appears to be likely between the carbonyl oxygen of Ala237 and the O1 boronic oxygen is consistent with the ability of this site to bind to protonated groups, such as hydroxyls, protonated acids, and waters. The O2 boronic oxygen corresponds to the position of the former lactam nitrogen, the leaving group in the acylation reaction. In the 0.85 Å structure, this proxy for the ring nitrogen may donate a proton to Wat1076 and accepts a proton from Ser130 (Figure 4B). Such a pattern would suggest that Ser130 is playing the role of the catalytic acid in the reaction, activating the leaving group, as has been previously suggested.20,51

Several aspects of this 0.85 Å resolution structure will interest the specialist. The ultrahigh resolution afforded us experimental evidence for positions of more than 70% of the protons on the protein. These positions are, of course, very important to know when modeling such things as catalytic activity and inhibition from a protein structure, but for many groups on a protein they are rarely observed and must instead be predicted. It may be interesting to compare the proton positions that we observe with those that would have been predicted by widely used prediction programs. These positions may be of direct interest to investigators interested in designing new inhibitors for TEM-1.2,52,53

The ultrahigh resolution also affords a view of protein motion that is not typically available in crystal structures. The active site, which is stabilized by a transition state analogue,19,34,54 appears to move as a group, may speak to fluctuation modes experienced by proteins that are relevant to activity.55,56 Other residues appear to sample side-chain rotamers, relatively independent of other side chains. For
these residues, the structure sometimes reveals multiple conformations where, at lower resolution, only a single conformation was modeled. An example of this may be seen for Glu58 (Figure 5B and C), which at lower resolution (1.73 Å) was fit as though it were in a single, apparently high-energy conformation.\textsuperscript{34} At 0.85 Å resolution, it is clear that this residue is sampling two low-energy conformations that were masked in the lower resolution structure. In this ultrahigh resolution structure, we observe 64% of all residues to occupy more than one conformation, which would suggest that in structures of lower resolution one is fitting only one out of several possible conformations or, occasionally, averaging several conformations together.

**Conclusion**

The observation of a protonated Glu166 at 0.85 Å resolution, and the hydrogen-bonding network in which it participates, is consistent with this residue acting as the catalytic base in the acylation reaction mechanism. According to such a mechanism, Glu166 would activate Wat1004, which, in turn, would activate Ser70, accepting the proton of the Ser70 nucleophile during the attack on the lactam carbonyl center (Figure 2B). In the transition-state analogue complex structure reported here, the proton has been shuttled from the serine through the water to end up on the glutamic acid. Meanwhile, the leaving group lactam nitrogen (mimicked by O2 of boronic acid) is activated by accepting a proton from Ser130, whose proton is also visible in this structure, hydrogen bonding to the boronic acid O2 (Figure 4B), which acts as a proxy for the lactam nitrogen. The role of Lys73, which in our structure and in previous structures hydrogen bonds with both Ser70 and Ser130,\textsuperscript{2} may be to activate both residues electrostatically. Lys73 may also act to transfer a proton to replace the one Ser130 has donated in activating the lactam nitrogen. Such a proton transfer might be essential for an efficient deprotonation of Glu166 for deacylation. Lys73 is optimally positioned to function in such a proton transfer, which can be accommodated to the observation that mutants at Lys73 disrupt enzyme activity, since such mutants would be expected to be less activated if a protonated Glu166 persists. We note that the possibility that Lys73 is acting as the catalytic base (Figure 2C) is not directly falsified by this structure; rather, such a mechanism would now have to bear the additional burden of explaining why Glu166 should be protonated in the acylation high-energy intermediate, and why it should participate in a hydrogen-bonding network that extends to the nucleophilic serine.

Taken together with the NMR pK\textsubscript{a} measurement of Lys73,\textsuperscript{10} this structure suggests that Glu166 is the most likely candidate for the general base role in acylation, acting through a conserved water. The same water later attacks Ser70 O\textsubscript{\textbeta} to release the product with activation again by Glu166, which would make the reaction cycle of class A β-lactamase symmetrical, similar to serine proteases. The high resolution of this structure affords an unusually detailed view of the proton positions on the enzyme overall, as well as some of the ranges of motion that the enzyme experiences. The structure may find broad use for investigators interested in predicting proton positions and protein movements, and find particular application among investigators interested in designing novel inhibitors for this key antibiotic resistance enzyme.

**Data Deposition.** The coordinates have been deposited in the Protein Data Bank (accession code for compound I/TEM-1 M182T complex is 1L7U).

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