Neutron and X-ray Crystal Structures of a Perdeuterated Enzyme Inhibitor Complex Reveal the Catalytic Proton Network of the Toho-1 β-Lactamase for the Acylation Reaction*

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Background: Antibiotic resistance from extended-spectrum β-lactamases (ESBLs) makes infections more dangerous and difficult to treat.

Results: Neutron and x-ray crystal structures were determined for an ESBL in complex with an acylation transition state analog.

Conclusion: Glu-166 is implicated as the general base in the acylation reaction.

Significance: Understanding the catalytic mechanism of β-lactamases will lead to improved antibiotics and β-lactamase inhibitors.

The mechanism by which class A β-lactamases hydrolyze β-lactam antibiotics has been the subject of intensive investigation using many different experimental techniques. Here, we report on the novel use of both neutron and high resolution x-ray diffraction to help elucidate the identity of the catalytic base in the acylation part of the catalytic cycle, wherein the β-lactam ring is opened and an acyl-enzyme intermediate forms. To generate protein crystals optimized for neutron diffraction, we produced a perdeuterated form of the Toho-1 β-lactamase R274N/R276N mutant. Protein perdeuteration, which involves replacing all of the hydrogen atoms in a protein with deuterium, gives a much stronger signal in neutron diffraction and enables the positions of individual deuterium atoms to be located. We also synthesized a perdeuterated acylation transition state analog, benzoithiophene-2-boronic acid, which was also isotopically enriched with 11B, as 10B is a known neutron absorber. Using the neutron diffraction data from the perdeuterated enzyme-inhibitor complex, we were able to determine the positions of deuterium atoms in the active site directly rather than by inference. The neutron diffraction results, along with supporting bond-length analysis from high resolution x-ray diffraction, strongly suggest that Glu-166 acts as the general base during the acylation reaction.

The production of β-lactamases is the predominant cause of bacterial resistance to β-lactam antibiotics (1). β-Lactamases catalyze hydrolysis of the critical β-lactam ring, thereby rendering the antibiotic harmless to bacteria. Except for class B metalloenzymes, β-lactamases belong to the family of serine-reactive hydrolases. Specifically, the class A β-lactamases are typified by the TEM, the SHV, and the emergent extended-spectrum β-lactamase CTX-M enzymes. The class A CTX-M extended-spectrum β-lactamases are most commonly encountered in clinical isolates associated with intra-abdominal and urinary tract infections from highly virulent bacteria. Due to their broad substrate profile, CTX-M extended-spectrum β-lactamases lead to treatment problems in many clinical settings and dramatically increase mortality rate. These enzymes exhibit increased hydrolytic activity against the first, second, and third generation extended-spectrum cephalosporins and monobactams (1–7). To develop new antibiotics and β-lactamase inhibitors, a greater understanding of both the catalytic mechanism and the structural features of these enzymes that contribute toward their broad substrate specificity is required.

The Toho-1 β-lactamase is classified as a CTX-M-type extended-spectrum β-lactamase on the basis of its amino acid sequence and its broad activity against the extended-spectrum cephalosporins. Toho-1 is composed of 262 amino acid residues and, like other Class A β-lactamases, is made up of two highly conserved domains (α/β and α), where the interface of these two domains forms the active site cavity (8, 9). All class A β-lactamases employ an active site serine nucleophile to cleave the lactam bond of the substrate in a two-step acylation-deacylation reaction cycle that leads to overall hydrolysis. The complete catalytic cycle for β-lactam hydrolysis is shown in Fig. 1. The acylation reaction initiates with the formation of a pre-covalent substrate complex (1). General base-catalyzed nucleophilic attack on the β-lactam carbonyl by the serine hydroxyl...
proceeds through a tetrahedral intermediate (2) to a transiently stable acyl-enzyme adduct (3). In the deacylation phase, the acyl-enzyme adduct (3) undergoes general base-catalyzed attack by a hydrolytic water molecule to form a second tetrahedral intermediate (4), which collapses to a postcovalent product complex (5), from which the hydrolyzed product is released.

Our present understanding of the cycle derives from a multitude of studies. These studies include mechanistic and mutagenesis studies (10, 11), computational simulation (12–14), and structural studies. X-ray crystal structures determined to date have spanned the reaction coordinate, from ligand-free enzymes to precovalent, acylation transition state analog, acyl-enzyme, deacylation transition state analog, and postcovalent complexes using the TEM, SHV, CTX-M, and Toho-1 β-lactamases (8, 15–25). In addition, we have recently published neutron crystallographic studies of ligand-free Toho-1 β-lactamase mutants (26, 27).

Despite this wealth of information, key aspects of the mechanism remain unresolved and controversial. Among them is the identity of the residue acting as the catalytic base in the acylation reaction. Two distinct mechanisms, with different residues serving as the general base, have been proposed. A number of studies have suggested that the highly conserved residue Lys-73 acts in its neutral form as the general base (11, 14, 24). In opposition, another hypothesis proposes that the highly conserved Glu-166, acting through the catalytic water, is the catalytic base during acylation (10, 13, 15, 19, 21, 26–28). Supporting this latter hypothesis are ultrahigh resolution (≤1.0 Å) x-ray structural studies of class A β-lactamases (15, 19, 21) and our neutron crystallographic studies (26, 27) that have revealed the location of proton positions and the resulting hydrogen-bonding interactions of key active site residues.

For resolving issues of this nature surrounding a catalytic mechanism, neutron crystallography is a powerful complement to x-ray crystallography because it can provide the locations of hydrogen atoms directly, rather than by inference. Because X-rays are scattered by electrons, hydrogen atoms scatter X-rays weakly and are usually not detected, even at high resolution. Neutrons are scattered by atomic nuclei, and the stable hydrogen isotope deuterium (^2H) has a similar neutron coherent scattering length to the heavier elements in proteins, such as carbon, nitrogen, and oxygen. Thus, the positions of deuterium atoms can be determined directly at resolutions up to 2.5 Å (29). Perdeuteration, the complete isotopic substitution of all
hydrogen atoms within a protein for deuterium atoms, provides an additional, powerful benefit in neutron crystallography by significantly increasing the signal-to-noise ratio of the diffraction data by greatly reducing the incoherent scattering background from hydrogen (29).

Although the benefits of perdeuteration in neutron crystallography are apparent, to date only a few examples of fully deuterated neutron structures have been reported (27, 30–32). By exploiting the sensitivity of neutron crystallography to deuterium, we were recently able to reveal unambiguously the ground-state active site protonation states and the resulting hydrogen-bonding network in the active site of the perdeuterated Toho-1 R274N/R276N double mutant (pd-Toho-1 R274N/R276N) (27). Here, we report the x-ray and neutron structures of the same protein in complex with a perdeuterated acylation transition state analog, benzothiophene-2-boronic acid (BZB)2 (Fig. 1), which reveals the changes likely associated with the acylation reaction. Of particular importance, the new structures show that Glu-166 is protonated in the complex, whereas it is deprotonated in the ligand-free protein. Further, the Ser-70 hydroxyl is deprotonated and has formed a tetrahedral boronate adduct with the inhibitor, analogous to the tetrahedral intermediate. Finally, the Lys-73 side chain is probably in the ammonium form. These results are in strong agreement with previous ultrahigh-resolution x-ray structures (15, 19, 21) that implicated Glu-166 as the catalytic base during acylation.

EXPERIMENTAL PROCEDURES

Materials—D₂O (99.8% D) was purchased from Cambridge Isotope Laboratories (Andover, MA). Benzothiophene, reagents, and anhydrous solvents for reactions were purchased from Sigma-Aldrich and used as received. Other chemicals and solvents were of A.C.S. reagent grade or better.

Benzothiophene-d₆—Benzothiophene was deuterated by metal-catalyzed exchange with D₂O under the conditions described by Clark and Primak (33) using the experimental apparatus described by Hawthorne et al. (34). In a typical reaction, 245 mg (1.8 mmol) of benzothiophene and 2.0 ml of degassed 6 mM FeCl₃ in D₂O were heated in a sealed glass tube apparatus described by Hawthorne described by Clark and Primak (33) using the experimental procedures.

Cate Glu-166 as the catalytic base during acylation.

Expression, Purification, and Crystallization of pd-Toho-1 R274N/R276N—The perdeuterated enzyme was purified and crystallized as described previously (26, 27). Briefly, using an Escherichia coli-based expression system, the pd-Toho-1 R274N/R276N was expressed to a high yield in a fully deuterated minimal medium according to a fed-batch fermentation protocol (35, 36). Large crystals suitable for neutron diffraction, along with smaller crystals suitable for x-ray diffraction, were grown at 20 °C with the batch crystallization method using 300 µl of a 10 mg/ml protein concentration in a solution containing 2.0 M ammonium sulfate and 0.1 M sodium citrate (pD 5.1) prepared in D₂O. For neutron diffraction, a large single crystal (~1.5 × 1.5 × 1.2 mm) was soaked for 1 h in a reservoir solution containing 5% dimethyl sulfoxide-d₆, 2.7 M ammonium sulfate, 0.1 M sodium citrate (pD 5.1), and 5.0 mM pd-[¹ⁱB]BZB immediately prior to mounting the crystal in a round quartz capillary and initiating data collection. For cryogenic x-ray diffraction data collection, a crystal (~0.8 × 0.8 × 0.5 mm) was soaked in the same reservoir solution for 12 h. The soaked crystal was then placed momentarily in a reservoir solution containing a cryoprotectant (30% w/v trehalose) and flash-frozen in liquid nitrogen.

Data Collection and Refinement—Monochromatic (2.67 Å) neutron diffraction data for the pd-Toho-1 R274N/R276N BZB complex were recorded to 2.0 Å resolution at room temperature using the BIODIFF diffractometer at the FRM II research reactor, whereas high resolution monochromatic (0.97 Å) x-ray diffraction data were collected at 100 K over a range of 180° (1° steps) using an Area Detector Systems Corp. Quantum 315r detector at the Advanced Photon Source (APS) on the ID19 beamline SBC-CAT to 1.2 Å resolution. Reduction of the neutron data was performed using the Denzo and Scalepack packages (37), whereas the x-ray data were reduced using programs from the CCP4 suite (38).

Refinement of both the x-ray and neutron models was completed using the phenix.refine program (39, 40) in the PHENIX suite (41). The x-ray structure was used as a starting model for neutron data refinement. All heterogen atoms (ligand, ions, and water molecules) were removed before using the ReadySet! program in PHENIX to substitute deuterium atoms in idealized geometrical positions for all hydrogen atoms within the model.

The final neutron model of the pd-Toho-1 R274N/R276N-BZB complex was obtained after several rounds of maximum likelihood-based refinement of individual coordinates, individual atomic displacement parameters, and occupancies against neutron diffraction data alone. D₂O molecules were added to the model using phenix.refine and were then adjusted manually during subsequent model building rounds based on analysis of σₓ-weighted Fₒ – Fc and 2Fₒ – Fc positive nuclear density maps. The final R_factor for the x-ray dataset was 11.7% with an R_free of 13.2%. For the neutron dataset, these figures were 21.9
TABLE 1

| X-ray and neutron data collection and crystallographic refinement |
|---------------------------------------------------------------|
| **X-ray data collection (at 110 K)** | 4bd0 |
| PDB accession code | |
| Unit cell parameters (Å) | |
| a, b, c | a = 72.50, b = 72.50, c = 97.67 |
| α, β, γ | α = β = 90° and γ = 120° |
| Space group | P3_2_1 |
| No. of unique reflections | 91,485 |
| Resolution range (Å) | 26.41-1.21 (1.27-1.21) |
| Multiplicity | 10.1 (9.3) |
| Rmerge (%) | 5.4 (2.9) |
| Rfree (%) | 12.3 (12.2) |
| Hydrogen atoms | 2033 |
| Solvent molecules | 394 |
| Atoms (nonhydrogen) | 2464 |
| r.m.s.d. angles (°) | 1.432 |
| r.m.s.d. bonds (Å) | 0.012 |
| PDB accession code | 4bd1 |
| Unit cell parameters (Å) | |
| a, b, c | a = 73.42, b = 73.42, c = 99.11 |
| α, β, γ | α = β = 90° and γ = 120° |
| Space group | P3_2_1 |
| No. of unique reflections | 19,436 |
| Resolution range (Å) | 50.00-2.00 (2.07-2.00) |
| Multiplicity | 4.0 (2.9) |
| Rmerge (%) | 7.8 (3.7) |
| Rfree (%) | 14.7 (27.9) |
| Hydrogen atoms | 2464 |
| Solvent molecules | 394 |
| Atoms (nonhydrogen) | 2,127 |
| r.m.s.d. angles (°) | 0.25 (0.25) |
| r.m.s.d. bonds (Å) | 0.001 |
| PDB accession code | 4bd2 |
| Unit cell parameters (Å) | |
| a, b, c | a = 73.42, b = 73.42, c = 99.11 |
| α, β, γ | α = β = 90° and γ = 120° |
| Space group | P3_2_1 |
| No. of unique reflections | 19,436 |
| Resolution range (Å) | 50.00-2.00 (2.07-2.00) |
| Multiplicity | 4.0 (2.9) |
| Rmerge (%) | 7.8 (3.7) |
| Rfree (%) | 14.7 (27.9) |
| Hydrogen atoms | 2464 |
| Solvent molecules | 394 |
| Atoms (nonhydrogen) | 2,127 |
| r.m.s.d. angles (°) | 0.25 (0.25) |
| r.m.s.d. bonds (Å) | 0.001 |
| PDB accession code | 4bd3 |
| Unit cell parameters (Å) | |
| a, b, c | a = 73.42, b = 73.42, c = 99.11 |
| α, β, γ | α = β = 90° and γ = 120° |
| Space group | P3_2_1 |
| No. of unique reflections | 19,436 |
| Resolution range (Å) | 50.00-2.00 (2.07-2.00) |
| Multiplicity | 4.0 (2.9) |
| Rmerge (%) | 7.8 (3.7) |
| Rfree (%) | 14.7 (27.9) |
| Hydrogen atoms | 2464 |
| Solvent molecules | 394 |
| Atoms (nonhydrogen) | 2,127 |
| r.m.s.d. angles (°) | 0.25 (0.25) |
| r.m.s.d. bonds (Å) | 0.001 |

**Neutron data collection (at 293 K)**

| Neutron crystallographic refinement (at 293 K) |
|------------------------------------------------|
| Rmerge (%) | 21.9 |
| Rfree (%) | 25.7 |
| r.m.s.d. bonds (Å) | 0.019 |
| r.m.s.d. angles (°) | 1.154 |
| Atoms (nonhydrogen) | 2,127 |
| Solvent molecules | 145 |
| Deuterium atoms | 2,238 |
| Neutron crystallographic refinement (at 293 K) |
| Rmerge (%) | 21.9 |
| Rfree (%) | 25.7 |
| r.m.s.d. bonds (Å) | 0.019 |
| r.m.s.d. angles (°) | 1.154 |
| Atoms (nonhydrogen) | 2,127 |
| Solvent molecules | 145 |
| Deuterium atoms | 2,238 |

and 25.7%, respectively (Table 1). Ramachandran plot quality assessment was done using Molprobity (42).

**RESULTS**

**Synthesis of Isotopically Labeled BZB**—To visualize the deuterons on the ligand, it was necessary to substitute all hydrogen atoms on the BZB with deuterium. Further, it was necessary to incorporate 11B-enriched boron because 10B (20% natural abundance) has a large thermal neutron absorption cross-section. Capture of a neutron by the 10B nucleus is followed by an energetic nuclear decay that would destroy the ligand and cause extensive damage in the surrounding crystal over a 10-μm range. The requisite pd-[11B]BZB was prepared in a two-step process that entailed perdeuteration of benzothiophene followed by lithiation at the 2-position and trapping with [11B]trimethyl borate. In this way, 250 mg of BZB with 92–93% enrichment of deuterium and 99% enrichment of 11B was obtained. Purification of the product by preparative HPLC afforded homogeneous material that was soaked into the protein crystals prior to the start of data collection.

**X-ray and Neutron Structures**—The high resolution x-ray structure of the pd-Toho-1 R274N/R276N in complex with BZB is very similar to previously reported structures of the Toho-1 enzyme and is characteristic of Class A β-lactamases (23, 27). As expected, both Fg − Fc and 2Fg − Fe positive electron and neutron density maps indicated that the BZB transition state analog is covalently bound to Oγ of Ser-70 in both the x-ray and the neutron models. The boron atom is tetrahedral, and all three B–O bonds have approximately equal lengths of ~1.47 Å, consistent with deprotonation of the Ser-70 hydroxyl and formation of an anionic boronate complex analogous to the tetrahedral intermediate for acylation.

**Active Site Hydrogen-bonding Network**—Of greatest interest was the proton (deuteron) inventory of the active site. To eliminate model bias and confirm the locations of deuterium atoms in the active site region, the initial neutron model was constructed with no deuterium atoms on the side chains of active site residues. Following initial refinement of the model against the neutron diffraction data, distinct positive peaks in the difference map were observed that corresponded to deuterium atoms on the side chains of the active site residues. Deuterium atoms were then added into these positive difference peaks, and the resulting nuclear density maps were analyzed following additional refinement (Fig. 2). Unambiguous nuclear density was present for all exchangeable deuterium atoms on the side chains of active site residues Glu-166, Lys-73, Asn-170, Asn-132, Ser-130, and Lys-234. Density corresponding to the conserved catalytic water molecule (wat1) adjacent to Glu-166 was also evident, allowing us to determine both its position and its relative orientation. Nuclear density from deuterium was clearly associated with the ε-nitrogens of Lys-73 and Lys-234, and three deuterium atoms are assigned to each in the model. The refined positions for these deuterium atoms are within acceptable hydrogen-bonding distances to their respective acceptor atoms in the active site (Fig. 3).

However, it was not possible to determine the exact position of the deuterons attached to the nitrogen atoms at 2.0 Å resolution, nor to establish unequivocally that these atoms were in the ammonium form (−ND3). As observed in our previous neutron structures of the Toho-1 β-lactamase (26, 27), the Lys-73 ammonium group in the BZB complex serves as a plag-
FIGURE 2. Protonation states of the active site residues and environment of BZB in the active site. In addition to BZB and selected protein residues, the catalytic water molecule (wat1) and a second bound water molecule (wat2) are shown. A and C, residues adjacent to the bound BZB (A) and Glu-166 (C) identified by χA-weighted 2Fo − Fc positive nuclear density maps colored in blue. The density for residues Lys-73, Glu-166, Asn-170, and the catalytic water molecule (wat1) has been omitted for clarity from panels A and B. Nuclear density corresponding to the BZB inhibitor (A) is contoured at 0.8σ (refined occupancy 48%). Nuclear density corresponding to the protonated Glu-166 (C), the catalytic water molecule (wat1), and the adjacent active site residues is contoured at positive 1.0σ (the density for the BZB molecule has been omitted for clarity). B and D, χA-weighted 2Fo − Fc positive electron density maps colored in blue are shown for residues adjacent to the bound BZB (B) and Glu-166 (D), respectively. Electron density corresponding to the BZB inhibitor (refined occupancy 67%) and adjacent sulfate ion (refined occupancy 33%) in B is contoured at positive 1.1σ. Electron density corresponding to Glu-166, the catalytic water molecule (wat1), and the adjacent active site residues is contoured at positive 1.1σ in D. The density for the BZB molecule has been omitted for clarity in D, E, the presence of a proton (Dε2) on Oε2 of Glu-166 identified by omit χA-weighted Fo − Fc positive nuclear density colored in green and contoured at 3.0σ; the final refined occupancy for this atom was 87%. Hydrogen-bonding interactions are shown with dashed lines.
sible hydrogen-bond donor to Ser-70, Asn-132, and Ser-130. The Lys-73 side chain assumes a conformation similar to conformation 2 of Lys-73 in the x-ray structure of the native Toho-1 enzyme (8) and is also the same as the conformations of Lys-73 in two x-ray structures of the CTX-M-9 β-lactamase (43, 44). The Glu-166 carboxyl serves as a hydrogen-bond donor to the catalytic water molecule, whereas Oγ of Ser-70 are acting as hydrogen-bond acceptors for water. Thus, the complete hydrogen-bonding pathway throughout the active site region can be deduced for a complex that mimics the acylation tetrahedral intermediate (Fig. 3).

The most striking result from this neutron crystallographic study was the strong positive nuclear density on O2 of Glu-166, which clearly indicates that Glu-166 is protonated (–COOD) in the BZB transition state analog complex. The refined occupancy for the deuterium atom attached to O2 of Glu-166 was 87%, whereas in the ligand-free protein, Glu-166 is deprotonated (–COO–). The C–O bond lengths in the high resolution x-ray structure of the BZB complex substantiate this finding. Bond lengths reflect protonation states because neutral carboxyls have unequal bond lengths of ~1.21 and 1.32 Å for the C=O and C–OH bonds, respectively. In contrast, negatively charged carboxyls have equal C–O bond lengths of ~1.26 Å.

Following refinement in phenix.refine, the x-ray structure was further refined using Shelx (45) with stereochemical restraints on all glutamate residues removed to optimize the carboxyl bond lengths according to electron density. The protonation states of the carboxyls were assigned by comparing the difference in C–O bond lengths with their associated estimated S.D. (46). Glutamates with differences in carboxyl bond lengths greater than three times estimated S.D. were assigned as protonated, and the others were assigned as deprotonated. In the case of Glu-166, the carboxyl bond lengths were refined to 1.21 and 1.28 Å (estimated S.D. 0.016 Å). These distances are consistent with the neutron structure and confirm that the Glu-166 is protonated on the oxygen closest to wat1.

DISCUSSION

Enzymes employ a wide variety of physical interactions to promote catalysis. In the case of β-lactamases and other serine hydrolases, general acid-base catalysis is a central feature. To achieve the required coordination of participating groups and stabilize charge, the catalytic residues are positioned to facilitate the seamless, rapid movement of protons and hydrogen bonds during catalysis. Understanding these networks is the key to understanding catalysis, but few experimental methods can directly reveal their structure. Neutron crystallography enables the locations of hydrogen atoms to be established consistently and reliably in macromolecules (29) and thus is an essential tool for revealing the organization of catalytic centers. In the present work, we have for the first time used neutron crystallography to study a fully deuterated protein-ligand complex. By deuterating the ligand, we greatly enhanced the scattering signals arising from it. This advantage both allowed us to see strong nuclear density from the ligand at partial occupancy and allowed us to connect the observed changes in the proton network of the protein to ligand binding.

The detailed picture of the hydrogen-bonding network that emerged from the x-ray and neutron structures allows us to suggest viable pathways of proton transfer throughout the active site region. The protonated Glu-166 side chain and the hydrogen-bonding network in which it participates with the inhibitor bound are consistent with this residue acting as the catalytic base in the acylation reaction mechanism where, upon binding to the BZB, the proton has been shuttled from...
Ser-70 through the catalytic water to Glu-166. Our results are consistent with earlier atomic resolution x-ray studies (10, 13, 15, 19, 21, 26, 27, 47).

Although our results are most consistent with Glu-166 serving as the general base in the acylation reaction, we cannot rule out the alternative mechanism in which Lys-73 acts as the general base. As general caveats, we note that the enzyme-inhibitor complex is not the true intermediate, and that low energy states observed in a crystal structure may not be mechanistically relevant. It is important in this respect that our neutron structure of the ligand-free protein, in which Glu-166 is deprotonated, was determined under the same conditions as the present structure, in which Glu-166 is protonated. This difference is consistent with a role for Glu-166 as the active-site general base but might alternatively be explained by a difference in pKa between ligand-free and inhibitor-bound enzymes.

The acylation reaction is completed by collapse of the tetrahedral intermediate to the acyl–enzyme adduct (Fig. 1, 2–3). It has been suggested that a second proton shuttle pathway, from Lys-73 to Ser-130 (13, 14, 24, 48–50), exists to facilitate this step through protonation of the departing β-lactam nitrogen. The present neutron structure, in which BZB mimics the acylation tetrahedral intermediate, fully supports the proposed proton shuttle pathway. In particular, the proposed pathway is in place as a network of hydrogen bonds from Lys-73 to Ser-130 to O1 of BZB, which is presumed to correspond to the β-lactam nitrogen (Fig. 3).

This crystallographic study of a perdeuterated enzyme–inhibitor complex thus fills in considerable detail on the structure of the proton network in the active site of a class A β-lactamase during the acylation reaction. In particular, the structures have revealed a change in the protonation state of Glu-166 upon binding of an acylation transition state analog and the presence of a hydrogen-bond network potentially capable of facilitating breakdown of the tetrahedral intermediate. Although deuteration of proteins and ligands can be technically challenging, this work demonstrates that there is potentially considerable value in pursuing it.

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