Biochemical Characterization of IMP-30, a Metallo-β-lactamase with Enhanced Activity toward Ceftazidime

Kevin M. Pegg, a Eleanor M. Liu, b Alecander E. LaCuran, b and Peter Oelschlaeger b

Supplemental Material

Department of Biological Sciences, College of Science, California State Polytechnic University, Pomona, California, USA a and Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, California, USA b

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Corresponding author:

Peter Oelschlaeger

Mailing address: 309 East Second St., Pomona, California 91766

Telephone: (909) 469-8232

Fax: (909) 469-5600

Email: poelschlaeger@westernu.edu
COMPUTATIONAL METHODS

β-Lactamase Preparation. The coordinates of IMP-1 (chain A) in PDB entry 1DD6 (1) were used and prepared for molecular dynamics (MD) simulations as follows. The mercaptocarboxylate inhibitor was removed and hydrogen atoms were added with the program protonate in the AMBER 10 software package (2). The zinc ions were represented using the cationic dummy atom approach (3, 4) using updated parameters and libraries available at http://mayoresearch.mayo.edu/mayo/research/camdl/zinc_protein.cfm. Consistent with this approach, the zinc-ligating residues His116, His118, His196, His263, and Cys221 according to the standard numbering scheme (5) were treated as histidinates or cysteinate. One residue in the second coordination sphere, Asp236, could be considered a proton acceptor of His118 and was treated as protonated. The central atoms of the zinc cationic dummy atom molecules were placed at the coordinates of the zinc ions in the PDB file, while the coordinates of the dummy atoms were calculated between the zinc ions and their ligands at a distance of 0.9 Å from the central atoms. IMP-30 was prepared by introducing the E59K mutation with the “mutate” tool in Swiss-PDB Viewer 4.0.1 (http://spdbv.vital-it.ch/) and selecting the most energetically favorable rotamer of K59. The library and force field parameter files for deprotonated ceftazidime were created using the program antechamber of AMBER 10 by applying the AM1-BCC atomic charge method (6) and General Amber Force Field (GAFF) (7). To mimic the polarizing effect of a zinc ion on the β-lactam carbonyl, the partial charge of the carbonyl oxygen (O8, refer to Fig. 1A in main text for atom labeling) was set to -1.0 e and a balancing positive charge was assigned to the carbonyl carbon (C8). The coordinates of ceftazidime were taken from the docked Michaelis complexes displayed in Figs. 1B and 1C in the main text. The tleap program of AMBER 10 was used to neutralize the IMP-30 system with Cl- counterions, solvate both complexes with a truncated octahedron of TIP3P water molecules (8) 10 Å around the protein, and create topology and coordinate files using the amino94 libraries and the GAFF and ff99SB force fields (9).

MD Simulations. Energy minimizations and MD simulations were carried out using sander of AMBER 10. First, the systems were minimized by 500 cycles of steepest descent followed by 500 cycles of conjugate gradient while constraining the zinc ions and ceftazidime (500 kcal mol⁻¹ Å⁻²), followed by heating to 300 K at constant volume with the positions of the aforementioned molecules constrained with 100 kcal mol⁻¹ Å⁻². Then, the systems were gradually equilibrated at 300 K and constant pressure with decreasing constraints on the zinc ions and ceftazidime (100, 50, 25, 10, and finally 0 kcal mol⁻¹ Å⁻²) for 5 ps each. Finally, the systems were submitted to 2 ns of unconstrained MD simulation at 300 K. The time step for all MD simulations was 1 fs and the SHAKE algorithm (10) was applied to all hydrogen bonds. Coordinates were saved every 1 ps. This protocol was carried out three times for each complex with different initial velocities at the beginning of the heating portion.
**MD Trajectory Analysis.** *Ptraj* was used to analyze root mean square deviation (RMSD) of the backbone heavy atoms, atom distances, angles, water occupancy in the first and second coordination sphere of the β-lactam C₈, and the radial distribution function of water molecules (center of mass) relative to C₈. Visualization of the MD simulations was performed using VMD software (11). Values reported are averages ± standard deviations of the three average values obtained from the three independent trajectories. Where values are reported as significantly different between the two complexes, *p* was smaller than 0.05 in a Student’s T test.

**RESULTS AND DISCUSSION**

**Overall Geometry of Michaelis Complexes.** Judged by the backbone RMSD values, both complexes were equilibrated after 150 to 850 ps of unconstrained MD simulation at 300 K, depending on the initial velocities. This allowed for the analysis of about 4 ns of unconstrained, equilibrated MD simulation for each complex. All zinc ligands maintained coordination to their respective zinc ion, indicating that the cationic dummy atom approach is a good model to maintain the active site geometry of MBL-substrate Michaelis complexes. To our knowledge, this is the first time that this approach has been used successfully for MBL Michaelis complexes as opposed to MBL-substrate intermediate complexes (12-14). While in both complexes O₈ remained coordinated to Zn1 at 1.9 ± 0.1 Å throughout the simulations (2.0 Å in the docked complexes), the distance between Zn2 and N₅ increased from 3.0-3.1 Å in the docked structures to 4.1-4.2 Å in both complexes. This increase in distance is consistent with the partial charge of -0.3623 assigned to N₅ by the AM1-BCC atomic charge method, which is not negative enough to keep it closer to the zinc ions. The Zn1-Zn2 distance had increased to 4.8-4.9 Å relative to the 3.6 Å in the crystal structure, consistent with previous computational observations with substrate intermediates (12) and spectroscopic studies of the MBL catalytic mechanism (15) that implied a movement of the zinc ions during the catalytic cycle. The cephem carboxylate maintained a stable salt bridge with Lys244 (defined as (O₁A---Lys244 Nζ + O₂B---Lys244)/2 < 4.5 Å) in 100% of the frames of the IMP-1 complex and in 93% of the frames of the IMP-30 complex, indicating productive Michaelis complexes over almost the entire simulation time.

**Electrostatic Interactions between Ceftazidime R₁ and Active Site Residues.** The docking calculations indicated that the R₁ carboxylate of ceftazidime interacted with Lys150a in IMP-1 and Lys59 in IMP-30 (Fig. 1B and 1C in the main text). The MD trajectories were analyzed to assess the stability and significance of these interactions. The average distance between Lys150a Nζ and the two oxygens of the R₁ carboxylate (O₂A and O₂B) was 5.2 ± 0.6 Å in IMP-1 and 6 ± 3 Å in IMP-30, and a stable salt bridge was observed in 30% of the frames of the IMP-1 simulations versus 24% of the IMP-30 simulations (Table S1). Interestingly, the salt bridge broke and formed again several times during the simulations of the IMP-1 complex (Fig. S1A).
and in the case of IMP-30 formed in one trajectory even though the R₁ carboxylate was initially oriented toward Lys59 (Fig. S1B).

The average distance between the ceftazidime R₁ carboxylate oxygens and Lys59 Nζ in IMP-30 was 7 ± 1 Å, in contrast to 3.5 Å in the docked structure (Fig. 1C in the main text) and a stable salt bridge was observed in only 13% of the MD simulation frames (Fig. S2A). This observation suggests that this salt bridge has little significance and is eventually replaced by the salt bridge between the ceftazidime R₁ carboxylate and Lys150a, just like in IMP-1. However, strong electrostatic interactions were also observed between the R₁ carbonyl oxygen (O₁₀) and Lys59 (Figure S2B) as well as the R₁ oxyimino oxygen (O₁₃) and Lys59 (Figure S2C), with both distances at 5 ± 2 Å on average and 43% and 24%, respectively, below 3.5 Å distance. So overall, R₁ tends to be oriented toward Lys59 in IMP-30 in a relatively high percentage of the MD simulation frames, whereas this group cannot undergo these interactions in IMP-1, where residue 59 is a glutamate. In cases where neither specific interactions with Lys150a or Lys59 are observed, such as the latter part of trajectory 2 of the IMP-30/ceftazidime complex (Figures S1B and S2A), the R₁ carboxylate interacts with bulk water molecules, as it reaches out all the way to the protein surface.

**Substrate Orientation and Water Accessibility.** The different orientation of the ceftazidime R₁ group could have an effect on the internal conformation of the substrate as well as its orientation within the active site. As indicators of these properties, we measured the angles O₈-C₇-C₁₁ (internal conformation) and Zn₁-C₇-C₁₁ (orientation). Both angles showed significant differences between IMP-1 and IMP-30 (angle O₈-C₇-C₁₁ = 108 ± 2° versus 116 ± 3°; angle Zn₁-C₇-C₁₁ = 101 ± 2° versus 110 ± 3°; Table S1). Essentially, the angle around C₇ opens up, which could pose a bigger strain on the β-lactam ring, thus facilitating hydrolysis, or allow for better water access to the amide bond. With the classical force field used here, we could not assess whether there is higher internal strain on the β-lactam ring, but we could assess the extent to which water can access the active site. Water is essential for nucleophilic attack on C₈ as well protonation of N₅. We did not explicitly model the hydroxide/water molecule bridging the two zinc ions in the free enzyme (1) in our simulations, but if this were the nucleophile, then water access would still be essential for replenishing the nucleophile after hydrolysis.

We analyzed the number of water molecules within certain radii around C₈ and found that they were slightly different in IMP-1 versus IMP-30: 0.4 ± 0.2 versus 0.6 ± 0.2 within a 3.4 Å radius and 2.5 ± 0.3 versus 3.0 ± 0.6 within a 5.0 Å radius (Table S1). We also calculated the radial distribution function of water molecules around C₈ (Fig. S3). Clearly, in IMP-30 the function has higher values at lower radii than in IMP-1. These data suggest that the β-lactam amide bond is more readily accessible to water in the IMP-30/ceftazidime complex than in the IMP-1/ceftazidime complex.

**Interpretation of Data.** In a high percentage of conformations, R₁ of ceftazidime specifically interacts with Lys59 in IMP-30, either via the R₁ carboxylate, the R₁ carbonyl oxygen, and/or the
R₁ oxyimino oxygen. This moves R₁ on average into a different orientation, which translates into a bigger angle around C₇ in IMP-30 relative to IMP-1 and better access of water molecules to the β-lactam amide bond, where hydrolysis takes place. This scenario is supported by the experimental steady-state kinetics, which reveals that the rate constant, $k_{\text{cat}}$, is increased six-fold in IMP-30 relative to IMP-1, whereas $K_m$ is nearly unchanged.
REFERENCES


**SUPPLEMENTAL TABLE**

**TABLE S1.** Measurements obtained from MD simulations distinguishing the IMP-30/ceftazidime complex from the IMP-1/ceftazidime complex.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>IMP-1/ceftazidime</th>
<th>IMP-30/ceftazidime</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_1$ carboxylate---Lys150a distance (Å)</td>
<td>5.2 ± 0.4</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>$%&lt;4.5$ Å</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>$R_1$ carboxylate---Lys59 distance (Å)</td>
<td>-</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>$%&lt;4.5$ Å</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>$R_1$ carbonyl---Lys59 distance (Å)</td>
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<td>5 ± 2</td>
</tr>
<tr>
<td>$%&lt;3.5$ Å</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>$R_1$ oxyimino---Lys59 distance (Å)</td>
<td>-</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>$%&lt;3.5$ Å</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>$O_8$-C7-C11 angle (°)</td>
<td>108 ± 2</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>$Zn_1$-C7-C11 angle (°)</td>
<td>101 ± 2</td>
<td>110 ± 3</td>
</tr>
<tr>
<td>Water molecules within 3.4 Å radius</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Water molecules within 5.0 Å radius</td>
<td>2.5 ± 0.3</td>
<td>3.0 ± 0.6</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL FIGURES

FIG S1. The average distance between Lys150 $\text{N}_\zeta$ and the two ceftazidime $R_1$ carboxylate oxygens is plotted over the course of the multiple MD trajectories of the IMP-1/ceftazidime complex (A) and the IMP-30/ceftazidime complex (B).
FIG S2. Distances between Lys59 Nζ and different oxygen atoms in the ceftazidime R₁ group are shown over the course of the MD trajectories of the IMP-30/ceftazidime complex. (A) Lys59 Nζ---R₁ carboxylate (O₂A and O₂B), (B) Lys59 Nζ---R₁ carbonyl oxygen (O₁₀), (C) Lys59 Nζ---R₁ oxyimino oxygen (O₁₃).
FIG S3. Radial distribution functions $g(r)$ of water molecules (center of mass) around $C_8$ in the IMP-1/ceftazidime complex (black line) and IMP-30/ceftazidime complex (red line).