Catalytic Mechanism of Class B2 Metallo-β-lactamase*

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The initial nucleophilic substitution step of biapenem hydrolysis catalyzed by a subclass B2 metallo-β-lactamase (CphA from Aeromonas hydrophila) is investigated using hybrid quantum mechanical/molecular mechanical methods and density functional theory. We focused on a recently proposed catalytic mechanism that involves a non-metal-binding water nucleophile in the active site of the monozinc CphA. Both theoretical models identified a single transition state featuring nearly concomitant nucleophilic addition and elimination steps, and the activation free energy from the potential of mean force calculations was estimated to be ~14 kcal/mol. The theoretical results also identified the general base for activating the water nucleophile to be the metal-binding Asp-120 rather than His-118, as suggested earlier. The protonation of Asp-120 leads to cleavage of the OAsp-Zn coordination bond, whereas the negatively charged nitrogen leaving group resulting from the ring opening replaces Asp-120 as the fourth ligand of the sole zinc ion. The electrophilic catalysis by the metal ion provides sufficient stabilization for the leaving group to avoid a tetrahedral intermediate. The theoretical studies provided detailed insights into the catalytic strategy of this unique metallo-β-lactamase.

The excessive use and abuse of β-lactam antibiotics have accelerated the spread of drug-resistant bacterial strains. The unprecedented level of antibiotic resistance threatens to destroy their efficacy in treating infectious diseases, posing a grand challenge to public health (1). The primary defense strategy adopted by bacteria is to deactivate the antibiotics by hydrolytic cleavage of the β-lactam ring, catalyzed by β-lactamases (2). These enzymes can be divided into four classes (3). Enzymes in classes A, C, and D utilize an active site serine in the covalent catalysis of the β-lactam hydrolysis, whereas class B consists of metalloenzymes with one or two zinc cofactors. Although the catalytic mechanism of the serine-based enzymes is relatively well established (2), our understanding of class B β-lactamases is less developed (4).

Metallo-β-lactamases often have very broad substrate spectra (5) stemming apparently from the metal-dependent catalytic mechanism. Despite much effort, no clinically effective inhibitor has been found. On the other hand, increasing evidence in recent years has pointed to rapid proliferation of these metalloenzymes in pathogenic microorganisms (6). Hence, these enzymes represent a potentially more potent threat to the existing arsenal of β-lactam antibiotics than other classes of β-lactamases (5, 6).

Class B β-lactamases can be further separated into three subclasses. Despite considerable sequence diversity (4), their catalytic scaffolds are relatively conserved. All class B β-lactamases have two potential metal binding sites (7–11). Protein ligands in the so-called Zn1 site include three His residues in the B1 and B3 subclasses, but a His residue is replaced by Asn in B2 subclass β-lactamases. The Zn2 site has an Asp-Cys-His triad in the B1 and B2 subclasses, and the Cys residue is substituted by His in the B3 subclass. It is well established that B1 and B3 subclass β-lactamases are catalytically active with one zinc cofactor, typically in the Zn1 site (7), and the second zinc ion typically enhances the catalytic activity (11–14). However, the second zinc ion inhibits, rather than enhances, the catalytic activity of subclass B2 enzymes (15), which are only found in Aeromonas and have a strong preference toward carbapenems (6). Interestingly, the catalytic zinc cofactor in B2 β-lactamases occupies the Zn2 site, as demonstrated by recent experiments (15–17).

Until recently, it was generally believed that the nucleophile in the hydrolysis reaction catalyzed by class B β-lactamases is a metal-bound hydroxide (4). This hypothesis is supported by several x-ray structures of B1 and B3 β-lactamases, in which an oxygen moiety was observed to coordinate with one or both zinc ions (7–10). However, a recent atomic resolution structure of a subclass B2 β-lactamase (CphA from Aeromonas hydrophila (15)) implicated a non-metal-binding active site water (17). Based on the structures of the enzyme in its native form and complexed with a hydrolysis intermediate, a mechanism advocating the nucleophilic role of this active site water was advanced by Garau et al. (17). This mechanism is very different from the conventional model based on a zinc-bound OH−. Given the unique sequence, substrate profile, and binding pattern of the sole zinc cofactor of subclass B2 β-lactamases, however, this alternative mechanism is not unreasonable but certainly requires more detailed investigations in both theoretical and experimental fronts.

We report here a detailed theoretical investigation on the catalytic mechanism of the CphA enzyme in hydrolyzing a carbapenem antibiotic (biapenem). It dovetails our recent work on the Michaelis complex of the same system using a quantum mechanical/molecular mechanical (QM/MM)2 method and density functional theory (DFT) (18). We focus on the initial nucleophilic substitution step of the catalytic hydrolysis reaction and examine the putative nucleophilic role of the non-metal-binding water in the proposed mechanism using the same theoretical methods.

MATERIALS AND METHODS

DFT Model—The truncated active site includes the zinc ion and its three protein ligands His-263, Cys-221, and Asp-120, approximated respectively by an imidazole, a methyl thiolate, and an acetate. The substrate was approximated by a biapenem analog in which the bicycletriazoliumthio group was replaced by −CH3. In addition, the model also

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2 The abbreviations used are: QM/MM, quantum mechanical/molecular mechanical; DFT, density functional theory; SSC-DFTB, self-consistent charge density functional tight binding; B3LYP, Becke-3-Lee-Yang-Parr; PMF, potential of mean force; MD, molecular dynamics; TS, transition state; EI, enzyme-intermediate; ES, enzyme substrate.
includes an H2O and a methyl imidazole representing His-118. The Becke-3-Lee-Yang-Parr (B3LYP) exchange correlation functional and a standard basis set (6–31G**) were used. The geometries of stationary points were fully optimized with no geometric constraints, and the default convergence criterion was used. This is followed by the calculations of their harmonic vibrational frequencies and electrostatic potential charges. Finally, solvent effects were approximately taken into account with the polarized continuum model. Model studies of the hydrolysis of a biapenem analog catalyzed by two general bases, namely acetate and imidazole, were also performed at the B3LYP/6–31+ +G** level of theory. All DFT calculations reported in this work were performed using Gaussian 03.3

**QM/MM Models**—A major disadvantage of the truncated active site model is its omission of the electrostatic and van der Waals microenvironment supplied by its surrounding protein residues and solvent. An approximate solution is to include the entire solvated protein but to partition the enzyme into a quantum mechanical region surrounded by a classical molecular mechanical region. This QM/MM approach (20) has been quite successful in studying enzymatic reactions (21, 22). The advantages are particularly conspicuous for metalloenzymes, because the metal-ligand bonds are notoriously difficult to model with force fields. Following our earlier work (18), a QM/MM method is used to study the catalytic mechanism of the CphA enzyme.

The model construction has been discussed in detail in our recent publication on the binding dynamics of CphA complexed with biapenem (18). Here, only a short description is given. Starting from the recent x-ray structure of the enzyme-intermediate complex (PDB code 1X8I) (17), the hydrolysis intermediate was removed, and hydrogens were added. The biapenem molecule was then manually docked at the enzyme active site in a manner consistent with the x-ray structures. After solvated in a pre-equilibrated sphere of TIP3P waters with a 25 Å radius, the enzyme-substrate complex was subjected to stochastic motions in the buffer zone supplied by its surrounding protein residues and solvent. An approximate solution is to include the entire solvated protein but to partition the enzyme into a quantum mechanical region surrounded by a classical molecular mechanical region. This QM/MM approach (20) has been quite successful in studying enzymatic reactions (21, 22). The advantages are particularly conspicuous for metalloenzymes, because the metal-ligand bonds are notoriously difficult to model with force fields. Following our earlier work (18), a QM/MM method is used to study the catalytic mechanism of the CphA enzyme.

The MM region in our simulations was characterized by the CHARMM all atom force field (24). The QM region contains Zn²⁺, its protein ligands Asp-120, Cys-221, and His-263, one crystal water (W11), and a potential general base His-118, as well as the biapenem substrate. The CHARMM van der Waals parameters were used for the protein ligands Asp-120, Cys-221, and His-263, one crystal water, and a potential general base His-118, as well as the biapenem substrate.

Metallo-β-lactamases, Enzyme Catalysis, QM/MM, DFT

**DFT Model**—As shown in our earlier work (18), the zinc binding site can be accurately described by a truncated active site model using DFT. Here, we have used a similar model to describe the nucleophilic substitution step of the hydrolysis reaction, with a larger basis set. The optimized ES complex in Fig. 1 was found to have a very similar geometry to that reported in Ref. 18. Some key geometric parameters are summarized in Table 1 along with average distances and angles obtained from our previous MD simulation of the enzyme-substrate complex. In particular, the zinc ion is tetracoordinated by four ligands, namely His-263, Cys-221, Asp-120, and the substrate carboxylate. A water molecule is located in a pocket formed by His-118, Asp-120, and the β-lactam ring, well positioned for the nucleophilic attack at the substrate carbonyl carbon (C₁). The O₁-water-C₁ distance is 3.38 Å, close to that observed in our

**RESULTS**

In the potential of mean force (PMF) calculations, umbrella sampling (32) with harmonic force constants in the 100–180 kcal/mol⋅Å² range was employed in twelve windows. The configuration space was sampled by classical molecular dynamics (MD). In each window, the minimal energy configuration was heated to 300 K in 30 ps followed by 30 ps of equilibration at the same temperature. The data were collected in the subsequent 40 ps. Finally, the PMF was obtained using the weighted histogram analysis method (WHAM) (33). During the MD calculations, the SHAKE algorithm (34) was applied to maintain all covalent bonds involving hydrogen atoms, except for those in the catalytic water. The time step was 1 fs.
general base, and its optimized bond lengths and angles are also listed in Table 1. This first-order saddle point, as evidenced by a single imaginary frequency, features nearly concerted nucleophilic addition and elimination steps, as evidenced by a shortened O$_{w}$-C$_{7}$ bond (1.68 \textit{versus} 3.38 Å in the ES complex) and an elongated C$_{7}$-N$_{4}$ bond (1.86 \textit{versus} 1.41 Å in the ES complex). The partial cleavage of the amide bond creates an anionic N$_{4}$, evidenced by its negative charge (−0.45) at the transition state, in sharp contrast with its charge (0.06) in the reactant. As the water nucleophile approaches C$_{7}$, one of its hydrogen atoms (H$_{1}$) starts to move toward O$_{w}$ of Asp-120. The O$_{w}$-H$_{1}$ and H$_{1}$-O$_{w}$ distances at the transition state are 1.05 and 1.50 Å, respectively. As a result, the metal-ligand bond between the Asp-120 carboxylate group and zinc ion becomes slightly impaired as the distance O$_{w2}$-Zn increases from 1.97 to 2.08 Å. In the meantime, the negative charge buildup at N$_{4}$ due to the C-N bond cleavage provides incentives for it to move toward the zinc ion. The corresponding N$_{4}$-Zn distance at the transition state becomes 2.21 from 3.44 Å in the ES complex. The zinc ion is pentacoordinated in the transition state.

No tetrahedral intermediate was found in this reaction pathway, and the intrinsic reaction coordinate leads directly to the ES complex in one direction and the EI complex in the other. The EI complex is displayed in Fig. 1, and selected bond lengths and angles are listed in Table 1 along with those for other stationary points. The procession from the transition state to the EI complex results in the complete cleavage of the lactam amide bond, accompanied by a concomitant proton transfer to the metal-binding Asp-120. The neutralization of the Asp-120 side chain renders it an ineffective ligand to the zinc ion, leading to a final O$_{w2}$-Zn distance of 4.10 Å, signifying the breaking of the bond. In the meantime, the negatively charged N$_{4}$ (q = −0.70) replaces Asp-120 as the fourth ligand to the zinc ion, as evidenced by the short (1.95 Å) N$_{4}$-Zn distance. The resulting EI complex can be converted to the enzyme product complex by proton transfer and possibly intramolecular rearrangements, as suggested by the recent x-ray structure (17). However, the latter processes were not studied here.

The energies, zero-point corrected energies, and free energies of all of the stationary points are summarized in Table 2. The reaction barrier
and exothermicity are 31.7 and 1.1 kcal/mol, respectively, at the B3LYP/6–31G** level. The basis set effect seems to be small as the corresponding values at the B3LYP/6–31G* are 32.0 and 1.2 kcal/mol, respectively. The activation free energy is estimated to be 35.0 kcal/mol. The relatively large reaction barrier is likely because of the lack of the protein/solvent environment in the truncated model. After the solvent effects are included using the polarized continuum model, for example, the barrier is reduced by ~6 kcal/mol.

However, repeated efforts to locate the transition state with His-118 as the general base failed. To understand the capacity of the His or Asp residue as a general base in catalyzing the hydrolysis of β-lactams, we examined simple model systems without the metal ion and its protein ligands in which the two amino acid side chains are approximated by an imidazole ring and acetate, respectively. Five stationary states were located for the acetate-catalyzed reaction, including the reactant complex, transition state for nucleophilic addition (TS1), tetrahedral intermediate, transition state for elimination (TS2), and product complex. They are displayed in Fig. 2 with key bond lengths indicated in the figure. The rate-limiting step is the formation of the tetrahedral intermediate, whereas the barrier for elimination is very small. These results are consistent with previous theoretical studies of β-lactam hydrolysis (35, 36).

When the imidazole group was used as the general base, on the other hand, no reactive transition state was located after an exhaustive search. Reaction path calculations showed a monotonically increased energy profile in the nucleophilic addition coordinate. These results indicate that Asp is a better catalyst than His in the gas phase. This issue will be further addressed below with a QM/MM model in which the protein/solvent environment is included in the simulations.

**QM/MM Models**—Although the truncated active site model provides insightful information about the catalytic reaction, it includes neither the protein/solvent environment nor the corresponding dynamics. To bridge the gap, we investigated the same nucleophilic substitution step of the β-lactam hydrolysis reaction employing two QM/MM models, namely the SCC-DFTB/CHARMM and B3LYP/CHARMM models.

The minimal energy profile along the reaction coordinate was first determined by adiabatic mapping. The geometric parameters of the transition state, which was obtained by conjugate peak refinement (37), are listed in Table 1, and their agreement with the DFT transition state is quite reasonable, although imperfect. As in the DFT model, the SCC-DFTB/MM results suggest a concerted SN2-type transition state featuring the nearly simultaneous addition of the water nucleophile and elimination of the nitrogen leaving group, although the QM/MM model has more advanced bond cleavage to the leaving group. As a result, the charge of the nitrogen leaving group (N) becomes increasingly more negative as the reaction proceeds (q = ~0.13, ~0.41, and ~0.51 for the ES, TS, and EI complexes, respectively). The SCC-DFTB/MM model also indicated that Asp-120 is the general base, which uses its metal-binding oxygen (O2) as the proton acceptor. This point will be further examined below. The proton transfer in the SCC-DFTB/MM model seems to be less advanced at the transition state than its DFT counterpart, as evidenced by a longer (1.77 Å) H1–O2 distance. The procession in the reaction coordinate did not lead a tetrahedral intermediate, further confirming the concerted nature of the reaction. The final EI com-

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**TABLE 2**

Energetics (kcal/mol) for the nucleophilic substitution step of the hydrolysis reaction

The truncated active site model at the B3LYP/6–31G** level of theory was used.

| Energetics | TS   | EI   |
|-----------|------|------|
| Energy    | 31.7 | 1.1  |
| Energy with ZPE correction | 31.2 | 2.2  |
| Free energy | 35.0 | 2.4  |
| Free energy (PCM) | 29.1 | 0.6  |

*a* All energies are given relative to ES.

*b* Aqueous solution (a = 80).

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**FIGURE 2.** Energy profile for acetate-catalyzed hydrolysis of a β-lactam analog and geometries of stationary points, namely the reactant complex (RC), first transition state (TS1), tetrahedral intermediate (TI), second transition state (TS2), and the product complex (PC). The energies are given in kcal/mol and distances in Å.
plex is characterized by a cleaved $C_7-N_4$ bond, with nitrogen ($N_4$) coordination to the zinc ion and protonated Asp-120 side chain, in good overall agreement with the DFT model.

The calculated PMF is shown in Fig. 3 as a function of the reaction coordinate. The reaction barrier height and exothermicity are 14.1 and 1.2 kcal/mol, respectively. The calculated activation free energy is consistent with experimentally observed rate constants ($300 \text{s}^{-1}$) (17), which gives a barrier height of 14.1 kcal/mol. The coincidence is likely to be superficial, however.

Snapshots of the active site in the ES, TS, and EI complexes are shown in Fig. 4. Several interesting observations of the TS complex are immediately in order. The zinc ion is pentacoordinated with His-263, Cys-221, Asp-120, as well as $N_4$ and $O_{13}$ of the substrate. The $O_{w-C_7}$ bond is partially forming, whereas the $C_7-N_4$ bond is partially breaking. A water proton is being transferred to the metal-binding oxygen ($O_{(H_2O)}$) of Asp-120. These observations are consistent with those made in the truncated active site model. In addition, a hydrogen bond network is clearly seen between the substrate and enzyme active site involving the Asn-233, Lys-224, and His-118 residues.

The EI complex, also displayed in Fig. 4, features an opened lactam ring with the nitrogen forming a coordination bond with the zinc ion. Asp-120 is released by the metal ion as a result of its protonation, so that the tetracoordination of the metal ion can be maintained. The newly formed carboxylate at $C_7$ is hydrogen-bonded with the imidazole ring of His-118. The overall configuration is also in keeping with the DFT model.

To ascertain the validity of the reaction mechanism, we have carried out single point B3LYP/MM calculations along the SCC-DFTB/MM reaction path. The results are also displayed in Fig. 3, showing reasonably good agreement with the PMF. Although the similarities between the two energy profiles provide strong supporting evidence for their validity, the B3LYP/MM energy profile should not be considered to be quantitatively accurate, because the basis set used in this calculation is small (6–31G) and because no sampling of the protein environment was carried out.

Let us now turn back to the identity of the general base. To confirm that His-118 is not a good acceptor of the proton from the nucleophile, two-dimensional minimal energy potentials in the nucleophilic addition and proton transfer coordinates were calculated using the SCC-DFTB/MM approach. The coordinate for nucleophilic addition is defined previously as the distance of $O_{w}$ and $C_7$ atoms ($R_{a1} = d_{Ow-C7}$), whereas that for proton transfer is represented by the asymmetric stretching coordinate of the transferring proton between the donor and acceptor. In particular, $R_{a2} = d_{H2O-Ow} - d_{H2-OH}$ for His-118 or $R_{a2} = d_{H1-Ow} - d_{H1-OH}$ for Asp-120. The latter coordinate forces the proton transfer in the desired direction. Two resulting potential energy surfaces are plotted in Fig. 5.
In both the upper and lower panels of Fig. 5, there is a potential minimum in the lower right corner, corresponding to the ES complex. In Fig. 5A, where Asp-120 is assumed to be the general base, the other potential minimum in the upper left corner corresponds to the EI complex. The minimal energy path connecting the two potential wells follows approximately a diagonal line, indicating concerted nucleophilic addition and proton transfer. This is consistent with the PMF discussed above. On the other hand, a different picture emerges in Fig. 5B, in which His-118 is considered as the proton acceptor. The minimal energy path from the ES complex at the lower right corner is primarily along the nucleophilic addition coordinate \( R_{62} \) leading to a local minimum in the lower left corner without too much change in the proton transfer coordinate \( R_{62} \). As expected, this potential minimum corresponds to the EI complex in which the C7–N4 bond is cleaved by the water. However, the water proton is transferred to the O1 of Asp-120 rather than His-118. This proton transfer accompanying the nucleophilic addition is apparently spontaneous with no barrier and not seen in \( R_{62} \). Interestingly, the system reaches another minimum from the EI complex.
There is ample experimental evidence for a metal-bound intermediate in metallo-β-lactamase-catalyzed hydrolysis (4). Benkovic and coworkers (47) have, for example, observed a strong transient absorption peak at 665 nm in the CcrA-catalyzed hydrolysis of nitrocefin, which was assigned to an anionic intermediate presumably stabilized by a metal ion. Similar observations have been made for model catalysts that mimic metallo-β-lactams (48). Evidence for changes of metal coordination during turnover was reported by Bicknell et al. (49) for the Bcll enzyme. More recently, Garrity et al. (19) presented direct spectroscopic evidence that the reaction intermediate of the L1 metallo-β-lactamase is metal-bound. Theoretically, the existence of an enzyme-intermediate complex featuring an N–Zn bond has been identified by the DFT study of Park et al. (46). The current theoretical results provide further evidence for the existence of such an intermediate.

The theoretical results presented in this work suggest several mutation experiments for the CphA enzyme. As indicated by the interaction pattern in Scheme 1, Asn-233 plays an important role in substrate binding, as noted previously (17, 18). The highly conserved Lys-224 is also seen in our simulations to provide hydrogen bond interactions to the substrate. Our results strongly imply that the mutation of Asp-120 would severely impair the catalytic activity of the enzyme. On the other hand, mutation of His-118 is predicted to have less impact on the catalysis. The influence on the catalysis by these mutations will provide necessary check for the proposed mechanism.

CONCLUSIONS

We have, in this work, investigated the catalytic mechanism of a subclass B2 metallo-β-lactamase (CphA) from A. hydrophila in hydrolyzing biapenem. Starting from the Michaelis complex derived from the x-ray structures, we studied the nucleophilic substitution step of the hydrolysis reaction using a truncated active site model and a full protein model. The results from both models indicate that the nucleophilic addition is in concert with the elimination step, characterized by a single S_n2-like transition state and no tetrahedral intermediate. We also identified the metal-binding Asp-120 as the general base that activates the water nucleophile. The protonation of Asp-120 results in the cleavage of its bond to the metal ion, which instead receives its fourth ligand from the negatively charged nitrogen leaving group. In addition to the metal
binding through its carboxylate at the C3 position, the substrate and the transition state is further stabilized by hydrogen bonding with Lys-224 and Asn-233. The consistency of the two complementary theoretical treatments is very encouraging.

The theoretical results provide strong support to the mechanism proposed by Garau et al. (17) but with a significant difference with regard to the identity of the general base. In particular, our model indicates that the metal-binding Asp-120, rather than His-118 as suggested earlier (17), is responsible for the activation of the water nucleophile via proton transfer. The proposed mechanism is very different from the commonly posited by Garau (17) but with a significant difference with regard to the identity of the general base. In particular, our model indicates that the metal-binding Asp-120, rather than His-118 as suggested earlier (17), is responsible for the activation of the water nucleophile via proton transfer. The proposed mechanism is very different from the commonly posited by Garau (17) but with a significant difference with regard to the identity of the general base. In particular, our model indicates that the metal-binding Asp-120, rather than His-118 as suggested earlier (17), is responsible for the activation of the water nucleophile via proton transfer. The proposed mechanism is very different from the commonly posited by Garau (17) but with a significant difference with regard to the identity of the general base. In particular, our model indicates that the metal-binding Asp-120, rather than His-118 as suggested earlier (17), is responsible for the activation of the water nucleophile via proton transfer. The proposed mechanism is very different from the commonly posited by Garau (17) but with a significant difference with regard to the identity of the general base. In particular, our model indicates that the metal-binding Asp-120, rather than His-118 as suggested earlier (17), is responsible for the activation of the water nucleophile via proton transfer. The proposed mechanism is very different from the commonly