Lactam Hydrolysis Catalyzed by Mononuclear Metallo-β-lactamases: A Density Functional Study

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Two central steps in the hydrolysis of lactam antibiotics catalyzed by mononuclear metallo-β-lactamases, formation of the tetrahedral intermediate and its breakdown by proton transfer, are studied for model systems using the density functional B3LYP method. Metallo-β-lactamases have two metal ion binding sites, one of which is occupied in the mononuclear species. In this work it is assumed that catalysis takes place at zinc site 1, which is modeled by the metal ion, three imidazole rings, and a hydroxide ion. The lactam ring, a minimal model of β-lactam antibiotics, is initially coordinating to the zinc ion. Potential proton shuttles from the second (unoccupied) metal-binding site (water, Asp, or Cys) are included in some calculations. The calculated reaction barrier for formation of the tetrahedral intermediate is 13 kcal/mol, close to what is observed experimentally for the rate-limiting step. The barrier for the breakdown of the intermediate is low, 0−10 kcal/mol, if it is assisted by a water molecule or by a Cys or Asp model. Thus, the results indicate that proton transfer is not rate-limiting, and that any of the residues from the second metal site may function as proton shuttle. For most studied systems, the tetrahedral structure is a stable intermediate. Moreover, the C−N bond in the lactam ring is intact in this intermediate, as well as in the following transition state—its cleavage is induced by proton transfer to the nitrogen atom in the lactam ring. However, for the model with Asp as a proton shuttle, attack of the zinc-bond hydroxide ion seems to be concerted with the proton transfer. We have also studied the effect of replacing one of the histidine ligands by an asparagine or glutamine residue, giving a zinc site representative of other subclasses of metallo-β-lactamases. This has only a small effect on the calculated reaction barriers. Likewise, if the zinc ion is replaced by cadmium, only small changes in the reaction barrier for proton transfer are seen, whereas the barrier for the formation of the tetrahedral intermediate increases by 3 kcal/mol and the intermediate is destabilized by 5 kcal/mol.

1. Introduction

β-Lactamases are bacterial enzymes that hydrolyze the C−N bond in β-lactam antibiotics, thereby inactivating these drugs. They have been divided into four classes, A−D.[1,2] Enzymes belonging to classes A, C, and D have a serine in the active site, which is responsible for catalysis, whereas enzymes from class B require metal ions for catalytic activity. In contrast to the enzymes from class A, C, and D, no clinically used inhibitors are available for the enzymes from class B. In addition, the class B β-lactamases have a broad substrate profile and some of the genes have spread to pathogenic bacteria,[3] causing a serious problem in treatments based on lactam antibiotics.

The metallo-β-lactamases have been further divided into three subclasses, B1−B3, on the basis of their amino acid sequences. For the enzymes of subclass B1, crystal structures have been determined for the enzymes from *Bacillus cereus*,[4−6] *Bactero-*

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ides fragilis,[7−11] and IMP-1 from *Pseudomonas aeruginosa*.[12,13] The structures show that there are two metal-ion binding sites. When two metal ions bind to the protein, a solvent molecule (probably a hydroxide ion) bridges the two metal ions and three histidine residues (His116, His118, and His196, following the numbering suggested by Galleni et al.[14]) coordinate to one of the metal ions (site 1), whereas an aspartate (Asp120), a cysteine (Cys221), a histidine (His263), and in some cases a water molecule coordinate to the other metal ion (site 2). The enzymes from *B. cereus* and *B. fragilis* both bind the first metal ion strongly, whereas the second one is more loosely bound to that from *B. cereus*.[15] In a crystal structure of the mononuclear enzyme, Asp120 and Cys221 form hydrogen bonds to the zinc-bound solvent molecule.[4]

No structure is yet available for any enzyme of subclass B2. The amino acid sequence indicates that His116 is replaced as a zinc ligand by an asparagine residue in this subclass. The β-lactamase from *Aeromonas hydrophila*, which belongs to this subclass, has two potential metal-binding sites,[16,17] but binding of the second zinc ion inhibits the enzyme.

For the enzymes from subclass B3, one crystal structure has been determined, viz. for the L1 enzyme from *Stenotrophomonas maltophilia*.[18] It showed that two zinc ions are bound, and that His121 coordinates the second zinc ion instead of Cys221, which is replaced by a serine residue in the amino acid sequence. For one of the enzymes of subclass B3, (GOB-1) His116 is replaced by a glutamine and might therefore be a metal ligand.

Most proposed mechanisms for mono- and dinuclear metallo-β-lactamases follow an overall scheme[3,19,20] in which the first step is substrate binding and structural reorganization. Next, a nucleophilic attack of the metal-bound hydroxide ion on the carbonyl carbon of the lactam ring leads to a tetrahedral oxyanion intermediate. This intermediate is broken down by hydrogen transfer to the amide nitrogen, and finally the product dissociates. A number of different pathways have been suggested, differing in what step is considered to be rate-limiting, in the source of the proton and shuttles for its transfer, and in what step the C−N bond is cleaved.

Bicknell et al.[19,20] have studied how Co-substituted mononuclear β-lactamase from *B. cereus* breaks down benzylpenicillin. They found that the C−N bond is cleaved at or after the rate-limiting step. Fitting this into the general scheme, it would indicate that either structural reorganization, nucleophilic attack, or C−N cleavage is rate limiting. For the mononuclear native zinc enzyme from the same bacterium, Bounaga et al.[21] proposed that the breakdown of the tetrahedral intermediate proceeds via a dianionic intermediate in which Asp120 has accepted a proton, facilitating the subsequent C−N bond fission, which occurs upon proton transfer to the amide nitrogen. They argue that either the formation of the dianionic intermediate or its breakdown is rate limiting. This is supported by a measured primary kinetic solvent isotope effect of 1.5 ± 0.1 for k_{cat}.

The pH rate profiles for hydrolysis of penicillins and cephalosporins have been studied for the mononuclear enzyme from *B. cereus*.[21,22] The observed rate-decrease at low pH (p$K = 5.6 \pm 0.2$) has been assigned to dissociation of zinc, probably as an effect of the protonation of the coordinating histidines. However, the decrease at high pH (p$K = 9.5 \pm 0.2$) has not yet been unambiguously assigned to any titratable group at the active site. The fact that no pK_a's below 9.5 can be assigned to the zinc-bound water molecule, has led to the assumption that it has a pK_a below 4.9 and is therefore a hydroxide ion at neutral pH.[22]

Recently Diaz et al.[23] presented a quantum mechanical study of the hydrolysis of benzylpenicillin by mononuclear β-lactamase. They suggest that the zinc-bound hydroxide, oriented by Asp120, performs a nucleophilic attack. Subsequently, His263 transfers a proton to the lactam ring and Asp120 accepts a proton from the hydroxide. Interestingly, their results indicate that tetrahedral intermediate does not exist on the B3LYP/6-31G-(d) potential energy surface, and that there is no barrier for the proton transfer, when His263 functions as proton donor. This raises the question whether the assumed tetrahedral structure actually is an observable intermediate.

Massova and Kollman[24] have performed quantum mechanical studies of hydrolysis of β-lactams in solution, that is, the uncatalyzed reaction. Their results show that the tetrahedral intermediate exists as a high-lying intermediate and that the nucleophilic attack is the rate-limiting step. In studies of model complexes that are functional synthetic analogues of the serine-β-lactamases, it was also found that the rate-limiting step is the nucleophilic attack.[25]

The structural and catalytic roles of the three residues in the second zinc-binding site, Asp120, Cys221, and His263, have been studied by mutagenesis experiments[15,26,27] on the enzyme from *B. cereus*. For the mononuclear enzyme, these residues are potential proton shuttles, and the mutants (Asp120Asn, Asp120Glu, His263Met, Cys221Ala, and Cys221Ser) are all inactive or severely impaired. Surprisingly, the dinuclear Cys221Ala mutant is catalytically active, indicating that the mono- and dinuclear enzymes function by different mechanisms.[15]

This is confirmed by a number of mechanistic studies that have been carried out for the dinuclear β-lactamases from *B. fragilis* and *S. maltophilia*.[28−31] McManus-Munoz[29] showed that the breakdown of an intermediate is rate-limiting for the enzyme from *S. maltophilia*. Likewise, Wang et al.[28] showed that for the dinuclear enzyme from *B. fragilis*, the rate-limiting step is breakdown of an intermediate with a zinc-acyl linkage and a negatively charged nitrogen in the lactam, i.e., an intermediate with a broken C−N bond. They propose that the second zinc ion plays a role in the stabilization of the negatively charged nitrogen in the intermediate, and that this intermediate may not exist for the mononuclear enzymes. This observation, however, seems to be specific for nitrocefin, because for two other substrates, cefaclor and meropenem, the intermediate is suggested to have an intact C−N bond, when the reaction is catalyzed by L1.[32]

The metallo β-lactamases are also active with other metal ions than zinc, for example cadmium.[17,33] For the mononuclear enzyme from *B. cereus*, the rate constant decreases by only a factor of 3 when zinc is replaced by cadmium.[33] A somewhat larger decrease is observed for the mononuclear enzyme of *A. hydrophila*.[17] Furthermore PAC, NMR, and EXAFS studies of the Cd-substituted enzyme from *B. cereus* suggest that in the mononuclear enzyme, the metal ion is dynamically exchanged between site 1 and 2.[33−35] This may have implications for the mechanism, but the issue of metal ion "jumping" will not be addressed here.

In this work, we study the hydrolysis of a lactam ring catalyzed by mononuclear metallo-β-lactamase, in which it is assumed that site 1 is responsible for activity. This is a generally accepted localization of the metal ion during catalysis.[3,21] We use small but realistic model systems for the studies and focus on nucleophilic attack and breakdown of the tetrahedral intermediate. The following points will be addressed: (1) Is creation of the tetrahedral intermediate or proton transfer to the

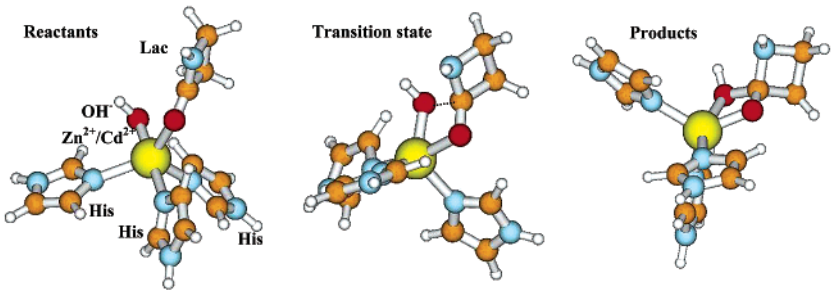

Figure 1. Formation of the tetrahedral intermediate in models 1 and 6. The reactants, transition state, and products are shown. Lac is an abbreviation for the lactam ring. The metal ion is either Zn^{2+} or Cd^{2+} (for models 1 and 6, respectively).

amide nitrogen rate-limiting? (2) Does the tetrahedral intermediate exist, that is, does it represent a local minimum on the potential energy surface? (3) Is the C−N bond intact in the tetrahedral intermediate? (4) Does the proton transfer to the nitrogen in the lactam ring lead to cleavage of the C−N bond? How is the reaction affected (5) when a solvent molecule or other amino acids assist the proton transfer, (6) when one of the histidine ligands is mutated to an asparagine or a glutamine, or (7) when zinc replaced by cadmium?

2. Methods

We use seven different models (denoted models 1−7) in this study of the formation and breakdown of the tetrahedral intermediate. In models 1−4, the model systems consist of a zinc ion, three histidines, and a solvent molecule coordinating the zinc ion, and a lactam ring. In addition, an extra solvent molecule is present in model 2, a cysteine in model 3, and an aspartic acid in model 4. Histidine residues are modeled by imidazole rings, the metal-bound solvent molecule by OH^-, the additional solvent molecule in model 2 by H_2O, the cysteine in model 3 by CH_3SH, and the aspartic acid in model 4 by CH_3COOH. In model 5, we replaced one of the three histidine ligands in model 2 by CH_3CONH_2 as a model of an asparagine or glutamine ligand. An amide may coordinate zinc by both the nitrogen and the oxygen atom, and both possibilities were investigated, models 5N or 5O. In models 6 and 7, we studied the effect of replacing Zn^{2+} with Cd^{2+} in models 1 and 2.

For the zinc-bound solvent molecule a hydroxide is used, following experimental evidence.[22] It is generally accepted that a hydroxide performs the nucleophilic attack.[3,21] The histidine residues are modeled by neutral imidazole rings, because there are no experimental indications that they should be deprotonated.

The model systems were constructed from the crystal structure of *B. fragilis*[7] using the Molden program.[36] The positions of the lactam ring (not present in the crystal) and the H_2O, Cys, or Asp (corresponding to Cys181 and Asp103 in 1ZNB) assisting in hydrogen transfer were determined by visual inspection. The binding mode of the lactam ring was verified by docking studies, and crystal structures and other theoretical work have shown that a water molecule can form hydrogen bonds to the Zn-bound water[7] and the positions of Asp and Cys are flexible.[37,38]

In all models, we started by optimizing the transition state and verified by a frequency calculation that the structure indeed is a first-order saddle point. Subsequently, the atoms were moved along the reaction coordinate in the direction of the reactants or products, and these were optimized. Frequency calculations were performed to verify that the structures represent true minima on the potential energy surface. No constraints were imposed in the optimizations. In the optimizations, the 6-31G(d)[39−43] basis was applied for all nonmetal atoms. For Zn^{2+}, the LanL2DZ basis[44−47] was used with an additional f function with the exponent 0.80. For Cd^{2+}, the LanL2DZ basis was also applied with additional p and f functions with the exponents 0.1172635 and 0.232832180, respectively.[48] Basis sets of this size have previously been shown to give reasonable structures.[49,50]

Accurate energies were calculated by single-point calculations on the optimized structures with the larger 6-311+G(2p,2d) basis.[51−58] For cadmium, the uncontracted basis set of Sadlej and Kellö[59] was used. Moreover, zero-point energy and thermodynamic corrections to the Gibbs free energy (at 298 K and 1 atm pressure) were calculated from the vibrational frequencies (with the 6-31G(d) basis set) determined using an ideal-gas approximation.[60] Finally, solvation effects were included in an approximate way by a set of conductor polarized continuum method (CPCM[61,62]) calculations as implemented in the Gaussian98 software.[63] We used the default settings for the these calculations, implying a water-like probe, a tesserae area of 0.4 Å^2, and a normalization of the calculated charge on each tessera by a constant factor to get the value predicted by Gauss' law. Repulsion, dispersion, and cavitation terms are included in the reported energies. The calculations were perfomed at two values of the dielectric constant, 4 and 78 using the 6-31G(d) basis set. The result of these calculations, together with that perfomed in a vacuum should include resonable solvation effects in the protein. The resulting activation and reaction energies for the two dielectric constants always differed by less than 2 kcal/mol. Therefore, we report only the results obtained with $\epsilon = 4$. In all calculations, we used the density functional method B3LYP[64−66] as implemented in the Gaussian98 program.

3. Results

3.1. Formation of the Tetrahedral Intermediate. The first step in the suggested reaction mechanisms is the formation of the tetrahedral intermediate by a nucleophilic attack of the zinc-bound hydroxide ion (O_{OH}) on the carbonyl atom of the lactam ring (C_{Lac}). Because this reaction does not involve any proton transfer, it was only studied with the small model 1 (i.e., without any proton-shuttle group); see Figure 1.

Before the nucleophilic attack, the $O_{OH}-C_{Lac}$ distance is 3.21 Å; see Table 1. This distance decreases to 1.85 Å in the transition state and to 1.50 Å when the tetrahedral intermediate has been formed. At the same time, the C−N bond length in the lactam ring increases slightly ($C_{Lac}-N_{Lac}$ is 1.35, 1.41, and 1.47 Å in the reactant, transition state, and product model, respectively). Also, the $Zn-O_{OH}$ distance increases because O_{OH} changes from a hydroxide ion to a hydroxyl oxygen, whereas the carbonyl oxygen in the lactam ring (O_{Lac}) binds stronger upon formation of the tetrahedral intermediate because it changes from a carbonyl to a deprotonated hydroxyl group ($Zn-O_{Lac}$ is 2.20, 2.17, and 1.95 Å in the three complexes). The

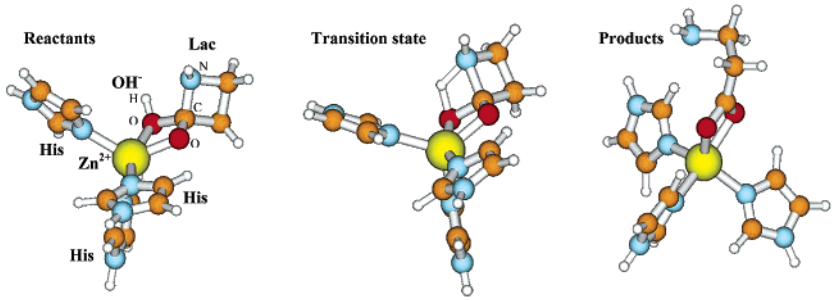

Figure 2. Breakdown of the tetrahedral intermediate in model 1 by direct proton transfer from O_{OH}. The reactants, transition state, and products are shown. Lac is an abbreviation for the lactam ring.

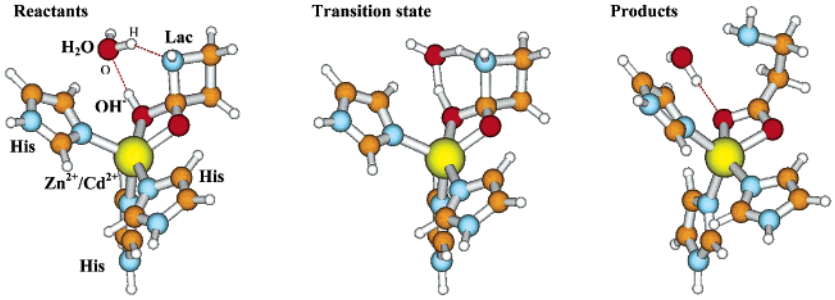

Figure 3. Breakdown of the tetrahedral intermediate in models 2 and 7 with a water molecule assisting the proton transfer. The reactants, transition state, and products are shown. Lac is an abbreviation for the lactam ring. The metal ion is either Zn^{2+} or Cd^{2+} (for models 2 and 7, respectively).

TABLE 1: Calculated Distances during the Formation of the Tetrahedral Intermediate Using Model 1[a]

	reactants	transition state	products
$O_{OH}-C_{Lac}$	3.21	1.86	1.50
$N_{Lac}-C_{Lac}$	1.35	1.41	1.47
$Zn-N_{His}$	2.13−2.20	2.13−2.20	2.13−2.18
$Zn-O_{Lac}$	2.20	2.17	1.95
$Zn-O_{OH}$	1.94	2.00	2.30

[a] Lac, OH, and His are abbreviations for the atoms in the lactam ring, the hydroxide, and the histidines, respectively. All distances are given in Å. See also Figure 1.

$Zn-N_{His}$ distances do not change much during the reaction (2.13−2.20 Å). In Table 9 it is seen that the reaction barrier for formation of the tetrahedral intermediate is 13 kcal/mol and that the tetrahedral intermediate is 8 kcal/mol higher in energy than the reactant model. Thus, these results indicate that the tetrahedral structure exists as a stable intermediate and that it has an intact C−N bond.

3.2. Breakdown of the Tetrahedral Intermediate. Figure 2 shows the next step of the reaction, the proton transfer from the hydroxyl group, formed from the hydroxide ion (for clarity we continue to call these atoms O_{OH} and H_{OH}, although they are no longer a hydroxide ion), to the nitrogen atom in the lactam ring (N_{Lac}). In the transition state, H_{OH} is positioned almost exactly between O_{OH} (1.25 Å) and N_{Lac} (1.30 Å), whereas it is fully transferred to the N_{Lac} in the product (1.02 Å); see Table 2. For the reactants, the $C_{Lac}-N_{Lac}$ bond length in the lactam ring is 1.47 Å. In the transition state, it is extended to 1.64 Å, and when the hydrogen is transferred to the nitrogen of the lactam ring it is broken (2.96 Å). Interestingly, the energy of the transition state for model 1 shown in Figure 2 is 23 kcal/mol above that of the tetrahedral intermediate (and therefore 31 kcal/mol above the substrate model) even if the product is 39 kcal/mol lower than the tetrahedral intermediate. Such a barrier is much too high to allow for an effective enzymatic reaction. Therefore, we investigated whether any of the nearby residues in the enzyme may reduce this barrier. We tested three possibilities: a water molecule, a cysteine, or an aspartic acid residue.

TABLE 2: Breakdown of the Tetrahedral Intermediate in Model 1 by Direct Proton Transfer from the O_{OH}[a]

	reactants	transition state	products
$O_{OH}-H_{OH}$	0.97	1.25	4.02
$H_{OH}-N_{Lac}$	2.23	1.30	1.02
$N_{Lac}-C_{Lac}$	1.47	1.64	2.96
$Zn-N_{His}$	2.13−2.18	2.13−2.19	2.11−2.17
$Zn-O_{Lac}$	1.95	2.01	2.08
$Zn-O_{OH}$	2.30	2.19	2.20

[a] Lac, OH, and His are abbreviations for the atoms in the lactam ring, the hydroxide, and the histidines, respectively. All distances are given in Å. See also Figure 2.

TABLE 3: Breakdown of the Tetrahedral Intermediate in Model 2 with a Water Molecule Assisting the Proton Transfer[a]

	reactants	transition state	products
$O_{OH}-H_{OH}$	0.99	1.12	1.82
$H_{OH}-O_{H_2O}$	1.75	1.33	0.98
$O_{H_2O}-H_{H_2O}$	1.00	1.29	2.20
$H_{H_2O}-N_{Lac}$	1.81	1.22	1.02
$N_{Lac}-C_{Lac}$	1.51	1.60	2.82
$Zn-N_{His}$	2.13−2.18	2.12−2.18	2.10−2.16
$Zn-O_{Lac}$	1.96	1.98	2.07
$Zn-O_{OH^-}$	2.27	2.22	2.21

[a] Lac, OH, H_2O, and His are abbreviations for the atoms in the lactam ring, the hydroxide, the solvent molecule assisting in the proton transfer, and the histidines, respectively. All distances are given in Å. See also Figure 3.

In model 2, a water molecule assists the proton transfer; see Figure 3. For the reactants, the water molecule forms hydrogen bonds to both O_{OH} ($H_{OH}-O_{H_2O}$ = 1.75 Å) and N_{Lac} ($H_{H_2O}-N_{Lac}$ = 1.81 Å); see Table 3. In the transition state, the $O_{OH}-H_{OH}$ bond is extended from 0.99 to 1.12 Å, whereas the $O_{H_2O}-H_{H_2O}$ bond is extended to 1.29 Å. The distance of the transferred hydrogen atom to N_{Lac} is shorter than for the direct proton transfer ($H_{H_2O}-N_{Lac}$ = 1.22 Å compared to $H_{OH}-N_{Lac}$ = 1.30 Å). In the transition state, the $C_{Lac}-N_{Lac}$ bond is longer than

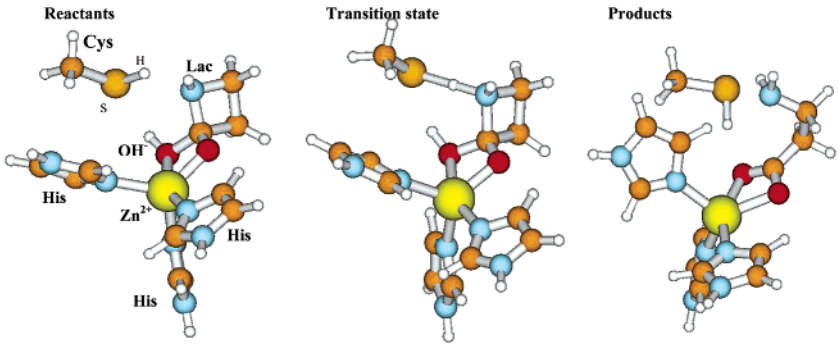

Figure 4. Breakdown of the tetrahedral intermediate in model 3 with a cysteine assisting the proton transfer. Lac is an abbreviation for the lactam ring.

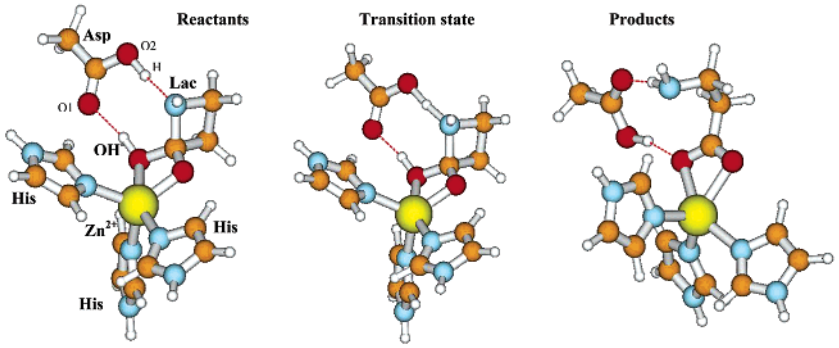

Figure 5. Breakdown of the tetrahedral intermediate in model 4 with an aspartic acid assisting the proton transfer. Lac is an abbreviation for the lactam ring.

TABLE 4: Breakdown of the Tetrahedral Intermediate in Model 3 with a Cysteine Assisting the Proton Transfer[a]

	reactants	transistion state	products
$O_{OH}-H_{OH}$	0.98	1.01	2.26
$H_{OH}-S_{Cys}$	2.43	2.12	1.35
$S_{Cys}-H_{Cys}$	1.37	1.66	2.81
$H_{Cys}-N_{Lac}$	2.16	1.31	1.02
$N_{Lac}-C_{Lac}$	1.49	1.55	2.89
$Zn-N_{His}$	2.13−2.19	2.12−2.18	2.12−2.18
$Zn-O_{Lac}$	1.95	1.98	2.06
$Zn-O_{OH}$	2.29	2.28	2.22

[a] Lac, OH, Cys, and His are abbreviations for the atoms in the lactam ring, the hydroxide, the cysteine assisting in the proton transfer, and the histidines, respectively. All distances are given in Å. See also Figure 4.

TABLE 5: Breakdown of the Tetrahedral Intermediate in Model 4 with an Aspartic Acid Assisting the Proton Transfer[a]

	reactants	transition state	products
$O_{OH}-H_{OH}$	0.99	1.01	1.71
$H_{OH}-O1_{Asp}$	1.72	1.61	1.00
$O2_{Asp}-H_{Asp}$	1.05	1.21	2.06
$H_{Asp}-N_{Lac}$	1.60	1.30	1.02
$N_{Lac}-C_{Lac}$	1.53	1.56	2.88
$Zn-N_{His}$	2.13−2.18	2.12−2.18	2.10−2.16
$Zn-O_{Lac}$	1.96	1.98	2.08
$Zn-O_{OH}$	2.29	2.28	2.23

[a] Lac, OH^-, Asp, and His are abbreviations for the atoms in the lactam ring, the hydroxide, the aspartic acid assisting in the proton transfer, and the histidines, respectively. All distances are given in Å. See also Figure 5.

for the reactants, as in model 1. However, it increases by only 0.09 Å, whereas an increase of 0.17 Å was observed for model 1. When the proton is transferred to N_{Lac}, the C−N bond breaks. From an energetic point of view, the water molecule strongly assists the proton transfer; the barrier is reduced to 10 kcal/mol.

In model 3, a cysteine residue assists the proton transfer to N_{Lac}; see Figure 4. In contrast to models 1 and 2, the $O_{OH}-H_{OH}$ bond length is almost the same in the transition state (1.01 Å) as in the reactants (0.98 Å); see Table 4. However, the $H_{Cys}-N_{Lac}$ distance is much shorter in the transition state (1.31 Å) than in the reactants (2.16 Å). Compared to model 2, a smaller extension of the $C_{Lac}-N_{Lac}$ bond is observed when going from the reactants to the transition state structure (an increase of 0.06 Å from 1.49 to 1.55 Å), but it is still broken in the products. This shows that the transition state is reactant-like and indicates that the reaction barrier is low. This is confirmed by a direct calculation of the energy; it is only 5 kcal/mol above the tetrahedral intermediate (13 kcal/mol above the initial substrate complex, calculated with model 1). In model 4, the reaction is assisted by an aspartic acid residue; see Figure 5. The same trends as for model 3 are observed. The $O_{OH}-H_{OH}$ bond is only 0.02 Å longer in the transition state than in the reactants, whereas the proton that is transferred is positioned at a distance of 1.30 Å from N_{Lac}; see Table 5. Compared to models 2 and 3, an even smaller extension of the $C_{Lac}-N_{Lac}$ bond is observed when going from the reactants to the transition-state structure; it increases by 0.03 Å from 1.53 to 1.56 Å. Again, it is broken for the products. This indicates that the energy barrier is even lower for this reaction than for model 3. This is also the case: Although there is a small barrier in a vacuum (1 kcal/mol), thermodynamic corrections led to a barrierless reaction, where the transition structure is slightly (2 kcal/mol) more stable than the reactants. This implies that the tetrahedral complex is not an intermediate when proton transfer is assisted by a protonated Asp residue (i.e., that the nucleophilic attack and the proton transfer is concerted). However, it must be remembered that an Asp residue is usually not protonated at neutral pH. We should therefore add the energy cost of forming a protonated Asp in the protein at neutral pH to this energy barrier.

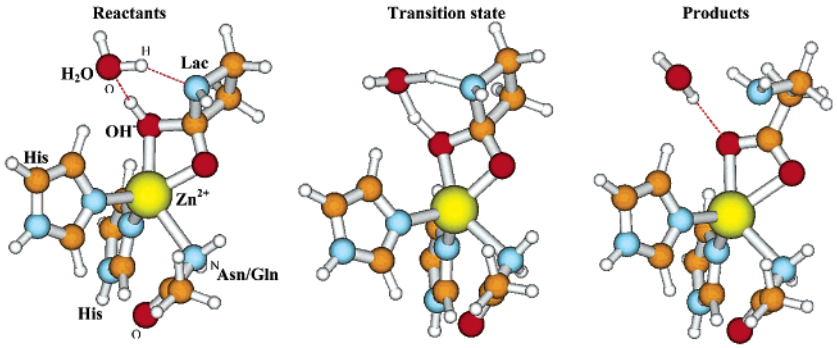

Figure 6. Breakdown of the tetrahedral intermediate in model 5N with water molecule assisting the proton transfer and the nitrogen of the Asn/Gln coordinating Zn^{2+}. The reactants, transition state, and products are shown. Lac is an abbreviation for the lactam ring.

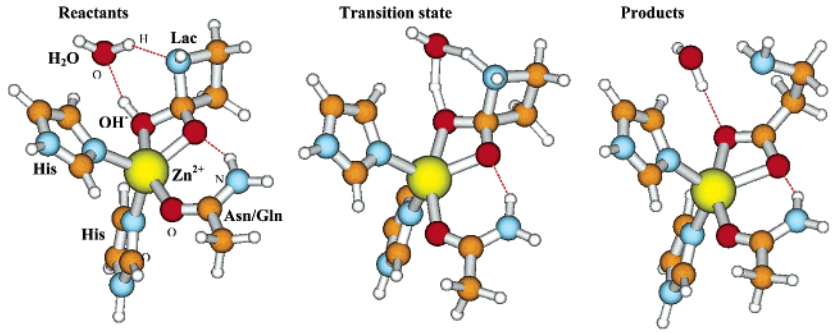

Figure 7. Breakdown of the tetrahedral intermediate in model 5O with a water molecule assisting in the proton transfer and the oxygen of the Asn/Gln coordinating Zn^{2+}. The reactants, transition state, and products are shown. Lac is an abbreviation for the lactam ring.

For all models 1−4, the $Zn-N_{His}$ bond lengths are almost the same for the reactant, transition-state, and product models, in general from 2.1 to 2.2 Å. A somewhat larger variation is observed for the distances to O_{OH} and O_{Lac}: The $Zn-O_{OH}$ distance is 2.27−2.30 Å for the reactants, and it decreases to 2.19−2.28 Å and 2.20−2.23 Å in the transition-state and product models, respectively, as an effect of its change from a hydroxyl to a carboxylate group. Similarly, the distance to the other carboxylate oxygen increases from 1.95−1.96 Å to 1.98−2.01 Å and 2.06−2.08 Å when going from the reactants via the transition state to the products.

3.3. Models with an Asn/Gln Zinc Ligand. As mentioned in the Introduction, some subtypes of metallo-β-lactamases probably have one of the histidine zinc ligands in site 1 replaced by an Asn or Gln ligand. We have therefore tested the effect of such a substitution on the reaction mechanism. Table 6 shows selected distances for model 5, where one of the histidine zinc ligands in model 2 has been replaced by CH_3CONH_2, as a model of Asn/Gln. This ligand may coordinate to zinc by either the amide oxygen ($O_{Asn/Gln}$) or nitrogen atoms ($N_{Asn/Gln}$). We have studied both possibilities, but the model where $O_{Asn/Gln}$ coordinates to the zinc ion was 14 kcal/mol more stable than the other complex, so we restrict the discussion to this complex (especially as the geometries and the energies of the two complexes are very similar). The reason this coordination mode was more stable is probably that the $Zn-O_{Asn/Gln}$ bond is stronger (around 2.1 Å compared to 2.4 Å) and that there is a hydrogen bond between the uncoordinated NH_2 group and O_{Lac}; see Figure 7.

Interestingly, the substitution of one of the His ligands by Asn/Gln does not change the geometry of the complexes significantly (compare Tables 6 and 3). The $Zn-O_{Asn/Gln}$ distance of 2.12−2.13 Å is very similar to the $Zn-N_{His}$ distances. Consequently, the energetics of model 5 is similar to that of model 2: The energy barrier is 8−9 kcal/mol (10 kcal/mol for model 2), whereas the reaction energy is −34 kcal/mol for both models.

TABLE 6: Breakdown of the Tetrahedral Intermediate in Models 5N and 5O with Water Molecule Assisting the Proton Transfer and the Nitrogen or Oxygen of the Asn/Gln Coordinating Zn^{2+} [a]

	reactants		transition state		products	
	N	O	N	O	N	O
$O_{OH}-H_{OH}$	1.00	0.99	1.15	1.15	1.86	1.84
$H_{OH}-O_{H_2O}$	1.74	1.72	1.29	1.29	0.98	0.98
$O_{H_2O}-H_{H_2O}$	1.00	1.00	1.25	1.27	2.16	2.17
$H_{H_2O}-N_{Lac}$	1.81	1.86	1.25	1.25	1.02	1.02
$N_{Lac}-C_{Lac}$	1.51	1.50	1.59	1.59	2.79	2.80
$Zn-N_{His}$	2.10	2.10, 2.11	2.10, 2.11	2.11	2.08, 2.09	2.09
$Zn-N/O1_{Asp}$	2.43	2.12	2.42	2.13	2.39	2.11
$Zn-O_{Lac}$	1.97	1.98	1.98	2.01	2.08	2.10
$Zn-O_{OH}$	2.16	2.23	2.12	2.19	2.13	2.20

[a] Lac, OH, H_2O, His, and Asn/Gln are abbreviations for the atoms in the lactam ring, the hydroxide, the solvent molecule assisting in the proton transfer, the histidines, and the asparagine/glutamine, respectively. The columns with N and O gives the numbers with the nitrogen or oxygen coordinating to Zn^{2+}; see Figures 6 and 7. All distances are given in Å.

3.4. Cadmium Instead of Zinc. Finally, we have examined the effect of replacing the zinc ion with Cd^{2+}. In model 6, we study the formation of the tetrahedral intermediate. The general structure of the models are very similar to those of the corresponding zinc complexes. However, as can be seen in Table 7, the Cd−ligand bonds are 0.1−0.2 Å longer than for zinc, reflecting the larger ionic radius of Cd^{2+}. Moreover, the $O_{OH}-C_{Lac}$ distance is 3.37 Å before the nucleophilic attack and 1.90 Å in the transition state, which is longer than in the zinc model; see Table 1. Therefore, it is not surprising that the reaction barrier is larger for cadmium than for zinc, 16 kcal/mol compared to 13 kcal/mol.

Furthermore, we have studied the breakdown of the tetrahedral intermediate, using model 7, the cadmium analogue of

TABLE 7: Formation of the Tetrahedral Intermediate in Model 6 Using Cadmium Instead of Zinc[a]

	reactants	transition state	products
$O_{OH}-C_{Lac}$	3.37	1.90	1.52
$N_{Lac}-C_{Lac}$	1.35	1.41	1.48
$Cd-N_{His}$	2.33−2.44	2.32−2.36	2.33
$Cd-O_{Lac}$	2.53	2.33	2.20
$Cd-O_{OH}$	2.09	2.17	2.34

[a] Lac, OH, and His are abbreviations for the atoms in the lactam ring, the hydroxide, and the histidines, respectively. All distances are given in Å. See also Figure 1.

TABLE 8: Breakdown of the Tetrahedral Intermediate in Model 7 with a Water Molecule Assisting the Proton Transfer Using Cadmium Instead of Zinc[a]

	reactants	transition state	products
$O_{OH}-H_{OH}$	0.99	1.12	1.81
$H_{OH}-O_{H_2O}$	1.76	1.35	0.98
$O_{H_2O}-H_{H_2O}$	1.00	1.35	2.20
$H_{H_2O}-N_{Lac}$	1.80	1.22	1.02
$N_{Lac}-C_{Lac}$	1.52	1.61	2.81
$Cd-N_{His}$	2.33−2.37	2.32−2.36	2.30−2.35
$Cd-O_{Lac}$	2.16	2.18	2.28
$Cd-O_{OH}$	2.38	2.35	2.33

[a] Lac, OH, and His are abbreviations for the atoms in the lactam ring, the hydroxide, and the histidines, respectively. All distances are given in Å. See also Figure 2.

model 2, where a water molecule assists the proton transfer. The result in Table 8 shows that there is no significant difference in the distances between the atoms directly involved in the reaction compared to the model with zinc (Tables 3). Therefore, the energy barrier (9 kcal/mol) is only 1 kcal/mol lower than that of the zinc model.

4. Discussion

We have studied two steps in the hydrolytic cleavage of the C−N bond in the lactam ring catalyzed by mononuclear metallo-β-lactamase, nucleophilic attack by the zinc-bound hydroxide ion on the lactam carbonyl carbon and the subsequent hydrogen transfer to the amide nitrogen. It is assumed that site 1 (modeled by a zinc ion, three neutral imidazole rings, and a hydroxide) is responsible for hydrolysis of the lactam ring. The antibiotic is modeled by a simple lactam ring, initially coordinating to the zinc ion. A neutral lactam ring is the simplest and most general model for antibiotics. Studies by Massova and Kollman[24] indicate that cleavage of a neutral lactam ring gives very similar reaction barriers as compared to a model, in which both the neighboring five-membered ring and the carboxylate group bound to the latter are included,[23] especially as the carboxylate group is neutralized in the protein by binding to a Lys residue.

The model systems are shown in Figures 2−7 and represent nucleophilic attack and different ways of transferring the proton, by either directly or assisted by H_2O or the side chains of Asp120 or Cys221. We analyze the most widely accepted mechanism.[3] This is analogous to the proposed mechanism for carboxypeptidase, where the metal ion is changing coordination number between 4 and 5 during catalysis.[67] A six-coordinate zinc ion is rarely encountered in catalytic sites of enzymes.[68] Theoretical calculations have shown that five- and four-coordination is the preferred coordination mode of a Zn^{2+} ion with the ligands encountered in proteins.[69,70] Diaz et al.[23] have recently investigated the mechanism of mononuclear zinc-β-lactamase, when the proton transfer is assisted by the third residue at site 2, His263. Thus, the two studies complement each other by testing various possible proton shuttles. Moreover, we have studied the effect of replacing zinc with cadmium and of replacing one of the coordinating histidines with asparagine or glutamine, a replacement assumed to occur in some metallo-β-lactamases.

TABLE 9: Energies for the Formation and Breakdown of the Tetrahedral Intermediate[a]

	transition state				products			
model	ΔE_{El}	$\Delta\Delta G_{Therm}$	$\Delta\Delta G_{Solv}$	ΔG_{Tot}	ΔE_{El}	$\Delta\Delta G_{Therm}$	$\Delta\Delta G_{Solv}$	ΔG_{Tot}
Formation of the Tetrahedral Intermediate								
1	11.8	2.2	−0.5	13.5	9.3	1.7	−3.0	8.0
6	14.7	2.0	−0.9	15.9	12.3	2.7	−1.5	13.5
Breakdown of the Tetrahedral Intermediate								
1	22.9	−1.4	1.6	23.1	−41.9	1.4	1.8	−38.8
2	12.7	−2.5	−0.6	9.6	−34.0	−0.4	0.8	−33.6
3	6.8	0.7	−2.9	4.6	−37.9	0.5	1.3	−36.1
4	0.6	−2.6	−0.3	−2.2	−33.9	−1.5	1.0	−34.4
5N	13.0	−3.4	−0.9	8.7	−32.7	−1.5	0.7	−33.6
5O	10.6	−2.6	−0.2	7.8	−34.5	−0.3	0.5	−34.3
7	12.2	−2.5	−0.9	8.7	−36.2	−0.8	0.3	−36.7

[a] All energy contributions (kcal/mol) are given relative to the reactants. $\Delta G_{Tot} = \Delta E_{El} + \Delta\Delta G_{Therm} + \Delta\Delta G_{Solv}$, where ΔE_{El} gives the electronic energy, $\Delta\Delta G_{Therm}$ the zero-point and thermal corrections, and $\Delta\Delta G_{Solv}$ the solvation energy. Note that for model 1, the products of the first reaction are the same as the reactants of the second reaction.

We will try to answer the questions posed in the Introduction with the help of the results of our calculations. The philosophy behind the calculations is to compare the calculated barriers with experimental kinetic data: If a calculated energy barrier is significantly higher than that observed experimentally, the tested reaction mechanism can be rejected. If the barrier is lower, this step in the reaction might occur, but it need not be rate-limiting. The k_{cat} values of 43 and 230 s^{-1} (for nitrocefin and benzylpenicillin, respectively)[71] for the mononuclear *B. cereus* enzyme, gives Gibbs free energy barriers of 14−15 kcal/mol. However, it should be kept in mind that the uncertainty on the calculated reaction barriers is 3−5 kcal/mol.[72]

The Asp120 and Cys221 residues have been shown to be mobile: Asp120 moved from the usual coordination at site 2 to coordination to site 1 in a crystal structure of a dicadmium enzyme (with a citrate molecule bound in the active site).[37] In a recent theoretical study,[38] it was shown that Cys221 can move relatively close to site 1, even though it usually coordinates to site 2. In addition, there is no unambiguous experimental information of how the substrate orients relative to these residues when it is bound to the enzyme. Therefore, we have chosen to optimize our geometries without any restraints in the model.

4.1. Formation of the Tetrahedral Intermediate. There is general consensus that the first chemical step in the hydrolysis of lactams is the attack of a hydroxide on the lactam carbonyl carbon, both in solution[24,73] and in metallo-β-lactamases.[3] In solution, this is also the rate-limiting step, but this need not be the case in the enzymes if they have effectively lowered the reaction barrier of this part of the reaction and increased the effective concentration of the OH^- ion by coordination to the zinc ion.

In our calculations, we find a reaction barrier of 13 kcal/mol for this step (see Table 9), which is slightly lower than the barrier of 14−15 kcal/mol derived from kinetic data. Consequently, this can be the rate-limiting step. The measured kinetic isotope effect of 1.5 for k_{cat} may seem a bit too large for a step not involving proton transfer, but it may originate from significant changes in the hydrogen-bond network upon formation of the transition state.

Diaz et al.[23] also concluded that the formation of the tetrahedral intermediate is rate-limiting, although they started from a different structure and found a slightly larger reaction

barrier, 17 kcal/mol. Using similar types of model systems and computational methods as applied in this study, it is found that the reaction barrier for nucleophilic attack of a zinc bound water molecule in thermolysin and stromelysin (with similar zinc ligands as in metallo-β-lactamase) on a small peptide is 15 and 13 kcal/mol, respectively.[74,75] For both enzymes, this step is rate-limiting.

4.2. The Tetrahedral Intermediate. Most proposed mechanisms for metallo-β-lactamases assume the existence of a tetrahedral intermediate, formed after the nucleophilic attack by the zinc-bound hydroxide ion on the carbon in the β-lactam ring. However, Diaz et al.[23] found that at the B3LYP/6-31G(d) level of theory, proton transfer is barrierless, using His269 as proton donor and Asp120 as a proton acceptor; that is, the nucleophilic attack is concerted with proton transfer and the tetrahedral intermediate does not exist. Yet, at the PM3 and HF/6-31G(d) levels of theory they find a stable tetrahedral intermediate.

We find something similar in our model 4, where Asp120 acts as a proton shuttle: the tetrahedral structure is a stable intermediate when optimized at the B3LYP/6-31G(d) level, but when zero-point, thermal, and solvation effects are added, the barrier for proton transfer disappears (Table 9). However, for the other models, we find a stable tetrahedral intermediate and a barrier for proton transfer of 5−23 kcal/mol, depending on the type of proton shuttle in the model.

Thus, it can still not be settled whether there is a stable tetrahedral intermediate or not in the reaction mechanism of metallo-β-lactamase. Instead, our results indicate that this critically depends on the presence and nature of a proton shuttle in the active site. Several amino acids in the active site could provide such a shuttle and further calculations are needed, involving appreciably larger models of the enzyme, to decide which residues have the correct position and protonation status to work as a proton shuttle in vivo. It is also probable that the shuttle, and therefore also the reaction mechanism, may depend on the source of the enzyme, the number of metal ions in the active site, and the type of substrate.

There have been different suggestions concerning the state of the C−N bond in the tetrahedral intermediate (for the binuclear enzyme). With nitrocefin, it is suggested to be broken both for L1[32] and the enzyme from *B. fragilis*.[76] However, for two other substrates, cefaclor and the meropenem, the C−N bond is suggested to be intact when the reaction is catalyzed by L1.[32] Because no experimental results about the state of the C−N bond in the tetrahedral intermediate have yet been reported for the mononuclear enzyme, it is not possible to make a direct comparison between experiment and theory. In our calculations, the C−N bond is always intact in the tetrahedral intermediate. Even in the following transition state it is intact, although clearly lengthened (1.5−1.6 Å). Similar results have also been observed in other theoretical investigations.[23] Thus, breakage of the C−N bond is triggered by proton transfer to the nitrogen atom. This indicates the stability of the C−N bond and it should be experimentally observable, provided that the tetrahedral intermediate exists.

4.3. Assisted Proton Transfer by Water, Cys, or Asp. For a zinc binding site with three histidines and a hydroxide ion, we have investigated four different ways of transferring the proton to the nitrogen of the lactam ring starting from the metal ion bound OH^-. Models with direct transfer have been considered, as well as models where the transfer is assisted by H_2O, Cys, or Asp. Thus, our investigations complement the work of Diaz et al.,[23] in which His269 and Asp120 assisted the proton transfer to the lactam nitrogen. The observed reaction barrier for model 1 is 23 kcal/mol, which is too high compared to experiments. Therefore, unassisted proton transfer can be rejected, and we do not comment further on this model. However, on the basis of the size of the energy barriers, models 2−4 might represent a realistic step in the catalytic process.

Model 2 has been constructed to test if a solvent molecule may assist the proton transfer. Second-sphere solvent molecules have been observed in the active site of metallo-β-lactamase by EXAFS[35] and in several X-ray structures (see, for example, 1ZNB[7]). Although these studies have been performed without substrate present, they indicate that a water molecule may well be present and available to assist the proton transfer. The calculated reaction barrier in model 2 is 10 kcal/mol. To this energy, we must add the energy required to form the tetrahedral intermediate (not determined for this model system, but we will assume that it is 8 kcal/mol as for model 1) to obtain the total barrier for the reaction, 18 kcal/mol. This is slightly larger than the observed barrier of 14−15 kcal/mol, but still a reasonable energy, considering the uncertainty of the method.

In model 3, a cysteine residue assists the proton transfer. Cys121 is a metal ligand of site 2 when both metal ions are present. Even though the cysteine belongs to site 2, which is not believed to be responsible for catalysis, this residue significantly influences the reaction rate: Both Paul-Soto et al.[15] and de Seny et al.[71] showed that mutations of this residue decrease the activity for the mononuclear enzyme of *B. cereus*, whereas the binuclear enzyme is almost unaffected by such mutations. There are several conceivable roles for this cysteine in the mononuclear enzyme: orienting the substrate, stabilizing the anionic tetrahedral intermediate, participating in hydrogen bonds essential for catalysis, activating the solvent molecule performing the nucleophilic attack, or mediating the proton transfer to the lactam ring, as we have investigated. The reaction barrier for the cysteine-assisted proton transfer is calculated to be 5 kcal/mol, and including the energy required for forming the tetrahedral intermediate of 8 kcal/mol gives a total barrier of 13 kcal/mol, which is the same as the barrier for formation of the tetrahedral intermediate and also close to what is observed experimentally.

Finally, in model 4, a protonated aspartic acid residue assists the proton transfer. It has been shown that mutation of Asp120 to an asparagine significantly decreases the activity of the enzyme (k_{cat} = 230 and 3.5 s^{-1} for the wild type and the mutant, respectively, with benzylpenicillin as substrate).[71] This has led to the suggestion that Asp120 plays a role in accepting a proton from the zinc-bound water molecule and orienting the so formed hydroxide before the nucleophilic attack.[1,3,21] However, Asp120 might also mediate the proton transfer to the lactam nitrogen atom. A similar role has been suggested for Glu270 in carboxypeptidase A.[67] For model 4, there is effectively no energy barrier for the proton transfer, as was discussed above. Thus, if the enzyme can protonate the aspartate residue (e.g., during the formation of the zinc-bound hydroxide ion) and orient the substrate so that the proton forms hydrogen bonds to the nitrogen in the lactam ring, the C−N bond is readily cleaved, probably in a concerted manner with the hydrophilic attack on the lactam carbonyl group.

In general, the energy barriers are small, when the amino acids in the active site mediate the proton to the lactam nitrogen atom. This is observed from our data involving the cysteine and the aspartic acid, as well as in the result presented by Diaz et al. involving both the histidine and aspartate residues.[23] This type of reaction has been discussed by Bounaga et al.,[21] who argue that all such proton-transfer steps would give rise to larger isotope effects than that observed experimentally (1.5 on k_{cat}).

Therefore, they conclude, the proton-transfer steps cannot be rate-limiting. On the other hand, the isotope effect is larger than what would be expected if no proton transfer was involved in the rate-limiting step, e.g., the formation of the tetrahedral intermediate.

Our calculations, can give a possible explanation to this dilemma. If the situation is close to what we find for model 3 (involving Cys), viz., that the barriers (including zero-point corrections) for the formation of the tetrahedral intermediate and the following proton transfer (calculated from the same reactants) are almost equal, such an effect can be expected, namely, in the case when the barrier for the proton transfer is lower but the barrier for deutron transfer is higher than that for the formation of the tetrahedral intermediate. Unfortunately, the accuracy of our calculations is far too low and the approximations involved are too crude to prove that this is actually the case for metallo-β-lactamase.

Alternatively, it has been suggested that steps later in catalysis are rate-limiting,[22] for example, product release, although this contradicts current proposals for the enzymatic mechanism. In such a case one might expect an influence of the type of metal ion (because the product coordinates to the metal), and no large isotope and pH effects. This is seen for example with liver alcohol dehydrogenase, for which release of NADH is rate-limiting in the oxidation of primary alcohols.[77]

4.4. Models of the Subclasses of Metallo-β-Lactamases: Mutating His to Asn/Gln. Metallo-β-lactamases from different subclasses have a surprisingly low sequence identity, and there are differences even in the metal ligands. These differences may increase the ability of the enzymes to hydrolyze different types of antibiotics, and it complicates the design of inhibitors. The metallo-β-lactamases of subclass B1 and some of those of subclass B3 have a metal-binding site with three histidine ligands. However, the enzymes of subclass B2 and at least one from subclass B3, have either an asparagine or a glutamine instead of His116 as metal ligand, according to amino acid sequence alignments.[14] It was shown recently that binding constants for formation of the mononuclear enzymes in the presence of substrates are very similar for all three metallo-β-lactamase subclasses, independent of ligand substitutions.[78] We have investigated the effect on the reaction barrier of mutating one of the histidine residues into an asparagine or glutamine by replacing an imidazole ring by acetamide. We study only the hydrogen-transfer step and only with a water molecule as a proton shuttle (model 5).

The results show that irrespectively of whether the amide ligand coordinates to zinc by the oxygen or nitrogen atoms, the reaction barrier for proton transfer (8−9 kcal/mol) is close to that observed with three histidine ligands (model 2). Thus, a His → Asn/Gln mutation does not significantly modify the ability of the metallo-β-lactamases to cleave the C−N bond. It also shows that minor modifications of the type of ligand do not change the reaction barriers dramatically, which may explain why a His → Ser mutation at site 1 does not significantly alter the activity of the enzyme from *B. cereus*.[71]

Even though our results show that His → Asn/Gln mutation of the metal ligand does not change the activation barriers for proton transfer significantly, it might still be important in other steps in the mechanism, e.g., in the formation of the tetrahedral intermediate, the release of the product, or by orienting the substrate. For example, the NH_2 group of Asn/Gln could attract the carbonyl oxygen of the lactam ring, activate the hydroxide before the nucleophilic attack, and decrease the strength of the $Zn-O_{Lac}$ bond, which would be advantageous for the product release.

4.5. Cadmium Substitution. In vivo, the metallo-β-lactamases bind one or two zinc ions. However, the enzymes are also active when zinc is substituted with other metal ions, e.g., Cd^{2+}.[17,33] The Cd-substituted enzyme is only slightly less active than the native enzyme. For example, the rate constant (k_{cat}) of the mononuclear enzyme from *B. cereus* when cleaving nitrocefin at pH 7.5 is 29 s^{-1} for Zn^{2+} and 8.8 s^{-1} for Cd^{2+},[33] indicating that the energy barrier is ∼1 kcal/mol higher for cadmium. However, recently Adolph et al. (unpublished results) have obtained much larger differences upon cadmium substitution, a factor of 1000 in k_{cat} for the mononuclear enzyme from *A. hydrophila*, which corresponds to an energy difference of over 4 kcal/mol.

We have replaced zinc with cadmium in models 6 and 7 to investigate the effect of metal substitution on the reaction barriers when forming the tetrahedral intermediate and transferring the proton. The calculations show that cadmium does not change the barrier of the second reaction, whereas that of the first reaction increases by 3 kcal/mol to 16 kcal/mol. This is probably owing to the poorer ability of Cd^{2+} to polarize the hydroxide ion. Thereby, the hydroxide becomes less nucleophilic. Interestingly, the tetrahedral intermediate is 13 kcal/mol higher in energy than the reactant model, which is 5 kcal/mol more than for the corresponding zinc model (model 1). This would increase the reaction barrier also for the proton transfer by this amount. Thus, our results seem to agree best with the recent results by Adolph et al., irrespective of whether the formation of the tetrahedral intermediate or the following proton transfer is the rate-limiting step.

5. Conclusion

By applying density functional theory on small model systems, we have investigated different ways for the mononuclear metallo-β-lactamases to catalyze hydrolytic cleavage of a lactam ring. Our results indicate that nucleophilic attack may be rate-limiting, as the calculated reaction barrier (13 kcal/mol) is comparable to that found experimentally for hydrolysis of β-lactam antibiotics. Other theoretical investigations have arrived at a similar conclusion.[23] The barrier for the breakdown of this intermediate by proton transfer to the lactam nitrogen atom strongly depends on how the proton is transferred. Without any assistance, it has a very high barrier. However, if it is assisted by a water, molecule, a cysteine or a protonated aspartic acid model, the barrier decreases so that it is slightly larger, equal, or lower than that for the formation of the tetrahedral intermediate. With the Asp model, the reactions are probably concerted so that there is no stable tetrahedral intermediate. This was also observed by Diaz et al.[23] for a model system with His263 acting as a proton shuttle.

Changing one of the metal ligands from imidazole to acetamide, modeling either an asparagine or a glutamine residue, has a small effect on the reaction barrier. This might have implications for some of the enzymes belonging to subclasses B2 and B3. Replacing zinc with cadmium increases the reaction barrier 3 kcal/mol when forming the tetrahedral intermediate, destabilizes the tetrahedral intermediate by 5 kcal/mol, whereas the barrier for the following proton transfer decreases by 1 kcal/mol.

Together, all these results show that interesting clues about the reaction mechanism for metallo-β-lactamases can be obtained with theoretical methods. However, more calculations are needed including the surrounding protein before any certain conclusions can be reached. Such investigations are currently performed at our laboratories.

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