**NDM-1, the ultimate promiscuous enzyme: substrate recognition and catalytic mechanism**

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**ABSTRACT** The specter of a return to an era in which infectious disease looms as a significant threat to human health is not just hyperbole; there are serious concerns about the widespread overuse and misuse of antibiotics contributing to increased antibiotic resistance in pathogens. The recent discovery of a new enzyme, first identified in *Klebsiella pneumoniae* from a patient from New Delhi and denoted as NDM-1, represents an example of extreme promiscuity: It hydrolyzes and inactivates nearly all known β-lactam-based antibiotics with startling efficiency. NDM-1 can utilize different metal cofactors and seems to exploit an alternative mechanism based on the reaction conditions. Here we report the results of a combined experimental and theoretical study that examines the substrate, metal binding, and catalytic mechanism of the enzyme. We utilize structures obtained through X-ray crystallography, biochemical assays, and numerical simulation to construct a model of the enzyme catalytic pathway. The NDM-1 enzyme interacts with the substrate solely through zinc, or other metals, bound in the active site, explaining the observed lack of specificity against a broad range of β-lactam antibiotic agents. The zinc ions also serve to activate a water molecule that hydrolyzes the β-lactam ring through a proton shuttle.—Kim, Y., Cunningham, M. A.; Mire, J., Tesar, C., Sacchettini, J., Joachimiak, A. NDM-1, the ultimate promiscuous enzyme: substrate recognition and catalytic mechanism. *FASEB J.* 27, 1917–1927 (2013). www.fasebj.org

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**Bacterial resistance to antibiotic agents is now recognized as an ancient evolutionary adaptation and not solely provoked by modern therapeutic practice (1). Nevertheless, the spread of pathogenic strains resistant to multiple drugs is a serious issue, and human activities strongly contribute to it, although the situation is not quite as desperate as depicted in the popular press (2). Microorganisms must continuously cope with microbial warfare and have developed a wide range of mechanisms to achieve antibiotic resistance (3). One of the most effective mechanisms employed by pathogens is the expression of enzymes known as β-lactamases that can hydrolyze the 4-membered β-lactam ring found in commonly used antibiotics like penams, cephalosporins, and carbapenems. These antimicrobials target bacterial cell wall synthesis. The β-lactamase enzymes are divided into 4 main classes (classes A–D) based on sequence homology (4). Class B enzymes utilize 1 or 2 zinc ions to catalyze the hydrolysis; the other 3 classes rely on an active-site serine residue (4).**

A recent addition to the list of class B enzymes is the New Delhi metallo-β-lactamase (NDM-1; refs. 5–7) from *Klebsiella pneumoniae* that has demonstrated an ability to proficiently hydrolyze nearly all β-lactam antibiotics (8). A number of X-ray crystal structures of the apo enzyme and the enzyme in complex with a variety of substrates and with varying occupation of the zinc binding sites have been reported (9–13). In addition, mechanistic studies have been conducted (14) in an attempt to understand how the enzyme achieves its catalytic capacity. A general framework for the activity of metallo-β-lactamases has been laid out previously (15). Briefly, it is presumed that the carbonyl carbon atom of the β-lactam ring is attacked by a hydroxide ion, forming an intermediate state that is stabilized by the zinc ions (16). Ring cleavage requires subsequent protonation of the nitrogen atom. However, at issue are the specifics of mechanistic and energetic details defining how precisely the enzyme environment facilitates the reaction.

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Theoretical calculations have been carried out for the monoozinc enzyme, CphA (17), with results that generally agree with the experimental data for that system (18, 19). Recent calculations in the dinuclear zinc L1 enzyme from \textit{Stenotrophomonas maltophilia} have also been reported (20) and offer insights into the catalytic mechanism, but these calculations do not provide evidence for protonation of the nitrogen in the final state of the reaction and presume that an active-site aspartic acid plays the key role of a general base in the reaction. This is contrary to mutational studies (18) of the CphA enzyme in which an aspartic acid to asparagine mutation resulted in a 100-fold reduction in catalytic efficiency but did not stop the reaction entirely, suggesting that the aspartic acid is important for zinc binding but does not serve as a general base (15).

Here, we report the results of a combined experimental/theoretical examination of the NDM-1 enzyme designed to address a number of outstanding issues regarding substrate recognition and the hydrolytic pathway. The experimental data suggest a multistep process (14), but the available crystal structures represent either the initial step of the reaction, prior to substrate binding or the (relaxed) product state subsequent to the catalytic reaction. As a result, definition of the Michaelis complex and the reaction pathway remain contentious subjects. In addition, different distances between zinc ions observed in several crystal structures and solution measurements suggest a possibility for alternative mechanisms (9–14). To resolve these issues, we have determined the crystal structure of NDM-1 with 2 zinc ions and hydrolyzed ampicillin at atomic resolution (1.05 Å, the highest resolution of an NDM-1 structure to date); the crystal structure with 2 manganese ions and hydrolyzed ampicillin at 1.50 Å resolution; the crystal structure with 2 cadmium ions and a mixture of hydrolyzed and unhydrolyzed ampicillin at 1.50 Å resolution; and the lower-resolution crystal structure with 2 cadmium ions and faropenem. The structure with ampicillin and cadmium corresponds to the Michaelis complex. We have utilized these structures as starting points for our numerical studies and employed molecular dynamics calculations to assess structural aspects of substrate binding. Subsequent hybrid quantum mechanics/molecular mechanics (QM/MM) calculations were performed to determine the energy surfaces along the putative reaction pathways. We have also determined the pH dependence and metal requirements of the NDM-1 catalyzed reaction.

**MATERIALS AND METHODS**

**Protein cloning, expression, and purification**

The Δ30 construct of the NDM-1 gene from \textit{K. pneumoniae} subsp. \textit{pneumoniae} MGH 78578 was cloned into the pMCSG7 vector using a modified LIC protocol as described by Kim \textit{et al} (21). This process generated an expression clone producing a fusion protein with an N-terminal His\textsubscript{6} tag and a tobacco etch virus (TEV) protease site. The expressed protein was purified using standard procedures on an ÄKTAexpress automated purification system (GE Healthcare Life Sciences, Piscataway, NY, USA) as described previously (22). The concentration of the purified protein was determined utilizing an ND-1000 spectrophotometer system (NanoDrop Technologies, Wilmington, DE, USA). The fusion tag was then removed by adding recombinant TEV protease at a ratio of \( \sim 1:75 \) (mg) and incubated for 48 h at 4°C. The cleaved protein was then separated using Ni-NTA affinity chromatography and the ÄKTAexpress system. The purified protein solution was dialyzed in a crystallization buffer [20 mM HEPES, pH 8.0; 250 mM NaCl; and 2 mM dithiothreitol (DTT)] and concentrated using an Amicon Ultra centrifugal filter device with a nominal molecular weight limit of 3000 (Millipore, Bedford, MA, USA).

**Protein crystallization**

The protein was crystallized using sitting-drop vapor diffusion at 16 and 4°C in 96-well CrystalQuick plates (Greiner Bio-One North America, Inc., Monroe, NC, USA). A 400-μl droplet of protein (40–80 mg/ml) was mixed with a 400-μl droplet of crystallization reagent and allowed to equilibrate over 135 μl of crystallization reagent. Nanopipetting was performed using the Mosquito nanoject liquid handling system (TTP LabTech, Cambridge, MA, USA). Several commercially available crystallization screens were used, including MCSG-1–4 (Microlytic Inc. Burlington, MA, USA) and Index (Hampton Research, Aliso Viejo, CA, USA). The plates were then incubated at 16 and 4°C in a RoboIncubator automated plate storage system (Rigaku, The Woodlands, TX, USA). Automated crystal visualization was utilized in locating several crystals (CrystalTrak; Rigaku). The best crystals of the Δ30 construct protein preincubated with 80 mg/ml of ampicillin (Δ30-amp-Zn) appeared at 16°C under the conditions of 0.2 M ammonium sulfate, 0.1 M Bis-Tris (pH 5.5), and 25% PEG3350, which corresponds to condition A6 from the MCGS-1 screen. The Δ30 protein preincubated with 80 mg/ml of faropenem and mixed with 10 mM cadmium chloride (Δ30-far-Cd) was crystallized in the same conditions, except for an additional 5 mM CdCl\(_2\). The Δ30, ampicillin, manganese (Δ30-amp-Mn) and cadmium (Δ30-amp-Cd) crystals were produced from the MCGS-1 screen condition B11, containing 0.2 M sodium chloride, 0.1 M HEPES (pH 7.5), and 25% (w/v) PEG3350, plus 5 mM MnCl\(_2\). The cocryystals of Δ30, ampicillin, and cadmium (Δ30-amp-Cd) were obtained from the MCGS-1 screen condition B11, containing 0.2 M lithium sulfate, 0.1 M Bis-Tris-HCI (pH 6.5), and 25% (w/v) PEG3350, plus 10 mM CdCl\(_2\). These crystals were cryoprotected and flash-cooled in liquid nitrogen before they were analyzed in the synchrotron X-ray beam at the Structural Biology Center (SBC), sector 19-ID at the Advanced Photon Source (APS; Argonne National Laboratory). The proteins with ampicillin and zinc, manganese, or cadmium were crystallized in the same space group (P2\(_1\)\_2\(_1\)\_2\(_1\)) with similar cell dimensions of \( a = 39.1–39.2, b = 78.6–79.2, c = 133.7–134.5 \) Å, although they were grown under two different conditions. However, Δ30-far-Cd crystallized in a different space group (C2) with cell dimensions of \( a = 145.63, b = 39.32, c = 75.37 \) Å and \( \gamma = 99.96^\circ \).

**Data collection**

All diffraction data were collected at 100 K at the 19-ID beamline of the SBC at the APS, using the ADSC Q315r detector. The high-resolution data to 1.05 Å resolution for the cocryystal of Δ30-amp-Zn were collected at 0.5166 Å with the detector distance of 200 mm from a rectangular crystal.
(0.1×0.06×0.03 mm) by exposing for 20 s/frame of 1° rotation on ω over 150° in 2 wedges (100 and 50°) from 2 different places of the same crystal. For the Δ30-amp-Mn crystal, the data at 0.9793 to 1.5 Å were collected from a single protein crystal (0.1×0.04×0.05 mm). The crystal was exposed for 3 s/1° rotation of ω with a crystal-to-detector distance of 280 mm. The data were obtained by scanning 160° on ω. The Δ30-far-Cd and Δ30-amp-Cd data were collected similarly at 0.9793 Å by exposing a crystal for 3 s/1° rotation frame over 200° on ω up to 2.5 Å for Δ30-far-Cd and by exposing a crystal for 3 s/1° rotation frame over 170° on ω for Δ30-amp-Cd. The detailed data collection statistics are shown in Table 1.

### Validation and deposition

The stereochemistry of the structures was checked with PROCHECK (25) and a Ramachandran plot. The main-chain torsion angles for nearly all residues are in allowed regions: for Δ30-amp-Zn, 98.7% of all residues in favored region with 1 outlier; for Δ30-amp-Mn, 98.7% with 2 outliers; for Δ30-amp-Cd, 98.9% with 3 outliers; for Δ30-far-Cd, 97.6% with 3 outliers.

### Molecular dynamics

Molecular dynamics simulations were performed with the code NAMD, (26) developed by the Theoretical and Computational Biophysics Group at the Beckman Institute for Advanced Science and Technology (University of Illinois at Urbana-Champaign, Urbana-Champaign, IL, USA). The CHARMM 27 force field (27) parameters were used. Parameters for the ampicillin and imipenem substrates were developed using the standard methodology. Analysis of dynamics trajectories and preparation of figures was conducted with the program VMD (28). Initial coordinates for protein atoms were obtained from the crystal structures. Missing atom coordinates (hydrogens) were defined with the PSFGEN module of VMD, which was also used to solvate the protein and neutralize the total charge of the simulation model. The solvation box was extended for 10 Å beyond the protein. With a 12-Å cutoff for electrostatic interactions, the box dimensions ensured a “diffuse” simulation model, in which no protein atoms interacted directly with protein atoms in the periodic images. Electrostatic interactions were computed using a smooth particle-mesh Ewald method (29), with a grid

### Table 1. Data collection, phasing, and refinement statistics

| Statistic              | Δ30-amp-Zn | Δ30-amp-Mn | Δ30-amp-Cd | Δ30-far-Cd |
|------------------------|------------|------------|------------|------------|
| Space group            | P2₁2₁2₁    | P2₁2₁2₁    | P2₁2₁2₁    | C2         |
| Cell dimensions        |            |            |            |            |
| a, b, c (Å)            | 39.19, 79.17, 134.47 | 39.10, 78.61, 133.71 | 38.99, 78.70, 134.38 | 145.63, 39.32, 75.37 |
| β (deg)                | 99.96      | 99.96      | 99.96      | 99.96      |
| Resolution (Å)         | 1.05       | 1.50       | 1.50       | 2.0        |
| Reflections (n)        | 185,255 (5746) | 67,113 (3259) | 66,056 (3509) | 28,649 (1438) |
| R_{merge} or R_{merge} | 0.089 (0.871) | 0.085 (0.664) | 0.096 (0.782) | 0.103 (0.629) |
| I/σI                   | 6.4 (2.3)  | 9.5 (2.7)  | 9.8 (2.1)  | 8.7 (2.3)  |
| Completeness (%)       | 94.9 (88.9) | 100 (100)  | 98.2 (95.5) | 99.5 (100) |
| Redundancy             | 5.5 (5.7)  | 7.8 (6.9)  | 6.2 (4.1)  | 3.9 (3.5)  |
| Refinement             |            |            |            |            |
| Resolution (Å)         | 1.05       | 1.50       | 1.50       | 2.0        |
| No. reflections        | 184,744    | 67,022     | 65,844     | 28,604     |
| R_{work}/R_{free}      | 0.133/0.157 | 0.147/0.168 | 0.137/0.149 | 0.204/0.251 |
| Residues (n)           | Protein 485 | 482        | 478        | 469        |
|                        | Ligand/ion 12 | 12        | 10         | 10         |
|                        | Water 612    | 469        | 469        | 136        |
| B factors              | 11.9       | 21.9       | 18.9       | 45.2       |
|                        | Protein 10.3 | 20.5       | 14.8       | 45.1       |
|                        | Ligand/ion 14.3 | 27.5       | 22.9       | 60.1       |
|                        | Water 21.7  | 32.5       | 30.7       | 39.0       |
| R.m.s. deviations      | Bond lengths (Å) 0.017 | 0.006     | 0.011     | 0.009     |
|                        | Bond angles (deg) 1.808 | 1.157     | 1.581     | 1.739     |
| Mc-Me distances        | 4.60/4.60  | 4.48/4.48  | 3.72/3.30  | 3.53/4.29  | 3.48/3.46  |
| pH                     | 5.5        | 7.5        | 6.5        | 5.5        |
| PDB ID                 | 4HL2       | 4H0D       | 4HL1       | 4HKY       |

Values in parentheses are for highest-resolution shell.
size of ~1-Å spacing. Simulations began with a small amount of minimization (~1000 steps) and then utilized typically 100,000 steps of NVT dynamics and 100,000 steps of NPT dynamics run with 1-fs time steps to equilibrate the system. Constant temperature was maintained by a Langevin method (30), and constant pressure conditions were enforced through a modified version of the Langevin piston (31) and Hoover (32, 33) methods. Typical production runs were of 10 ns duration and were conducted using NPT dynamics with 2-fs time steps, recording coordinate information at 1-ps intervals. Simulations were conducted for the dimeric conformation of the protein, as observed in the crystal structure. There were ~50,000 atoms in the simulations, in a box of nominal dimensions, 65 × 68 × 102 Å.

QM/MM studies

To study energetics along the proposed reaction pathway, we performed QM/MM calculations with the program NWChem (34). We utilized a nudged elastic band (NEB) method (35) implemented recently. Initial coordinates for reactant states were taken from snapshots of the dynamics trajectories in which the attacking water molecule was positioned in what we would describe as a near attack conformation (36). The quantum partitions for all simulations included the side chains of residues His120, His122, Asp124, His189, Cys208, and His250. Also treated quantum mechanically were the penem moiety of the imipenem substrate (excluding the R1 and R2 substituents), the zinc or cadmium ions, the attacking water, and additional waters that were coordinated to the zinc or cadmium ions. Atoms beyond 15 Å from the target carbon atom of the substrate were frozen in place, and only atoms within that spherical region were allowed to move. The reactant state was defined by optimizing the original model using the density functional method B3LYP (37) and a 6–31+ + G* basis set for all atoms except zinc and cadmium and a 6–31G*+(2s,2p) basis set for the zinc ions and a def2-tzvp basis set for the cadmium ions. The def2 basis set includes an effective core potential (ECP) for 28 electrons. An initial estimate of the reaction pathway was constructed by optimizing intermediate structures subject to constraints. For example, the distance between the water molecule oxygen atom and the substrate carbonyl carbon atom would be systematically reduced from its original value of ~3 Å to a final value of 1.4 Å. The product-state geometry was then obtained by removing all constraints and reoptimizing the geometry, starting from the last step along the pathway. The pathways produced utilized between 10 and 15 intermediate structures to represent the initial pathway guess.

The initial pathways were subjected to further NEB optimizations to define an optimal QM pathway. Free energy calculations were then conducted for some pathways, using the (static) reference geometries of the QM partition atoms and allowing the MM partition atoms to move dynamically. Typical simulations utilized 30 ps of dynamics, and the free energy values reported represent the average values obtained by neglecting the initial (equilibration) 10 ps of the trajectories. Trajectories were examined visually for stability and sd of all results were <2 kJ/mol.

Enzymatic studies

pH dependence of NDM-1 activity was assayed at 25°C in a reaction buffer containing 50 mM Bis-Tris (pH 5.5, 6.5, 7.5) or 50 mM Tris (pH 8.5), 100 mM NaCl, and 50 μM ZnCl2, varying concentrations of Centa substrate (EMD Biosciences, San Diego, CA, USA), and 2 nM NDM-1. Steady-state hydrolysis of the β-lactam ring by NDM-1 was monitored by increase in λmax: 405 nm during the linear portion of the reaction initial velocity. The extinction coefficient of Centa was experimentally determined (ε=1767 M−1 cm−1). The rate of spontaneous hydrolysis of the β-lactam ring was determined in absence of enzyme at each pH and was subtracted from the initial velocities determined with NDM-1. Metal dependence of NDM-1 activity was assayed at 25°C using ampicillin as a substrate in reaction buffer containing 50 mM Tris (pH 6.5), 100 mM NaCl, and varying concentrations of ZnCl2 and CdCl2. Hydrolysis of the β-lactam ring by NDM-1 was monitored by decrease of the lactam chromophore λmax: 235 nm (ε = 900 M−1 cm−1) during the linear portion of the reaction initial velocity. Kinetic parameters were determined by plotting the initial velocities against substrate concentration and curve fitting with the Michaelis-Menten equation using KaleidaGraph 4.0 (Synergy Software, Reading, PA, USA).

RESULTS

Active site organization

The NDM-1 active site contains 2 zinc-binding sites. One notable discrepancy among the experimental crystal structures is the observed distance between the zinc ions, which ranges on average from 3.5 to 4.6 Å (Supplemental Fig. S1 and refs. 9–14). It was shown earlier that the zinc ion (Zn1 site) coordinated by 3 histidine residues is occupied before the second zinc ion (Zn2 site) coordinated by Asp124, Cys208, and His250. It appears that both zinc ions have the flexibility to move considerably within the active site (11). All of the structures confirm electron density between the zinc ions that is interpreted variously as a water molecule or hydroxide ion. We have conducted a number of molecular dynamics simulations with and without substrates for typical times of 10–15 ns duration. In the simulations, the site between the zinc ions was populated with alternatively, a water molecule, a hydroxide ion, or a chloride ion. For the cases where the site was occupied by water, we observed the Zn-Zn distance to be 5.6 Å; for Cl−, the distance is 4.6 Å; and for OH−, the distance is 3.6 Å. Averaged over several nanoseconds of simulation time, the standard deviations are in the range of 0.10–0.15 Å and are largely independent of the presence or absence of the substrate (imipenem). The shortened distances observed in our simulations with ions present between the two zinc ions reflect the strong electrostatic interactions between the positively charged zinc ions and the negatively charged hydroxide or chloride ion.

In our atomic resolution 1.05-Å structure of the NDM-1 protein, depicted in Fig. 1 (similar but higher-resolution structure to PDB 3Q6X; ref. 9), in which a hydrolyzed ampicillin substrate is present, the observed Zn-Zn distance is 4.6 Å, suggesting that the active site is partially occupied by a water molecule and partially by a hydroxide ion (electron density is consistent with both). In one of the NDM-1 structures published recently (ref. 10; PDB 3SPU), Zn-Zn distances are substantially shorter (3.8 Å) and consistent with the presence of a bridging hydroxide ion. These crystals
were obtained at a high pH. Interestingly, our atomic resolution structure with ampicillin was crystallized at a pH of 6.5, and the crystals leading to the 3SPU structure were obtained at a pH of 7.5. Similarly, EXAFS solution scattering data (14), suggesting a 3.38-Å spacing between the zinc ions, were obtained at a higher pH (pH 7.6). The pH dependence of the zinc spacing from 21 high-resolution models is illustrated in Supplemental Fig. S1. We suggest that the different experimentally observed zinc distances reflect the pH conditions of crystallization: occupation by a water molecule at low pH and a hydroxide ion at higher pH.

*K. pneumoniae* is a gram-negative bacterium, and it belongs to the Enterobacteriaceae family that can grow in a variety of environments in a pH range of 5 to 8. Its intracellular pH has not been measured, but we can expect it to be similar to other bacteria in this family. Intracellular pH for *Escherichia coli* is very well established using several methods, and its value (7.6 ± 0.1) is quite independent of the external medium pH (38, 39). The pH of the periplasm, where NDM-1 resides, is more variable, more closely reflecting the pH of the extracellular environment. Assuming a similar intracellular pH for *K. pneumoniae*, we anticipate that the active site of NDM-1 is populated partly by a water molecule and partly by a hydroxide ion. This finding is consistent with the observation that the distance between zinc ions at a pH range of 6.5 to 7.5 varies between 3.9 and 3.6 Å, respectively.

**Promiscuity of substrate recognition**

The active site in NDM-1 is a narrow slot defined by the two mobile loops, ASL1 and ASL4 (9), which we illustrate in Fig. 2A. At the bottom of the slot, 2 zinc ions are each coordinated by 3 protein side chains: His120, His122, and His189 for Zn1, and Asp124, Cys208, and His250 for Zn2, as depicted in Fig. 1. The zinc ions held by the protein side chains appear to provide the essential capability to recognize β-lactam substrates and define what we term the catalytic core of the active site. The β-lactam substrates that are hydrolyzed by NDM-1 all possess carboxyl and carbonyl groups that are spaced by their similar, roughly planar core geometries. Oxygen atoms from these groups coordinate with the zinc ions to form the principal substrate-recognition unit of the enzyme. The distance between zinc atoms (4.1 Å; an average from available crystal structures; Supplemental Fig. S1) matches well with the distance range of the β-lactam carbonyl and carboxyl (3.45–4.42 Å) oxygen atoms. Other potential substrates with a nonplanar core are selected against by the action of the slot narrowing. The various substitutions to the R1 and R2 moieties extend into larger, open, and solvent-filled spaces on the protein surface (9). Crystal structures with ligands and modeling show that R1 and R2 generally do not interact with the protein directly, thus explaining the enzyme’s capacity for hydrolyzing a broad range of substrates, all contain-
conducting a β-lactam core. These observations are also supported by the crystal structure of NDM-1 with ampicillin and 2 manganese ions (Δ30-amp-Mn). This structure obtained at pH 7.5 shows a metal distance of 4.5 Å and a completely hydrolyzed ampicillin. Similarly, structures with ampicillin, faropenem, and 2 cadmium ions show partly disordered ligands with exception of the β-lactam core. Our data clearly suggests that NDM-1 can substitute zinc with manganese, or cadmium, as was reported earlier (40), and bind substrates using different metals in the catalytic core.

In our molecular dynamics simulations with imipenem and ampicillin bound to NDM-1, we found persistent interactions only between the β-lactam carbonyl and carboxyl oxygen atoms of the substrate and the zinc ions (Fig. 2). The binding cleft in NDM-1 is lined with nominally hydrophobic residues, so no specific hydrogen-bonding interactions occurred between the β-lactam core and the protein. The $R_1$ and $R_2$ substituents of imipenem and ampicillin extend into larger cavities and are primarily solvated by water. For imipenem, we observed a hydrogen bond formed between Asp124 and the hydroxyethyl group ($R_2$) that can persist for hundreds of picoseconds, but no long-lived bonds were observed between the protein and the $R_2$ moiety. No such interactions were observed in simulations with ampicillin, where the $R_2$ moiety made sporadic interactions with the protein. $R_1$ and $R_2$ substituents are partly disordered in the crystal structures with unhydrolyzed β-lactams (see below).

What we have also observed in our simulations, as illustrated in Fig. 2B, is that the cavity can widen due to the relative motion of the mobile ASL1 and ASL4 loops. When this happens, although this does not necessarily always occur in the time scales accessible in our simulations, a monolayer of water molecules can intrude between the substrate and the protein. The hydrogen-bonding network of the bulk water is disrupted within the monolayer, with hydrogen bonds forming preferentially with the ligand and not the (hydrophobic) protein residues. We believe that the motion of the active site loops provides a mechanical filter of sorts that selects substrates with a core geometry that is nearly planar.

**Reaction pathways**

Previous discussions of the enzyme mechanism have focused on the oriented water molecule (hydroxide ion) identified between the two zinc ions, where it is presumed that this molecule will serve as the nucleophile that initiates the hydrolytic reaction. We have investigated several possible reaction pathways by identifying conformations from the molecular dynamics simulations where molecules were aligned appropriately for an attack of the C7 carbon atom of the substrate to be productive. That is, we initiated the attacks from what would be described as near-attack conformations (36). The QM/MM calculations were conducted by computing optimized structures along a putative reaction pathway, defined by placing constraints on key distances like that between the C7 carbon atom of the substrate and the $O_\text{w}$ oxygen atom of the attacking water (hydroxide) and changing the constraints in a stepwise fashion. This initial guess for the reaction pathway was then optimized using a nudged elastic band method, (35) in which no atoms were constrained, producing an estimate of the potential energy surface along the path. Subsequent dynamics calculations on the MM partition allowed us to estimate the free energy along the pathway; given the computational requirements for such calculations, free energy calculations were only performed for the most favorable pathways. In our methodology, the estimate of the free energy is dominated by the quantum component, with the MM component providing corrections typically of the order of 20–50 kJ/mol. In our experience, the MM component will not lower a 200 kJ/mol barrier to a 100 kJ/mol barrier.

On analyzing all of the different reaction pathways, the subset of pathways studied in which the oriented water or hydroxide ion served as the nucleophile was energetically unfavorable. For the attack of an hydroxide ion, we observed a potential energy surface that rises monotonically to 170 kJ/mol at the intermediate state (see Supplemental Fig. S2 and Supplemental Movie S1). Such a state would not be stable, so we have also considered attacks using an initial oriented water molecule and a pathway that utilized Asp124 as a general base (Supplemental Fig. S3 and Supplemental Movie S1); a concerted attack in which the N4 nitrogen atom was directly protonated by the attacking water molecule (Supplemental Fig. S4 and Supplemental Movie S1); and a pathway in which a bulk water molecule served as a base (Supplemental Fig. S5 and Supplemental Movie S1). In each of these scenarios, we found barriers of 200 kJ/mol or more, suggesting that the oriented water molecule is not the nucleophile. Previous experimental results in the CphA system, which is a class B2 β-lactamase, have excluded Asp124 as a base. It is possible that the mechanisms in the two enzymes differ, but we believe the experimental findings lend credence to our computational modeling results, at least in terms of excluding Asp124 as the general base.

We have identified conformations from the molecular dynamics simulations in which the substrate was solvated by bulk water. We have also examined pathways in which the oriented water or hydroxide ion served as a general base and the nucleophilic water originated from the bulk solvent. In Fig. 3A–D, we depict the proposed mechanism of action for a pathway in which a bulk water molecule transfers a proton to the oriented hydroxide ion and then attacks the carbonyl group of the substrate (Supplemental Fig. S6 and Supplemental Movie S1). Formation of the intermediate (bead 6 in Supplemental Fig. S6) is the rate-limiting step of the reaction; subsequent proton translocations involving a second bulk water molecule result in protonation of the N4 nitrogen atom of the substrate and
ring cleavage. Subsequent steps along the pathway are energetically downhill.

Finally, we have also studied the pathway possibility where a water molecule occupies the site between the zinc ions. (Supplemental Fig. S7 and Supplemental Movie S1). In this case, the initial proton transfer to the oriented water results in protonation of the carboxyl group of the substrate. Subsequent proton translocations also result in cleavage of the N4-C7 bond. The observed barrier is \( \sim 80 \text{ kJ/mol} \). By comparison, in Fig. 3, we compare the relative energetics along the pathways involving bulk water serving as the nucleophile and one of the cases in which the oriented water served as the nucleophile. We observed a relatively large barrier for the latter pathway, typical of all the pathways in which the oriented water or hydroxide ion served as the nucleophile. These results suggest that the most probable reaction mechanism involves a bulk water molecule serving as the nucleophile, and either the oriented water molecule or hydroxide ion, serving as the general base. The mechanism for an oriented water molecule is depicted in Supplemental Fig. S8. Our proposed mechanism is consistent with an increased volume of the active site, high flexibility of NDM-1 and its chemical environment.

**pH and metal dependence of NDM-1 activity**

To gain further structural insights into the mechanism of binding and hydrolysis of β-lactam substrates by NDM-1, we sought to trap the Michaelis complex of NDM-1 with various β-lactam substrates. Several attempts were made by cocrystallization, as well as crystal soaking, but structures revealed only product bound in the active site when NDM-1 was liganded with zinc or manganese. To facilitate trapping of the Michaelis complex of NDM-1 with β-lactam substrates, we sought to identify conditions that would decrease the turnover number of NDM-1. We approached this from the perspective of pH and selection of divalent metal cation.

We have determined the turnover number of NDM-1 as a function of pH, and in the presence of varying concentrations of divalent cations zinc and cadmium. The results reveal a direct dependence of turnover number \( k_{\text{cat}} \) on pH (Fig. 4). As the pH is increased from 5.5 to 8.5, \( k_{\text{cat}} \) increased \( \sim 4 \)-fold from 26 ± 2 to 101 ± 5 s\(^{-1}\), respectively. The results are consistent with an increasing frequency of zinc-liganded hydroxide ion as the pH increased.

The dependence of \( k_{\text{cat}} \) on divalent cation concentration (Fig. 5) showed that in the presence of 1–200 μM zinc, the turnover number increased from \( \sim 23 \pm 3 \) to \( 110 \pm 4 \text{ s}^{-1} \), with a plateau between 100 and 200 μM zinc. In the presence of 1–200 μM cadmium, \( k_{\text{cat}} \) decreased from 6 ± 1 to 1 ± 1 s\(^{-1}\), with a plateau between 100 and 200 μM. Although the activity of NDM-1 was severely attenuated when liganded with cadmium, a basal level of hydrolysis was maintained, which is consistent with partially hydrolyzed faropenem and ampicillin molecules in the active site of cadmium liganded NDM-1 (see below).

**Structure of the Michaelis complex**

As shown in Fig. 4, cadmium ion is a very poor substituent for zinc, consistent with earlier reports that
substituting cadmium for zinc inhibits enzyme activity (40). We have obtained 2 crystal structures of the enzyme with cadmium: a high-resolution structure in complex with ampicillin (\textit{H9004}30-amp-Cd) and a lower resolution structure in complex with faropenem (\textit{H9004}30-far-Cd). Unlike the \textit{H9004}30-amp-Zn structure that shows a well-defined electron density for hydrolyzed ampicillin bound to the active site containing 2 Zn ions, only a partial electron density (2\(F_o-F_c\)) for the ligand was observed in the presence of cadmium ions. In the case of the high-resolution \textit{H9004}30-amp-Cd structure (Fig. 6), Cd in the primary metal site (Cd1; ref. 11) is split into 2 positions in both protein chains in the asymmetric unit, Cd1a and Cd1b, that are 1.01–1.18 Å apart and have 64–65 and 35–36% occupancies, respectively. Both metal ions are coordinated by the same set of interactions with active site residues. The second metal site (Cd2) maintains its usual position and shows two different distances between the two metal sites, 3.53–3.72 and 4.29–4.50 Å, respectively. The electron density for the ligand is somewhat different from both hydrolyzed ampicillin and unhydrolyzed ampicillin; while the 5-membered ring in the lactam moiety and its carboxyl group are well-defined, the 4-membered ring (unhydrolyzed) or carboxyl group (hydrolyzed) are partly disordered, and the phenyl peptide assumes a distinct conformation from that of a hydrolyzed ampicillin, albeit not well-defined (Fig. 6C). This electron density was interpreted as being partially occupied by unhydrolyzed and hydrolyzed ampicillin. This crystal structure indicates that on hydrolysis, one part of the ligand is very well ordered, and the other part undergoes conformational changes. The 5-membered ring of the \(\beta\)-lactam and the \(R_1\) moiety of the phenyl peptide show an ensemble of unhydrolyzed and hydrolyzed intermediates and appear as disordered regions in the ligand. We also noted that the position of the ligand in the \textit{H9004}30-amp-Cd structure is slightly different from that of the hydrolyzed ampicillin in the \textit{H9004}30-amp-Zn structure (Fig. 6A, B). Perhaps the action of active site narrowing and widening by ASL1 and ASL4 as the catalytic reaction progresses forces the ligand (substrate, intermediate, or product) to move about, rotation of a few degrees counterclockwise in this case, for a higher catalytic efficiency.

In the lower-resolution \textit{H9004}30-far-Cd structure, the density for the ligand is not as well defined as that of other structures of NDM1-hydrolyzed ampicillin complexes (3Q6X, 4H0D, 4HL2) and is only visible for the \(\beta\)-lactam moiety. Faropenem is smaller than ampicillin, but the electron density is different from what was predicted for hydrolyzed faropenem. We modeled the electron density as a mixture of hydrolyzed and unhydrolyzed faropenem; however, only a portion corresponding to the \(\beta\)-lactam moiety refined well, and it is difficult to resolve the two conformations. The metal-metal distance is significantly shortened (3.46–3.48 Å); no well-ordered oriented water molecule is present between the cadmium ions, and the Asp124 carboxyl group coordinates both cadmium ions (2.80–2.83 and 3.44–3.82 Å). With this rearrangement, the active site appears to be in a conformation that is not quite optimal for binding \(\beta\)-lactam antibiotics (faropenem) and hydrolyzing it. We also note that the furyl group (\(R_1\)) of the substrate is poorly resolved in the structure, consistent with our observations that the \(R_1\) and \(R_2\) moieties of ampicillin were only partially ordered in the other NDM1-hydrolyzed ampicillin structures.

Using the cadmium-bound structures observed experimentally as a starting model we have conducted a number of molecular dynamics simulations. From these simulations, we have obtained a structure in which the active site is organized in a conformation like the proton shuttle-starting configuration that was described...
for our zinc containing structures. The potential energy along a proposed proton shuttle pathway computed through QM/MM yields a high barrier of 200 kJ/mol. Results of the QM/MM simulations for cadmium are displayed in Supplemental Fig. S9 and Supplemental Movie S1. The high-energy barrier is consistent with experimental observations that the enzyme efficiency is greatly reduced when zinc is substituted by cadmium. In the structure depicted in Fig. 6, we interpret the density to indicate that the ampicillin is only partially hydrolyzed, suggesting that cadmium replacement does not completely disable the enzymatic activity of the protein and this is confirmed experimentally (Fig. 4). These results show that the dimetal ion core is essential for ligand binding and that very specific framework of that core is needed for efficient substrate hydrolysis.

**DISCUSSION**

Metallo-β-lactamases represent a family of enzymes capable of evolving multiple catalytic activities and accepting a wide range of substrates (3). Therefore, it is not very surprising that the NDM-1 represents an evolutionary step that can bind and hydrolyze a broad range of β-lactam-based antibiotics. The enzyme shows a high flexibility of loops forming the active site and, with exception of the β-lactam moiety, does not seem to make extensive interactions with the substrates, which is a characteristic of promiscuous enzymes (41–43). Recognition of the substrate is provided almost entirely by what we can call the catalytic core of the active site; the two zinc ions interact with the carboxyl and carbonyl oxygen atoms of the β-lactam. The presence of divalent metal cations seems to be essential for binding. The zinc ions can be replaced with other metals (Mn$^{2+}$, Cd$^{2+}$) that provide an equivalent β-lactam binding framework, but not necessarily the same catalytic potential as Zn$^{2+}$. Modest substrate specificity is provided by the mechanical action of the mobile loops, ASL1 and ASL4, which select planar core ligand structures.

When the mostly hydrophobic trench widens, a single layer of water molecules is admitted into the space between the substrate and protein, and it is these water molecules that are apparently recruited to hydrolyze the β-lactam ring. As the protein surface is predominantly hydrophobic, the water molecules preferentially coordinate with the substrate and catalytic core. The dinuclear zinc catalytic core is coordinated by 6 side chains that form 2 catalytic triads. Interestingly, the distance between the metal ions can vary considerably...
NDM-1 is liganded with Cd$_2^+$ and the shuttle pathway would encounter a high barrier when bound to systems, where no crystal structures are available from our simulations correspond closely with the liganded structures. We find the near-attack conformation with EXAFS data (14) that suggest a multistep reorganization of the active site is observed in Fig. 6, the Michaelis complex for the reaction. A significant role of specificity for substrate recognition, with exception of the β-lactam moiety, and can use multiple metal cofactors and switch catalytic mechanisms based on pH.

We believe that the cadmium structures represent the Michaelis complex for the reaction. A significant reorganization of the active site is observed in Fig. 6, and the shorter Cd-Cd distance does not admit an oriented water molecule as was observed in the zinc liganded structures. We find the near-attack conformations from our simulations correspond closely with the observed crystal structures with cadmium. We believe that this lends credibility to the Michaelis complexes that we identified from our simulations in the zinc-bound systems, where no crystal structures are available. Moreover, our simulations indicate that a proton shuttle pathway would encounter a high barrier when NDM-1 is liganded with Cd$^{2+}$, in agreement with our observation of partially hydrolyzed ampicillin in the crystal structure, and the known much lower enzyme activities. From these results we would predict a significant isotopic effect for the reaction.

Development of inhibitors for the NDM-1 enzyme has proven challenging. A structure of NDM-1 in complex with L-captopril (IUPAC (2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid) has been reported recently (13). In this structure, the sulfur atom binds between the two zinc ions, with the remainder of the molecule extending beyond the catalytic core of the active site. Our results suggest that other planar scaffolds that contain oxygen atoms to coordinate the zinc ions should also prove to be successful inhibitors.

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