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Structural and Mechanistic Insights into NDM-1 Catalyzed Hydrolysis of Cephalosporins

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Supporting Information

ABSTRACT: Cephalosporins constitute a large class of β-lactam antibiotics clinically used as antimicrobial drugs. New Dehli metallo-β-lactamase (NDM-1) poses a global threat to human health as it confers on bacterial pathogen resistance to almost all β-lactams, including penicillins, cephalosporins, and carbapenems. Here we report the first crystal structures of NDM-1 in complex with cefuroxime and cephalexin, as well as NMR spectra monitoring cefuroxime and cefixime hydrolysis catalyzed by NDM-1. Surprisingly, cephalosporoate intermediates were captured in both crystal structures determined at 1.3 and 2.0 Å. These results provide detailed information concerning the mechanism and pathways of cephalosporin hydrolysis. We also present the crystal structure and enzyme assays of a D124N mutant, which reveals that D124 most likely plays a more structural than catalytic role.

β-Lactam antibiotics such as penicillins, cephalosporins, and carbapenems constitute the largest class of clinically used antimicrobial agents worldwide.[1] All these compounds contain a four-membered β-lactam ring at the structure core that mimics a substrate of bacterial transpeptidases, but actually serves as a long-lived inhibitor of the enzyme and sterically hinders cross-linking of adjacent peptidoglycan chains during cell wall synthesis. Cephalosporins, which are structurally characterized by a β-lactam-fused six-membered dihydrothiazine ring, remain among the most often prescribed antibiotics for the treatment of bacterial infections. Tens of cephalosporin compounds containing diverse side chain groups at positions R1 and R2 (Figure 1) with different antimicrobial properties have been developed during past decades.

The emergence of resistant bacteria carrying β-lactamase genes is a growing clinical concern. Of the four classes of β-lactamases, class A, C and D enzymes use a catalytic serine to hydrolyze the β-lactam ring via an enzyme-acyl intermediate, while the class B metallo-β-lactamases (MBL) utilize bound zinc atoms in the active site to mediate catalysis without proceeding via a covalent intermediate.[2,3] As shown in Figure 1, cephalosporin hydrolysis begins with the amide bond cleavage upon the attack of a hydroxide ion to the carbonyl at position 8 (compound 1), with the generation of an anionic nitrogen intermediate (2). The hydrolyzed compound then undergoes

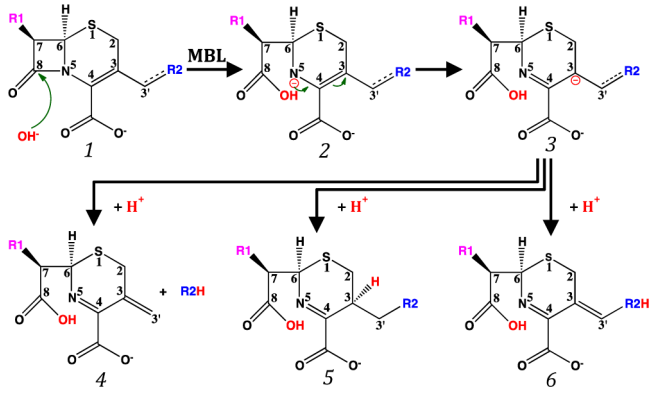

Figure 1. Cephalosporin hydrolysis mediated by MBLs.

tautomerization of the double bond in the dihydrothiazine ring from position 3−4 to 4−5, with a consequent anion at position 3 (3). The following reaction depends on the chemical nature of the R2 functional group and the bond valence between C-3′ and R2.[4,5] Cephalosporins containing good leaving R2 groups such as cephalothin,[4] ceftazidime,[6] and cefoxitin[7] yield a product with elimination of the R2 group and formation of new double bond at position 3−3′ (4).[8] Those without leaving groups, e.g., cephalexin, yield a cephalosporoate intermediate with a proton uptake at position 3 from the α-face (5)[8] due to steric constraints resulting from the folding of the aromatic ring and the dihydrothiazine ring.[5] The generated intermediate is subjected to several subsequent reactions according to the pH in aqueous media.[5] As for other cases where the R2 group is connected to the six-membered ring through a double bond, e.g., cefixime and cefdinir from the third-generation of cephalosporins, a detailed hydrolytic mechanism has not been reported, but generation of a product with a double bond shifted to 3−3′ (6) seems, in principle, reasonable.

The recent discovered NDM-1 poses a global threat to human health due to its ability to hydrolyze nearly all β-lactam antibiotics and its rapid worldwide dissemination. NDM-1 belongs to the B1 subclass of MBL that requires a dinuclear zinc center to catalyze hydrolysis of a variety of substrates. Since the first identification in 2009,[9] a number of crystal

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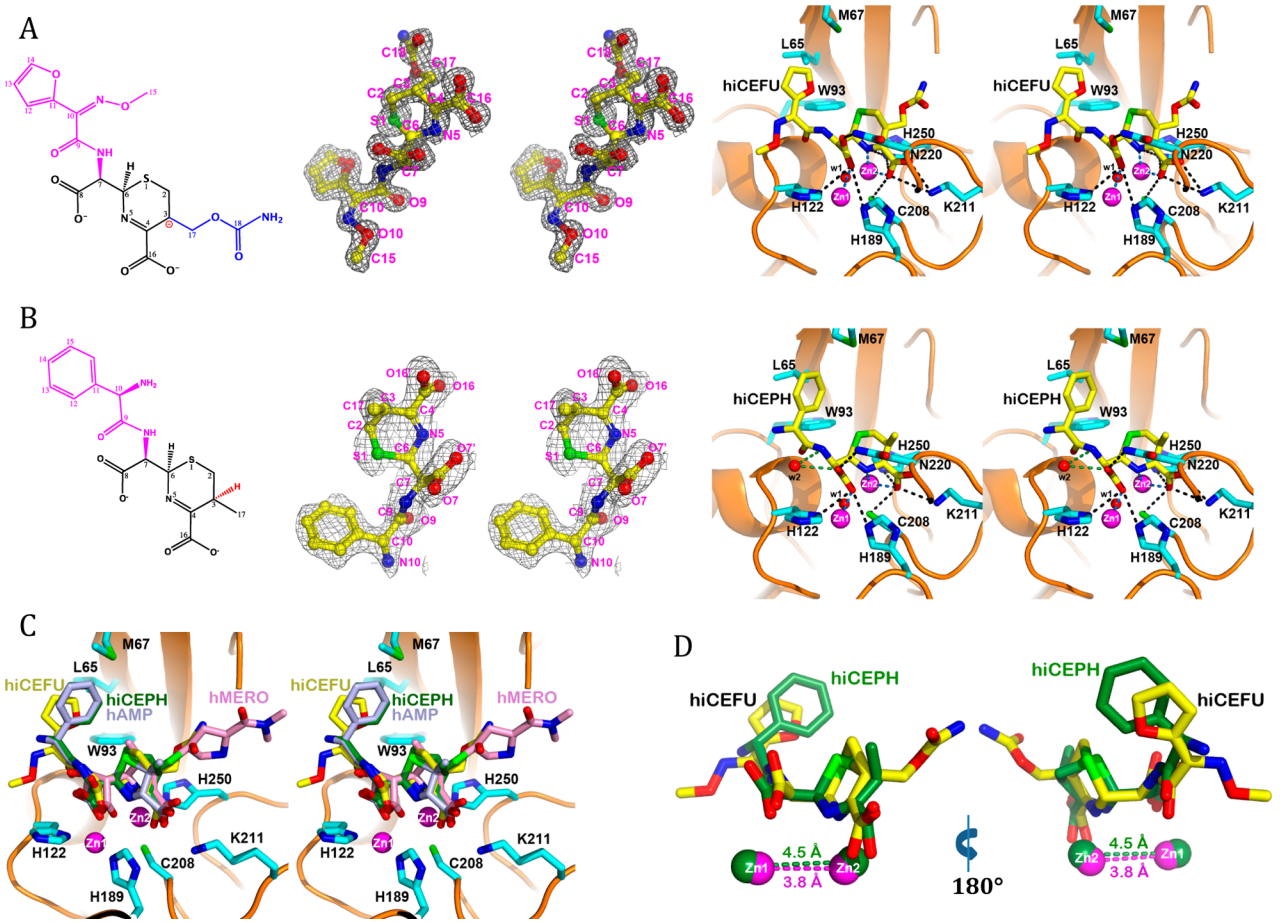

Figure 2. Crystal structures of NDM-1 in complex with hydrolytic intermediate of cefuroxime (hiCEFU) and cephalexin (hiCEPH). (A,B) Structures of hiCEFU and hiCEPH, respectively. The R1 and R2 groups are colored in pink and blue, respectively. The shown $2F_o - F_c$ density is contoured at 1.0σ. Coordination bonds and hydrogen bonds are denoted by dashed lines in blue and black, respectively. (C) Overlay of hiCEFU and hiCEPH with hydrolyzed ampicillin (hiAMP) and meropenem (hMERO). (D) hiCEFU and hiCEPH overlay close-up to zinc coordination. The zinc ions in hiCEFU and hiCEPH bound structures are represented by magenta and green spheres, respectively.

structures of the apoenzyme and complexes with substrates or potential inhibitors have been reported.[10−14] These structures together with modeling studies[15,16] reveal that NDM-1 contains a shallower and wider substrate binding groove, which more easily accommodates most β-lactam antibiotics than other B1MBLs. The antibiotics crystallized with NDM-1 include several penicillins[12−14] and meropenem,[13] but none of cephalosporins is in the list.

To gain structural and mechanistic insights into NDM-1 mediated hydrolysis of cephalosporins, we chose cephalexin, cefuroxime, and cefixime as our research targets in this study. Their molecular structures represent the three classes with different chemical context of R2 groups shown in Figure 1, and they are also representatives of the first, second, and third generation of cephalosporin antibiotics, respectively.

We first needed to know the exact structure of each hydrolyzed product. Cefuroxime contains a carbamoyl R2 group (Figures 2A and S1A, Supporting Information) structurally similar to the acetyl-CH_3 group in well-studied cephalothin,[4,8] suggesting it to be a good leaving group. In addition, the eliminated carbamate group is supposed, on theory, to decompose into CO_2 and NH_4^+ in aqueous media. Consistently, severe bubbling in the reaction solution by mixing excess of cefuroxime with NDM-1 was observed as expected. By contrast, no bubble at all was produced during hydrolysis of cephalexin or cefixime, which contain no group or a methylene at R2, respectively.

To demonstrate if cefuroxime was hydrolyzed in a similar pathway as cephalothin, the enzymatic reaction was monitored by ^{1}H NMR following the published protocols.[4,8] The spectroscopic data clearly showed a significant downfield shift in the protons bonded to C17 (C-3′ in Figure 1) from δ 4.76, 4.58, 2d to δ 5.57, 5.49, 2s, and upfield shifts of the signals assigned to protons bonded to C6 and C7 within 10 min after hydrolysis started (Figure S2 and Table S2, Supporting Information). The changes in spectrum definitely indicate (i) tautomerization of the double bond in the dihydrothiazine ring from position 3−4 to 4−5; and (ii) a time-dependent development of a methylene signal at C17, which means the hydrolyzed product of cefuroxime is chemically identical to that of cephalothin[4,8] (Figure S1A, Supporting Information).

The ^{1}H NMR spectra of cefixime acquired in the same time window as cefuroxime was characterized by (i) loss of the C18 methylene resonance (δ 5.33, 5.16, 2d) and (ii) development of a methyl signal (δ 1.72, 3d) (Figure S3, Supporting Information), both of which are clearly indicative of a double bond shift from position 17−18 to 3−17 with a proton uptake on C18 (Figure S1C, Supporting Information). This result confirmed our speculation (compound 6 in Figure 1).

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The crystal structures of complexes with cefuroxime and cephalexin were solved at 1.3 and 2.0 Å, respectively (Table S1, Supporting Information). In the cefuroxime bound structure, clear $F_o - F_c$ density was observed at the substrate-binding site close to the dinuclear zinc center, allowing us to confidently model the opened lactam core and the R1 group of cefuroxime. We at first expected to model a hydrolyzed product (compound 3 in Figure S1A, Supporting Information) at this site, as the reported structures of penicillin[12−14] and meropenem.[13] Density for the carbamoyl group, however, was surprisingly present and well connected with the rest of the model of the ligand, suggesting the enzymatic reaction suspended at a certain intermediate stage. Studies on CcrA, IMP-1, and BcII all suggested that protonation of the anionic nitrogen was the rate limiting step of β-lactam hydrolysis.[3,17] In this case, good planarity among C6, N5, C4, and C16 remained during refinement even with loose restraints applied on these atoms, indicative of a shifted double bond from position 3−4 to 4−5. Additionally, tetrahedral bonding to C3 clearly visible in density suggests the complete transition of hybridization orbit from sp^2 to sp^3 of this atom. Taken together with all these observations, we finally modeled an intermediate containing a negatively charged C3 with perfect density fitting and reasonable stereochemical configuration of itself and neighboring atoms in this structure (Figures 2A and S4A, Supporting Information).

Ligand modeling of cephalexin in the other complex structure was straightforward, as the conformation of protonated intermediate (compound 5 in Figure 1) was already known.[5] Same as the cefuroxime-bound structure, electron density of the ligand clearly reveals complete sp^3 hybridization of C3, suggestive of full protonation of cephalexin in this structure. Notably, the chirality of this atom differs from the hydrolysis intermediate of cefuroxime but remains consistent with that proposed from the NMR spectra[5] (Figures 2B and S4B, Supporting Information).

No cephalosporoate intermediate has ever been captured *in crystallo*, and neither have NDM-1 mediated hydrolytic intermediate of penicillins or carbapenem. The two structures obtained in the current study provide the first crystallographic evidence for presence of stable enzyme−intermediate complex during cephalosporin hydrolysis, which also agrees well with a presteady state kinetic study of NDM-1.[18] In particular, the snapshot of an anionic carbon intermediate of cefuroxime provides undeniable evidence for protonation being the rate-limiting step in the hydrolytic process and supports an argument that the C−N bond breaking is likely coincident with the double bond tautomerization in the dihydrothiazine ring.[3]

As for the protonation following hydrolysis, the source of proton was unclear. Structural and computational studies on penicillins or carbapenems have proposed that the proton from the newly formed carboxylate bound to C8,[12] an apical water molecule bound to Zn2,[19] or a bulk water entering the active site[14] may serve to protonate the nitrogen on the cleaved β-lactam ring. The capture of a protonated intermediate of cephalexin with a proton uptake from the α-face (Figures 2B and S4B, Supporting Information) seems favorable to the second possibility given the orientation of the dihydrothiazine ring and the Zn2−C3 distance of 4.0 Å.

Exactly like the reported structures with hydrolyzed penicillins and meropenem,[12−14] the cephalosporoate core of both intermediates form extensive interactions with the zinc ions and neighboring amino acids such as K211 and N220, whereas the R1 and R2 groups are relatively poorly contacted (Figures 2, S5, and S6, Supporting Information). The indole ring of W93 in all solved NDM-1 structures is oriented roughly perpendicular to the five- or six-membered ring juxtaposed to the cleaved amide bond. Given the presence of a sulfur atom and a double bond in the dihydrothiazine ring, good $\pi-\pi$ interaction is formed between W93 and the cephalosporoate core. This may partly account for higher affinity of the enzyme toward cefuroxime (K_m = 8 μM) or cephalexin (K_m = 5.6 μM) than ampicillin (K_m = 310 μM) or meropenem (K_m = 54 μM).[9,20]

Hydrolyzed ampicillin and meropenem were observed to be bound at the active site of NDM-1 in somewhat different positions.[12,13] Structure superimposition shows that the binding scheme of either cefuroxime or cephalexin gets convergent with ampicillin (Figure 2C). In particular, cephalexin perfectly overlays with ampicillin (Figure S7A, Supporting Information). Different from the C7 carboxylate in meropenem that intercalates between Zn1 and Zn2, the C8 carboxylate in our structures is positioned within hydrogen bond distance with Zn1 only (Figure S7B, Supporting Information).

Zn−Zn distances vary from 3.5 to 4.6 Å among solved NDM-1 structures.[10−14] A proposed pH-dependent mechanism suggests that the distance is probably governed by pH of crystallization conditions, i.e., higher pH leading to shorter distance.[14] Inconsistently, our crystals were obtained at pH 5.8 and 6.0, but the distances are 3.8 vs 4.5 Å in cefuroxime and cephalexin bound structures, respectively (Figure 2D). Structure comparison between them reveals considerable conformational difference on the cephalosporoate core. Apparently, the cephalexin intermediate adopts more extended conformation than cefuroxime, probably attributable to protonation of C3 in cephalexin. By comparison of crystallization conditions among published structures and ours, we reason that it is not pH but rather the conformation of the two carboxylates (C4 and C8) in bound antibiotics that determines the Zn−Zn distance. In this scenario, the two zinc ions are actually pushed away by the carboxylates of hydrolyzed penicillin or cephalexin, which adopts extended conformations of the β-lactam core (Figure S7A, Supporting Information). This hypothesis agrees well with the distances listed in Table S4, Supporting Information, except the structure with hydrolyzed meropenem, in which both Zn1 and Z2 are laterally dragged by the carboxylates occupying distinctive positions from other structures (Figure S7B, Supporting Information).

The catalytic role of D124 in NDM-1 and its counterpart in other MBLs has been extensively studied.[3] Several models have been proposed to suggest that the aspartic acid at this position may, apart from being a ligand of Zn2, function as a general base to stabilize the catalytic hydroxide between Zn1 and Zn2, or a proton receptor/donor for protonation of the anionic intermediate.[14,19] To further investigate its role in catalysis, we crystallized a D124N mutant. In the structure refined at 2.1 Å, both zinc ions are present in the active site, albeit much weaker density of Zn2 than the wild-type enzyme, indicating significantly lower occupancy of Zn2 in this structure (Figure S8A, Supporting Information). Correspondingly, the distance between Zn2 and N124 is almost doubled compared with that in the structures with cephalexin or cefuroxime bound or the apoenzyme (PDB code 3spu)[11] (Figure S8B,C, Supporting Information). Apparently, the affinity of this residue to Zn2 considerably decreases upon mutation. Notably, the hydroxide

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(w1), between Zn1 and Zn2 is well conserved in this structure by perfectly fitting into density (Figure S8A, Supporting Information), suggesting that substitution from D to N on this residue makes little contribution in orienting and stabilizing the catalytic hydroxide, which agrees well with a recent computational study denying D124 as a general base.[14]

Consistent with previous mutational analyses of this residue in other MBLs,[21,22] the D124N mutant remains detectable, albeit with much reduced activity on hydrolysis of cefuroxime, ampicillin, and meropenm when using a D124A mutant as the negative control (Figure S9, Supporting Information). Conclusively, all our experimental results argue for the possibility that D124 serves only as a ligand of zinc coordination.

Lack of efficient inhibitors to NDM-1 has become a big clinical concern. In this context, the structural and mechanistic understanding of NDM-1 mediated cephalosporin hydrolysis, including the chemical nature of reaction intermediates and knowledge of the rate-limiting steps, obtained in this study are important for the rational design of inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods of protein expression, purification, and crystallization, NMR spectrometry, structure determination and mutagenesis study; tables of X-ray and NMR data and summery of Zn−Zn distances; NMR spectra, figures showing structure representations, and enzyme assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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