Supporting Information

Structural and mechanistic insights into NDM-1 catalyzed hydrolysis of cephalosporins

Han Feng^{1‡}, Jingjin Ding^{1‡}, Deyu Zhu^{2‡}, Xuehui Liu¹, Xueyong Xu¹, Ying Zhang¹, Shanshan Zang¹, Da-Cheng Wang^{1*} and Wei Liu^{3*}

¹National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

²State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, Jinan 250100, China

³Institute of Immunology, The Third Military Medical University, Chongqing 400038, China

[‡] These authors contributed equally.

* Corresponding emails: dcwang@ibp.ac.cn and wei.liu.2005@gmail.com

MATERIALS AND METHODS

Protein expression and purification

The nucleotide sequence encoding NDM-1 from G29 to R270 was synthesized using GENEWIZ (Sangon Biotech, Shanghai, China) and inserted into a pET28a vector with an N-terminal His₆-tag. The recombinant protein was produced in *Escherichia coli* strain BL21(DE3) at 22°C with an incubation for 16~20 h after the induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Harvested cells were resuspended in lysis buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol and 1 mM ZnCl₂. Bacteria were lysed by sonication on ice at 200 W using 3 s pulses with 7 s intervals for 16.5 min before the removal of insoluble debris by centrifugation for 30 min at 13000 g and 4°C. The supernatant was immediately loaded onto a Ni²⁺-NTA chromatography column (Novagen), followed by column washing and elution with 250 mM imidazole added in the same buffer. The His₆-tag was subsequently removed with thrombin digestion for 12 h at 4°C prior to reloading the protein solution onto the same Ni-NTA column. NDM-1 without the His₆-tag eluted in the flow-through fraction was further purified by anion exchange (HiTrap Q 5 ml column, GE healthcare) and size exclusion (HiLoad superdex75 16/600 column, GE healthcare) chromatography. Purified protein were stored in 20 mM Tris pH 8.0, 150 mM NaCl and 2 mM DTT and were frozen at -80°C until further use.

In order to investigate the structural determinants of NDM-1-antibiotics recognition and binding, mutants of D124A and D124N were produced by means of site-directed mutagenesis. Expression and purification of these mutants were carried out using the same protocol as the wild-type protein.

NMR experiments on hydrolysis of cefuroxime and cefixime

The NMR samples of cefuroxime and cefixime were prepared by dissolving the antibiotic compounds in D₂O and DMSO (> 99.9% proton deuterated), respectively. The catalytic reaction started by mixing 50 nM NDM-1 and 500 μM cefuroxime/cefixime in 20 mM Tris, pH 8.0 and 150 mM NaCl with the solvent of

D₂O. The hydrolytic intermediate and product was subjected to NMR spectrometry at every 5 min after the reaction started to monitor the chemical change in the reactive solution. All NMR experiments were carried out on an Agilent 500 MHz spectrometer at room temperature. The assignment of the NMR signals were performed using the ¹H and ¹³C spectra and confirmed by checking with a set of 1D and 2D experiments including distortionless enhancement by the polarization transfer (DEPT) spectrum, ¹H-¹H correlation spectroscopy (COSY) spectrum, ¹H-¹³C heteronuclear single quantum correlation (HSQC) spectrum, ¹H-¹³C heteronuclear multiple bond correlation (HMBC) spectrum and ¹H-¹H rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY, mixing time 200 ms) spectrum. The ¹H and ¹³C spectra were collected with a spectrum width of 4496.4 Hz and 31250 Hz respectively. The spectrum widths of the 2D spectra were as follows, 2717.4 Hz for the ¹H dimension and 25141.4 Hz (HSQC) or 30165.9 Hz (HMBC) for the ¹³C dimension.

Crystallization and diffraction data collection

All crystallization trials were carried out using the hanging-drop vapor-diffusion method. Purified NDM-1 was concentrated to 60 mg/ml before mixing with cephalexin (11mg/ml) or cefuroxime (12.5 mg/ml) in 1:1 ratio. Both complexes were crystallized at almost identical conditions. Crystals of NDM-1-cephalexin were obtained in 28% (w/v) PEG3350, 0.1 M Bis-Tris, pH 5.8 and 0.2 M ammonium sulfate; crystals of NDM-1-cefuroxime were grown in 30% (w/v) PEG3350, 0.1M Bis-Tris pH 6.0 and 0.2 M lithium sulfate; the D124N mutant was crystallized in 27% (w/v) PEG3350, 0.1 M Tris, pH 7.5.

Crystals used for diffraction data collection were soaked in cryoprotectant (100% paraffin oil for the D124N mutant and reservoir solution supplemented with 15% glycerol for cefuroxime or cephalexin bound crystals) for 10 s before flash cooling in streams of liquid nitrogen. Data of cefuroxime bound crystals were collected at a wavelength of 0.97915 Å on beamline BL17U of Shanghai Synchrotron Radiation Facility (SSRF), China, while those for cephalexin bound and D124N crystals were collected using an in-house Rigaku R-Axis IV++ image plate and a Cu K_α radiation source (λ = 1.5418 Å). Crystals of NDM-1-cefuroxime, NDM-1-cephalexin and D124N diffracted to 1.3, 2.0 and 2.1 Å respectively. All X-ray data were indexed, integrated

and scaled using *iMosflm*¹ and *SCALA* from the *CCP4* program suite².

Structure determination and refinement

All structures were determined by the molecular replacement using the program *Phaser*³, with chain A of the ampicillin bound NDM-1 structure (PDB 3q6x)⁴ as a search model. After density modification and automatic model building using the *PHENIX* program suite⁵, the structure was refined using *phenix.refine*⁶ with several rounds of manual remodeling in *coot*⁷ between refinement cycles. Models of hydrolytic intermediate of cefuroxime and cephalexin were manually built using ligand builder in *coot*⁸. The resultant models were sent to the *PRODRG* server⁹ for generating the topological dictionary files, which were later manually modified for use in further refinement. After manually modeling of the ligands in an omit difference map ($F_{\text{obs}} - F_{\text{calc}}$) contoured at 3.0 σ , ten cycles of full model refinement were done. The final ligand modeling was manually checked by both electron density fitting and stereochemistry of its own and nearby atoms, while the overall structure was validated using *MolProbity*¹⁰. Statistics of data collection and structure refinement was summarized in Table S1. All figure showing structure representations were prepared using the molecular-visualization program *Pymol*¹¹.

Enzymatic activity assay

Hydrolysis of cefuroxime, ampicillin and meropenem catalyzed by NDM-1 was monitored on UV-Star 96 well microplate reader (Tecan Group Ltd.) at room temperature in 20 mM Tris, pH 8.0. The hydrolytic activity of both the wild-type enzyme and the D124N mutant was measured in terms of the decrease of UV absorption at the wavelength of 260 nm for cefuroxime, 235 nm for ampicillin, and 300 nm for meropenem. The D124A mutant with completely abolished activity¹² was used as the negative reference in our experiment, i.e. the relative enzymatic activity of wild-type NDM-1 and D124N was calculated by subtracting the UV absorption of D124A from the experimentally measured values.

Table S1. Summary of X-ray data collection and refinement

	hiCEFUS [§]	hiCEPH [¶]	D124N
Data collection			
Wavelength (Å)	0.97915	1.5418	1.5418
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P1
Cell parameters			
a (Å)	39.18	38.62	46.10
b (Å)	78.89	77.57	68.12
c (Å)	133.06	132.31	68.91
α (°)	90.00	90.00	87.62
β (°)	90.00	90.00	88.34
γ (°)	90.00	90.00	77.42
Resolution (Å)*	29.364-1.30	38.785-2.01	33.985-2.10
	(1.37-1.30)	(2.12-2.01)	(2.21-2.10)
Completeness (%)*	91.9 (94.0)	97.8 (99.8)	94.5 (97.6)
No. unique reflections*	94679	26996	45119
Redundancy*	7.1 (6.4)	6.1 (5.7)	4.0 (3.8)
R _{sym} (%)*	9.8 (28.4)	8.6 (25.3)	4.2 (21.1)
Average I/σ(I)*	5.7 (2.6)	6.6 (2.9)	14.2 (3.3)
Structure refinement			
R _{work} (%)	13.07	14.62	15.89
R _{free} (%)	15.67	19.26	19.22
RMSD of bond angles (deg.)	1.433	1.111	0.844
RMSD of bond lengths (Å)	0.011	0.009	0.004
Atoms in model			
Protein	3499	3598	6652
Ligand	90	86	-
Zinc ion	4	4	8
Water	649	438	588
Ramachandran plot			
Favored region (%)	99.13	97.91	98.63
Allowed region (%)	0.87	1.88	1.14
Outliers (%)	0.0	0.21	0.23

[§] X-ray data from a crystal containing the hydrolytic intermediate of cefuroxime

[¶] X-ray data from a crystal containing the hydrolytic intermediate of cephalexin

* Values in parentheses are statistics of the highest resolution shell

Table S2 ^1H NMR data for cefuroxime and hydrolyzed product

Cefuroxime				Hydrolyzed product			
<i>No.</i>	Chemical shift	Integration	Mult	<i>No.</i>	Chemical shift	Integration	Mult
2	3.32, 3.57	2	d, d	2	3.63, 3.41	2	d, d
6	5.12	1	d	6	water signal [*]	N/A	
7	5.72	1	d	7	5.58	1	d
12	6.79	1	b	12	6.91	1	b
13	6.53	1	b	13	6.49	1	b
14	7.59	1	b	14	7.55	1	b
15	3.90	3	s	15	3.85	3	s
17	4.76, 4.58	2	d, d	17	5.57, 5.49	2	s, s

^{*} Proton signal merged with water signal

Table S3 ^1H NMR data for cefixime and hydrolyzed product

Cefixime				Hydrolyzed product			
<i>No.</i>	Chemical shift	Integration	Mult	<i>No.</i>	Chemical shift	Integration	Mult
6	5.13	1	d	6	water signal [*]	N/A	
7	5.70	1	d	7	water signal [*]	N/A	
13	6.93	1	s	13	7.17	1	s
14	4.45	1	s	14	4.40	1	s
17	6.64	1	d, d	17	6.16	1	q
18	5.33, 5.16	2	d, d	18	1.72	3	d

^{*} Proton signal merged with water signal

Table S4 Zn-Zn distances in NDM-1 structures

Protein/Ligand	PDB ID	pH [§]	Zn-Zn distance [¶]	Reference
Apo enzyme	3SPU	8.5	3.82	King & Strynadka ¹³
D124N mutant		7.5	4.08	
Ampicillin	3Q6X	5.5	4.59	Zhang & Hao ⁴
Ampicillin	4HL2	5.5	4.60	Kim <i>et al.</i> ¹⁴
Methicillin	4EY2	5.5	4.58	King <i>et al.</i> ¹⁵
Oxacillin	4EYB	5.5	4.55	King <i>et al.</i> ¹⁵
Benzylpenicillin	4EYF	5.5	4.62	King <i>et al.</i> ¹⁵
Cephalexin		5.8	4.52	
Cefuroxime		6.0	3.82	
Meropenem	4EYL	6.5	3.97	King <i>et al.</i> ¹⁵
Ethylene glycol	4EXY	7.5	3.65	King <i>et al.</i> ¹⁵
Captopril	4EXS	7.5	3.54	King <i>et al.</i> ¹⁵

[§] pH values used in crystallization conditions

[¶] Zn-Zn distance averaged from all subunits in the asymmetric unit

References:

- (1) Battye, T.G., Kontogiannis, L., Johnson, O., Powell, H.R. and Leslie, A.G. *Acta crystallographica. Section D, Biological crystallography* **2011**, 67, 271-81
- (2) Winn, M.D., Ballard, C.C., Cowtan, K.D., Dodson, E.J., Emsley, P., Evans, P.R., Keegan, R.M., Krissinel, E.B., Leslie, A.G., McCoy, A., McNicholas, S.J., Murshudov, G.N., Pannu, N.S., Potterton, E.A., Powell, H.R., Read, R.J., Vagin, A. and Wilson, K.S. *Acta crystallographica. Section D, Biological crystallography* **2011**, 67, 235-42
- (3) Bunkoczi, G., Echols, N., McCoy, A.J., Oeffner, R.D., Adams, P.D. and Read, R.J. *Acta Crystallogr D Biol Crystallogr* **2013**, 69, 2276-86
- (4) Zhang, H. and Hao, Q. *FASEB J* **2011**, 25, 2574-82
- (5) Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., McCoy, A.J., Moriarty, N.W.,

- Oeffner, R., Read, R.J., Richardson, D.C., Richardson, J.S., Terwilliger, T.C. and Zwart, P.H. *Acta Crystallogr D Biol Crystallogr* **2010**, 66, 213-21
- (6) Afonine, P.V., Grosse-Kunstleve, R.W., Echols, N., Headd, J.J., Moriarty, N.W., Mustyakimov, M., Terwilliger, T.C., Urzhumtsev, A., Zwart, P.H. and Adams, P.D. *Acta Crystallogr D Biol Crystallogr* **2012**, 68, 352-67
- (7) Emsley, P., Lohkamp, B., Scott, W.G. and Cowtan, K. *Acta Crystallogr D Biol Crystallogr* **2010**, 66, 486-501
- (8) Debreczeni, J.E. and Emsley, P. *Acta Crystallogr D Biol Crystallogr* **2012**, 68, 425-30
- (9) Schuttelkopf, A.W. and van Aalten, D.M. *Acta Crystallogr D Biol Crystallogr* **2004**, 60, 1355-63
- (10) Chen, V.B., Arendall, W.B., 3rd, Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S. and Richardson, D.C. *Acta Crystallogr D Biol Crystallogr* **2010**, 66, 12-21
- (11) Schrodinger, LLC **2010**, The PyMOL Molecular Graphics System, Version 1.3r1
- (12) Liang, Z., Li, L., Wang, Y., Chen, L., Kong, X., Hong, Y., Lan, L., Zheng, M., Guang-Yang, C., Liu, H., Shen, X., Luo, C., Li, K.K., Chen, K. and Jiang, H. *PLoS One* **2011**, 6, e23606
- (13) King, D. and Strynadka, N. *Protein Sci* **2011**, 20, 1484-91
- (14) Kim, Y., Cunningham, M.A., Mire, J., Tesar, C., Sacchettini, J. and Joachimiak, A. *FASEB J* **2013**, 27, 1917-27
- (15) King, D.T., Worrall, L.J., Gruninger, R. and Strynadka, N.C. *J Am Chem Soc* **2012**, 134, 11362-5
- (16) Laskowski, R.A. and Swindells, M.B. *J Chem Inf Model* **2011**, 51, 2778-86

Figure S1. The hydrolysis process of cefuroxime (A), cephalixin (B) and cefixime (C) catalyzed by NDM-1. The hydrolytic intermediates of cefuroxime (A2) and cephalixin (B2) were captured in our crystals; the hydrolyzed products of cefuroxime (A3) and cefixime (C2) were observed in the NMR spectra shown in Figure S2 and S3.

Figure S2. ^1H NMR spectra of the hydrolytic reaction of cefuroxime in D_2O . (A), Spectra recorded at 0 (a), 5 (b) and 10 (c) min after addition of the enzyme. (B), Enlarged NMR spectra with ppm between 4.5 and 6.0.

A

B

Figure S4. Enlarged view of hydrolytic intermediate of cefuroxime (A) and cephalixin (B) structures shown in Fig. 2A and 2B, and with a different view angle. The shown $2F_o - F_c$ density is contoured at 1.0σ .

Figure S5. Ligand-protein and ligand-Zn²⁺ interactions between the hydrolytic intermediate of cefuroxime (Fig. 2A) in subunit A of NDM-1 schematically depicted using *LIGPLOT*¹⁶. Hydrogen bonds and amino acids involved in forming hydrophobic interactions with the bound ligand are shown as dashed lines and indented curves, respectively. The numbers labeled at dashed lines are the length of hydrogen bonds (Å).

A

B

Figure S7. Ligand overlay with close-up of Zn^{2+} coordination. (A), Superimposition of the hydrolytic intermediate of cephalexin (hiCEPH) (yellow) and hydrolyzed ampicillin (hAMP) (cyan); (B), Superimposition of the hydrolytic intermediate of cefuroxime (hiCEFU) (yellow) and hydrolyzed meropenem (hMER) (cyan).

Figure S8. Structure of the D124N mutant. (A), The $2F_o - F_c$ density contoured at 1.0σ of the active site. (B), Structure comparison among D124N (orange), the cephalalexin bound structure (green) and the apoenzyme (PDB 3spu)¹³ (cyan). The zinc ions in D124N, cephalalexin bound structure and the apoenzyme are shown as magenta, green and cyan spheres, respectively. (C), Structure comparison among D124, cefuroxime bound structure (green) and the apoenzyme (PDB 3spu)¹³ (cyan). The zinc ions in D124N, the cephalalexin bound structure and the apoenzyme are shown as magenta, green and cyan spheres, respectively.

Figure S9. Hydrolysis activity of NDM-1 measured by enzymatic assays upon the mutation of D124N towards cefuroxime (A), ampicillin (B) and meropenem (C). The relative enzymatic activity was calculated by subtracting the UV absorption of D124A from the experimentally measured values.