Investigating the position of the hairpin loop in New Delhi metallo- β -lactamase, NDM-1, during catalysis and inhibitor binding

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Abstract

In an effort to examine the relative position of a hairpin loop in New Delhi metallo- β -lactamase, NDM-1, during catalysis, rapid freeze quench double electron electron resonance (RFQ-DEER) spectroscopy was used. A doubly-labeled mutant of NDM-1, which had one spin label on the invariant loop at position 69 and another label at position 235, was prepared and characterized. The reaction of the doubly spin labeled mutant with chromacef was freeze quenched at 500 μ s and 10 ms. DEER results showed that the average distance between labels decreased by 4 Å in the 500 μ s quenched sample and by 2 Å in the 10 ms quenched sample, as compared to the distance in the unreacted enzyme, although the peaks corresponding to distance distributions were very broad. DEER spectra with the doubly spin labeled enzyme with two inhibitors show that the distance between the loop residue at position 69 and the spin label at position 235 does not change upon inhibitor binding. This study suggests that the hairpin loop in NDM-1 moves over the metal ion during the catalysis and then moves back to its original position after hydrolysis, which is consistent with a previous hypothesis based on NMR solution studies on a related metallo- β -lactamase. This study also

demonstrates that this loop motion occurs in the millisecond time domain.

Keywords: DEER spectroscopy, rapid freeze quench, metallo- β -lactamase, site specific spin labeling

1. Introduction

A recent study conducted by the British government concluded that the failure to fight antibiotic resistance would lead to 10 million deaths annually and would cost up to \$100 trillion globally by the year 2050.[1] According to a 2013 CDC report, bacterial resistance to antibiotics is a major global threat, and in 2013 alone, more than 2 million people in the United States were infected with antibiotic-resistant infections, resulting in over 23,000 deaths.[2] By far, the largest class of antibiotics contains β -lactam containing compounds, such as penicillins, cephalosporins, and carbapenems. The most common resistance pathway is bacterial production of β -lactamases, which hydrolyze and inactivate most β -lactam containing antibiotics. In the early 1990s extended-spectrum β -lactamases (ESBLs) were isolated from certain *Enterobacteriaceae* strains, and most of clinical strains responded to carbapenems. However within a decade, carbapenemase-producing *Enterobacteriaceae* and *Klebsiella pneumonia* strains have appeared throughout the world.[3, 4] Over the last 20 years, bacterial strains, which produce metallo- β -lactamases (MBLs), have appeared, and these strains have caused mortality rates as high as 67% and high morbidity rates.[5-7] Of the >200 MBLs (including MBL variants) that have been isolated, IMiPenemase (IMP), Verona Integron-encoded Metallo- β -lactamase (VIM), and New

Delhi Metallo- β -lactamase (NDM) appear to be most clinically important due to rapid dissemination of the genes to many organisms, to the high relative mortality rates caused by bacteria that harbor the genes for these enzymes, and to the rapid proliferation of variants of these enzymes.[8, 9] These three MBLs belong to the B1 subclass of β -lactamases and contain 2 Zn(II) binding sites. To date no clinical inhibitors are available to combat the MBLs, and MBL-producing bacteria have been declared to be a serious medical threat by the Centers for Disease Control and Prevention. To date there are a total of 16 variants of NDM (NDM-1 - NDM-16), which have been isolated from clinics throughout the world.[10]

Most of the MBLs contain a hairpin loop (6 to 10 residues) directly above the invariant zinc binding site(s), and this loop has been implicated in catalysis.[11] Deletion of the entire hairpin loop or mutations on the loop resulted in significantly lower activities of MBLs CcrA, IMP-1, and BcII.[12-15] Previous studies also suggested that this loop is dynamic during catalysis;[12, 13] however, it is not clear how the loop moves during catalysis. In our recent studies on CcrA, the loop appears to move about 10 Å away from the metal center in the sample quenched at 10 ms, presumably to accommodate substrate binding in the active site.[16] Given the importance of the loop in catalysis,[16] we speculated that the loop might be targeted

for the generation of an inhibitor. However before the loop is targeted, a better understanding of the role of the loop is necessary. To probe the motion of the hairpin loop (8 amino acid residues in length) in NDM-1 during catalysis and inhibitor binding, RFQ-DEER experiments were conducted.

2. Experimental

2.1. Materials.

Site-directed mutagenesis kits were purchased from Stratagene (Carlsbad, CA). *E. coli* strains DH5 α and BL21(DE3) cells were purchased from Novagen (Madison, WI). Sequencing and mutagenesis primers were purchased from Integrated DNA Technologies. The mutagenic primers were: G69C Forward: 5'-atctcgacatgccgtgtttcggggcagtc-3'; G69C Reverse: 5'-gactgccccgaaacacggcatgtcgagat-3'; A235C Forward: 5'-ccgcgtcagcgcgctgctttggtgcggcggtt-3'; A235C Reverse: 5'-aacgccgcaccaaagcagcgcgctgacgcgg-3'. Isopropyl- β -D-

thiogalactopyranoside (IPTG) was purchased from Anatrace (Muamee, OH). Q-Sepharose and Sephacryl S-200 chromatographic media were purchased from GE Healthcare. S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methylmethanesulfonothioate (MTSL) was purchased from Toronto Research Chemicals (Toronto, Canada). The substrate chromacef was a gift from Sopharmia, Inc. Captopril was purchased from Fisher Scientific, and inhibitor

G11 was provided by Professor Walt Fast from the University of Texas at Austin. All buffer solutions and growth media were prepared with Barnstead Nanopure water.

2.2. Protein purification, characterization, and spin labeling.

Protein over-expression and purification were performed using previously-described procedures.[17] Steady-state kinetic studies, metal analyses, site directed mutagenesis and spin labeling, and determination of spin efficiency were conducted as previously described.[11, 17, 18]

2.3. Molecular dynamics.

Molecular modeling and molecular dynamics studies were performed using nanoscale molecular dynamics (NAMD) with the molecular graphics software VMD. The crystal structure of NDM-1 (PDB ID: 3SPU) was used as the starting coordinates for distance prediction studies. The "mutate residue" option of the VMD was used to mutate Gly69 and Ala235 of NDM-1 to cysteines. Spin label (MTSL) was incorporated in the structure at the G69C and A235C mutations using CHARMM force-field topology parameters in the NAMD software. The resulting spin-labeled protein was solvated in a water box, and equilibration and energy minimizations of the solvated spin-labelled protein was performed using NAMD simulations. Molecular dynamic simulations were collected out to 1 ns at room temperature using Langevin dynamics in the NAMD software. The trajectory data were recorded

in 1 ps increments. The possible distance distribution between the spin labels was obtained from the analysis of the trajectory data file using VMD.

2.4. Sample preparation for RFQ-DEER.

Initial concentrations of doubly spin-labeled enzyme and substrate were 160 μ M, and the enzyme and substrate stock solutions were prepared in 50 mM HEPES, pH 7.0, and containing 10% v/v glycerol. Freeze-quenched EXAFS samples were generated using a modified Update Instruments (Madison, WI) freeze quench system.[19, 20] A model 715 Update Instruments manual ram controller was used to drive a PMI-Kollmorgen stepping motor connected to a ram that in turn drove Update Instruments syringes. The samples that were quenched at 10 ms were generated using a commercially-available mixer (Update Instruments) and an isopentane bath that was maintained at -130 °C using liquid nitrogen. The 500 μ s samples were generated using a home-made sample collection unit, which consisted of a stainless steel funnel cooled with liquid nitrogen.[21] Q-Band EPR tubes (Bruker) were packed using a 0.97 mm diameter metal string. All samples were stored in liquid nitrogen until data collection.

2.5. DEER experiments.

Four-pulse DEER experiments were performed at 80 K using Bruker ELEXSYS E580 pulsed EPR spectrometer equipped with SuperQ-FT pulse Q-band system with a 10 W amplifier and EN5107D2

resonator (34.2 GHz). All DEER samples were prepared at a spin concentration of 80-100 μM containing 20% glycerol as a cryoprotectant agent. A four pulse $[(\pi/2)_{v1} - \tau_1 - (\pi)_{v1} - t - (\pi)_{v2} - (\tau_1 + \tau_2 - t) - (\pi)_{v1} - \tau_2 - \text{echo}]$ DEER sequence was employed at Q-band with a probe pulse width of 10/20 ns, pump pulse width of 24 ns, 80 MHz of frequency difference between probe and pump pulse, shot repetition time (1013 μs) determined by spin-lattice relaxation rate (T_1), 100 echoes/point, and 2-step phase cycling at 80 K collected out to 2.3 to 2.7 μs for 8 to 12 hours data acquisition time.[22]

Fits presented were obtained using DEERAnalysis v.2013.[23] The distance distributions $P(r)$ were obtained by Tikhonov regularization in the distance domain, incorporating the constraint $P(r) > 0$. A homogeneous three-dimensional model was used for background correction (240 ns was used for starting value for background correction). The regularization parameter in the L curve was optimized by examining the fit of the time domain spectra. Error in distance was determined by observing the shift in peak maxima upon fitting the data with different background starting values (shown in Figure S8) and fittings at different L-curve inflection points(Figures S1-S6).[24]

3. Results

In order to use DEER spectroscopy to evaluate intramolecular distances in a single enzyme, a site-specifically labeled enzyme must be generated with two spin labels roughly 25-45 Å apart. By using a crystal structure of NDM-1,[25] we identified Gly69 (Gly63 in the standard numbering scheme)[26] on the hairpin loop and Ala235 (Ala248 in the standard numbering scheme) on a remote α -helix as optimal sites for MTSL labeling (Figure 1). Molecular dynamics calculations[27] showed that the MTSL labels (distance between the nitrosyl oxygens) in these positions are 38 Å apart.

The G69C and G69C/A235C mutants (hereafter referred to as NDM-1* and NDM-1**, respectively) were over-expressed and purified using previously described procedures.[18, 28] Expression levels, steady-state kinetic constants, k_{cat} and K_m , and metal content of NDM-1* and NDM-1** were found comparable with those of wild-type NDM-1 (Table 1). NDM-1* and NDM-1** were labeled with MTSL as previously described,[11] and the resulting spin-labeled mutants exhibited metal content and steady-state kinetic constants almost identical with those of the unlabeled enzymes (Table 1). The spin labeling efficiencies of NDM-1* and NDM-1** were 96% and 94%, respectively, as determined by use of continuous wave-EPR spectroscopy.[11]

The time domain traces with background fittings for the resting enzyme (enzyme with no substrate), the samples quenched

at 500 μ s and at 10 ms, and the product complex of doubly spin-labeled NDM-1** are shown in the Figure 2A. The distance distribution of the resting sample showed a broad peak centered at 38 Å (Figure 2B), which nicely matches the distance calculated by molecular dynamics. The distance distribution of the NDM-1**/chromacef sample quenched at 500 μ s showed a broad peak centered at 34 Å, while the sample quenched at 10 ms exhibited a distance distribution centered at 36 Å. The enzyme product complex prepared by incubating doubly spin labeled 160 μ M NDM-1** and 160 μ M chromacef on ice for one hour showed a broad peak centered at 39 Å, corresponding to a distance similar to that of the resting enzyme. Previous studies on MBL L1 showed that the RFQ process does not alter the DEER results on the enzyme-product complex (Figure S7). The error in the distance distributions (peak maxima) was less than 2 Å in all samples. Previous crystal structures of unbound NDM-1 and NDM-1-product complexes showed that the distance between the metal center and Gly69 on the hairpin loop in fact increased 1-2 Å in the product complexes.[29-31] While small, the changes in average distance determined from these DEER experiments are similar to the changes reported in earlier crystallographic studies. On the other hand, the distances between the metal center and Ala235,

found on α -helix 7 of NDM-1, remained essentially constant upon product binding.[32]

These data strongly suggest that the hairpin loop in NDM-1 moves over the metal binding site during catalysis and/or substrate binding, thereby closing the active site. This movement supports previous hypotheses that the hairpin loop, particularly in CcrA, closes down on the substrate and potentially activates the substrate for nucleophilic attack.[16] In NDM-1, by 10 ms of reaction time, the loop has started to move back to a position similar to that of the resting enzyme, possibly to allow product to leave the active site.

To evaluate whether loop dynamics are also important for inhibitor binding, doubly spin labeled NDM-1** was incubated with captopril and G11 (structures shown in Scheme S1), which are competitive inhibitors of NDM-1. Interestingly, the distance distributions of both enzyme-inhibitor complexes revealed peaks centered at 39 Å (Figure 3), which is almost identical to the resting enzyme. These data suggest that the loop movements observed with the RFQ samples are associated with catalysis, rather than substrate binding.

4. Discussion

Recently, we reported RFQ-DEER studies on MBL CcrA, and our data showed that the hairpin loop in CcrA moves 10 Å away from the metal site at 10 ms of reaction time.[16] This result did

not support previous hypotheses on CcrA that suggested that the hairpin loop "clamps" down on substrate during catalysis.[16] We interpreted our DEER results to suggest that the loop in CcrA needed to "move out of the way" so that catalysis could occur. The studies described herein on NDM-1 suggest that the loop does in fact "clamp down" on the substrate during catalysis (Figure 4), suggesting that the loop may play different roles in the different MBLs. However pre steady-state kinetic experiments showed that more than 90% of the substrate was consumed in the CcrA-catalyzed reaction at 10 ms (M. Aitha and M. Crowder, unpublished results),[33, 34] indicating that the previous RFQ-DEER experiments on CcrA were investigating a sample that was mostly enzyme-intermediate complex. On the other hand, stopped-flow studies show that only 5% of chromacef is hydrolyzed by NDM-1 at 10 ms, indicating that the DEER experiments described herein are investigating samples that are mostly enzyme-substrate complex with some enzyme-intermediate present.[28,33]

In the preparation of our spin-labeled NDM-1 samples, we chose a mutant with a DEER-accessible distance between the spin labels. This mutant was shown to bind the same amount of Zn(II) and to exhibit steady-state kinetic constants that were indistinguishable from the wild-type, recombinant enzyme. This mutant also could be spin-labeled with high efficiency. The time domain DEER trace of this sample had a DEER modulation depth of

4% and very little signal modulation between 0.5 and 2.0 μ s (Figure 2). The resulting distance distribution, using an L-curve inflection point of 10,000, was broad, with an average distance of 38 Å. The breadth of the distance distribution was 7 Å using the statistics of the Tikhonov regularization, and 5 Å based on the full-width at half-maximum of the distribution. Both methods to determine distance distribution are used in the literature [16, 35], but not in all studies.[22, 24, 36-39]

There appear to be at least three contributing factors that lead to broad distance distributions: (1) shallow modulation amplitudes, (2) low signal to noise, and (3) the conformational space sampled by the spin labels and/or the part of the protein that is spin-labeled.[22, 38, 40-44] There does not appear to be a very good understanding of what sample properties improve these data, and in many cases, multiple samples with spin labels in different positions are prepared until good data (large DEER modulation amplitudes, showing multiple oscillations, leading to narrow distance distributions) are obtained.[22, 24, 45] This approach is straightforward for some systems;[16] however, the present system requires that we recover double mutants with appropriate metal content and catalytic activity. In our DEER study on MBL L1, we tried 9 different double mutants before we found one that exhibited the correct biochemical properties.[11] The application of DEER for probing intramolecular electron-

electron dipolar couplings during catalysis will always have this issue. There are some reports that higher microwave power and the use of spin labels with restrictive motion can result in narrower distance distributions,[46-50] and we are testing these strategies with NDM-1. There is considerable variability in the literature regarding fitting DEER data,[36-40] and data fitting can significantly affect the resulting distance distribution spectra (see Figures S1-S6). Complicating the technical issues above, the hairpin loops in the MBLs are known to be very flexible. Previous studies suggested that the hairpin loop in NDM-1 appears to be more flexible than the loops in other MBLs and that the increased flexibility may explain NDM-1's extended spectrum of activity[51], as well as the broad distance distributions we observed here.

It is not clear what induces the movement of the loop upon substrate and product binding; however, previous studies have shown that there is a reorganization of the active site upon binding. A crystal structure of NDM-1 bound to hydrolyzed ampicillin showed a long metal-metal distance.[30] RFQ-EXAFS studies on ZnZn-L1[20, 52] and ZnCo-L1 (Aitha, Tierney, and Crowder, unpublished data) showed longer metal-metal distances in the 10 ms intermediate compared with the resting enzyme, and this distance shortened in the enzyme-product complex. It is likely that the changes in the metal-metal distances are

coordinated with loop movements. A recent crystal structure of NDM-1 complexed with a reaction intermediate of hydrolyzed cephalixin showed a metal-metal distance of 4.48 Å. However, the loop was not resolved in the structure of the resting enzyme in this study making a comparison of the relative positions of the loops impossible.[53] We are currently working to shorten the dead time of our RFQ apparatus, in order to better correlate DEER and EXAFS spectra of an NDM-1/substrate reaction, quenched very early in the catalytic cycle, to further probe whether the metal-metal distance can be correlated with the movement of the loop.

5. Conclusions

In summary, this study probed the relative position of a hairpin loop in NDM-1 during catalysis and inhibitor binding. Our data suggest that this loop moves over the active site in NDM-1 during catalysis, but not during inhibitor binding. Studies are on-going to improve the sensitivity of our RFQ-DEER studies.

Abbreviations

Double Electron Electron Resonance (DEER)

Metallo- β -lactamase (M β LS)

Extended-spectrum β -lactamases (ESBLs)

New Delhi Metallo- β -lactamase (NDM)

Verona Integron Metallo- β -lactamase (VIM)

Site-directed spin labeling (SDSL)

Rapid Freeze Quench (RFQ)

S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl
methanesulfonothioate (MTSL)

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Appendix A, Supplementary data

References

[1] J. O'Neill, AMA Review on Antimicrobial Resistance by United Kingdom Government, 2014.

- [2] Antibiotic Resistance Threat Repost, CDC, Eurosurveillance, vol. 18, 2013, pp. 28-28.
- [3] P.A. Bradford, C. Urban, N. Mariano, S.J. Projan, J.J. Rahal, K. Bush, Antimicrob Agents Chemother, vol. 41, 1997, pp. 563-569.
- [4] M. Watanabe, S. Iyobe, M. Inoue, S. Mitsuhashi, Antimicrob. Agents Chemother., vol. 35, 1991, pp. 147-151.
- [5] P. Nordmann, T. Naas, L. Poirel, Emerging Infect. Dis., vol. 17, 2011, pp. 1791-1798.
- [6] D. Marchaim, T. Chopra, F. Perez, K. Hayakawa, P.R. Lephart, S. Bheemreddy, C. Blunden, A.M. Hujer, S. Rudin, M. Shango, M. Campbell, J. Varkey, J. Slim, F. Ahmad, D. Patel, T.Y. Chen, J.M. Pogue, H. Salimnia, S. Dhar, R.A. Bonomo, K.S. Kaye, Infection control and hospital epidemiology, vol. 32, 2011, pp. 861-871.
- [7] M.J. Satlin, D.P. Calfee, L. Chen, K.A. Fauntleroy, S.J. Wilson, S.G. Jenkins, E.J. Feldman, G.J. Roboz, T.B. Shore, D.C. Helfgott, R. Soave, B.N. Kreiswirth, T.J. Walsh, Leukemia & lymphoma, vol. 54, 2013, pp. 799-806.
- [8] M. Aitha, A.R. Marts, A. Bergstrom, A.J. Moller, L. Moritz, L. Turner, J.C. Nix, R.A. Bonomo, R.C. Page, D.L. Tierney, M.W. Crowder, Biochemistry, vol. 53, 2014, pp. 7321-7331.
- [9] P. Nordmann, L. Poirel, T.R. Walsh, D.M. Livermore, Trends Microbiol., vol. 19, 2011, pp. 588-595.

- [10] K. Bush, T. Palzikill, G. Jacoby,
<http://www.lahey.org/studies/> Jürgen Pleiss.
- [11] M. Aitha, T.K. Richmond, Z. Hu, A. Hetrick, R. Reese, A. Gunther, R. McCarrick, B. Bennett, M.W. Crowder, J. Inorg. Biochem., vol. 136C, 2014, pp. 40-46.
- [12] S.D.B. Scrofani, J. Chung, J.J.A. Huntley, S.J. Benkovic, P.E. Wright, H.J. Dyson, Biochemistry, vol. 38, 1999, pp. 14507-14514.
- [13] J.J. Huntley, S.D. Scrofani, M.J. Osborne, P.E. Wright, H.J. Dyson, Biochemistry, vol. 39, 2000, pp. 13356-13364.
- [14] C. Moali, C. Anne, J. Lamotte-Brasseur, S. Groslambert, B. Devreese, J. Van Beeumen, M. Galleni, J.M. Frere, Chem. Biol., vol. 10, 2003, pp. 319-329.
- [15] J.M. Gonzalez, A. Buschiazzi, A.J. Vila, Biochemistry, vol. 49, 2010, pp. 7930-7938.
- [16] M. Aitha, M. Mortiz, D.A. Sahu, O. Sanyurah, Z. Roche, R. McCarrick, G.A. Lorigan, B. Bennett, M.W. Crowder, J. Biol. Inorg. Chem., vol. 20, 2015, pp. 585-594.
- [17] H. Yang, M. Aitha, A.M. Hetrick, T.K. Richmond, D.L. Tierney, M.W. Crowder, Biochemistry, vol. 51, 2012, pp. 3839-3847.
- [18] H. Yang, M. Aitha, A.R. Marts, A. Hetrick, B. Bennett, M.W. Crowder, D.L. Tierney, J Am Chem Soc, vol. 136, 2014, pp. 7273-7285.

- [19] Z. Hu, G. Periyannan, B. Bennett, M.W. Crowder, J. Am. Chem. Soc., vol. 130, 2008, pp. 14207-14216.
- [20] R.M. Breece, Z. Hu, B. Bennett, M.W. Crowder, D.L. Tierney, J. Am. Chem. Soc., vol. 131, 2009, pp. 11642-11643.
- [21] M. Tanaka, K. Matsuura, S. Yoshioka, S. Takahashi, K. Ishimori, H. Hori, I. Morishima, Biophys J., vol. 84, 2003, pp. 1998-2004.
- [22] I.D. Sahu, B.M. Kroncke, R. Zhang, M.M. Dunagan, H.J. Smith, A. Craig, R.M. McCarrick, C.R. Sanders, G.A. Lorigan, Biochemistry, vol. 53, 2014, pp. 6392-6401.
- [23] G. Jeschke, Annu. Rev. Phys. Chem., vol. 63, 2012, pp. 19.11-19.28.
- [24] I.D. Sahu, R.M. McCarrick, K.R. Troxel, R. Zhang, H.J. Smith, M.M. Dunagan, M.S. Swartz, P.V. Rajan, B.M. Kroncke, C.R. Sanders, G.A. Lorigan, Biochemistry, vol. 52, 2013, pp. 6627-6632.
- [25] V.L. Green, A. Verma, R.J. Owens, S.E. Phillips, S.B. Carr, Acta crystallographica. Section F, Structural biology and crystallization communications, vol. 67, 2011, pp. 1160-1164.
- [26] G. Garau, I. Garcia-Saez, C. Bebrone, C. Anne, P. Mercuri, M. Galleni, J.M. Frere, O. Dideberg, Antimicrob. Agents Chemother., vol. 48, 2004, pp. 2347-2349.

- [27] J.C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R.D. Skeel, L. Kale, K. Schulten, *J. Comput. Chem.*, vol. 26, 2005, pp. 1781-1802.
- [28] H. Yang, M. Aitha, A.M. Hetrick, T.K. Richmond, D.L. Tierney, M.W. Crowder, *Biochemistry*, vol. 51, 2012, pp. 3839-3847.
- [29] D. King, N. Strynadka, *Protein Sci.*, vol. 20, 2011, pp. 1484-1491.
- [30] H. Zhang, Q. Hao, *FASEB J.*, vol. 25, 2011, pp. 2574-2582.
- [31] Y. Kim, M.A. Cunningham, J. Mire, C. Tesar, J. Sacchettini, A. Joachimiak, *FASEB J.*, vol. 27, 2013, pp. 1917-1927.
- [32] D.T. King, L.J. Worrall, R. Gruninger, N.C. Strynadka, *J. Am. Chem. Soc.*, vol. 134, 2012, pp. 11362-11365.
- [33] Z.G. Wang, S.J. Benkovic, *J. Biol. Chem.*, vol. 273, 1998, pp. 22402-22408.
- [34] Y. Yang, D. Keeney, X.J. Tang, N. Canfield, B.A. Rasmussen, *J. Biol. Chem.*, vol. 274, 1999, pp. 15706-15711.
- [35] I.D. Sahu, R.M. McCarrick, G.A. Lorigan, *Biochemistry*, vol. 52, 2013, pp. 5967-5984.
- [36] M.C. Puljung, H.A. DeBerg, W.N. Zagotta, S. Stoll, *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, 2014, pp. 9816-9821.
- [37] S. Stoll, Y.T. Lee, M. Zhang, R.F. Wilson, R.D. Britt, D.B. Goodin, *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, 2012, pp. 12888-12893.

- [38] W. Wang, S.J. Lippard, J. Am. Chem. Soc., vol. 136, 2014, pp. 2244-2247.
- [39] N.K. Kim, M.K. Bowman, V.J. DeRose, J. Am. Chem. Soc., vol. 132, 2010, pp. 8882-8884.
- [40] S. Dunkel, L.P. Pulagam, H.J. Steinhoff, J.P. Klare, Phys. Chem. Chem. Phys., vol. 17, 2015, pp. 4875-4878.
- [41] M. Sippach, D. Weidlich, D. Klose, C. Abe, J. Klare, E. Schneider, H.J. Steinhoff, Biochim. Biophys. Acta., vol. 1838, 2014, pp. 1760-1768.
- [42] B. Joseph, V.M. Korkhov, M. Yulikov, G. Jeschke, E. Bordignon, J. Biol. Chem., vol. 289, 2014, pp. 3176-3185.
- [43] Y. Shenberger, A. Shimshi, S. Ruthstein, J. Phys. Chem. B, vol. 119, 2015, pp. 4824-4830.
- [44] N. Krause, C. Engelhard, J. Heberle, R. Schlesinger, R. Bittl, FEBS Lett., vol. 587, 2013, pp. 3309-3313.
- [45] C.J. Lopez, Z. Yang, C. Altenbach, W.L. Hubbell, Proc. Natl. Acad. Sci. U. S. A., vol. 110, 2013, pp. E4306-4315.
- [46] P.P. Borbat, E.R. Georgieva, J.H. Freed, J. Phys. Chem. Lett., vol. 4, 2013, pp. 170-175.
- [47] Y. Polyhach, E. Bordignon, R. Tschaggelar, S. Gandra, A. Godt, G. Jeschke, Phys. Chem. Chem. Phys., vol. 14, 2012, pp. 10762-10773.

- [48] P.A. Cruickshank, D.R. Bolton, D.A. Robertson, R.I. Hunter, R.J. Wylde, G.M. Smith, *Rev. Sci. Instrum.*, vol. 80, 2009, pp. 103102.
- [49] P.P. Borbat, R.H. Crepeau, J.H. Freed, *J. Magn. Reson.*, vol. 127, 1997, pp. 155-167.
- [50] S. Pistolessi, R. Pogni, J.B. Feix, *Biophys. J.*, vol. 93, 2007, pp. 1651-1660.
- [51] Y. Kim, C. Tesar, J. Mire, R. Jedrzejczak, A. Binkowski, G. Babnigg, J. Sacchettini, A. Joachimiak, *PLoS One*, vol. 6, 2011, pp. e24621.
- [52] A. Costello, G. Periyannan, K.W. Yang, M.W. Crowder, D.L. Tierney, *J. Biol. Inorg. Chem.*, vol. 11, 2006, pp. 351-358.
- [53] H. Feng, J. Ding, D. Zhu, X. Liu, X. Xu, Y. Zhang, S. Zang, D.C. Wang, W. Liu, *J. Am. Chem. Soc.*, vol. 136, 2014, pp. 14694-14697.

Table 1. Metal content and steady state kinetic constants of NDM-1 mutants (chromacef was used as substrate).

Enzyme (Abbreviation)	Metal content (eq)	K_m (μM)	k_{cat} (s^{-1})	Metal content after spin- labeling	K_m (μM) after spin-labeling	k_{cat} (s^{-1}) after spin labeling
Wild-type (NDM-1)	1.9 ± 0.1	5.1 ± 0.9	4.2 ± 1	1.8 ± 0.1	5.0 ± 0.5	4.0
G69C (NDM-1*)	1.9 ± 0.1	5.3 ± 0.5	4.0 ± 0.5	1.9 ± 0.1	4.8 ± 0.5	4.1
G69C/A235C (NDM-1**)	2.0 ± 0.1	4.9 ± 0.8	3.9 ± 0.6	1.9 ± 0.1	5.1 ± 0.3	3.7

Figure 1. NDM-1 crystal structure with the positions Gly69 and Ala235, (PDB id: 3ZR9 was used). Previously-described procedures were used to generate this figure.[11]

Figure 2. A. Unprocessed time domain decay data of double MTSL-labeled NDM-1** samples and corresponding background fits. B) Processed time domain DEER traces and corresponding fits (left) and distance distribution spectra (right) of the resting enzyme, enzyme product complex, and samples quenched at 500 μ s and at 10 ms (right).

Figure 3. Q-band DEER spectra of double MTSL-labeled NDM-1** samples. Time domain traces with corresponding fit of the resting enzyme, enzyme captopril and enzyme G11 complexes (left). Distance distribution spectra of the resting NDM-1** (black), complex with captopril (blue), and complex with G11 (green), (right).

Figure 4. Proposed model based on our results. Previously described procedures were used to generate the figure.[11]

Pictogram

Rapid-freeze quench double electron electron resonance spectroscopy (RFQ-DEER) was used to probe the position of an invariant loop in New Delhi metallo- β -lactamase, NDM-1, during catalysis and inhibitor binding. The results show that the loop moves over the active site during catalysis but stays in the same position during inhibitor binding.