Supporting Information for

Evolution of New Delhi metallo-β-Lactamase (NDM) in the clinic: Effects of NDM mutations on stability, zinc affinity, mono-zinc activity

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Table S1. Clinically Isolated Variants of NDM.

NDM variants	Amino acid substitutions	Reference	UniProt ID
NDM-2	P28A	(1)	F2YZ26
NDM-3	D95N	(2)	I3VKD5
NDM-4	M154L	(3)	H6WZS9
NDM-5	V88L, M154L	(4)	G3K399
NDM-6	A233V	(5)	G9JVE6
NDM-7	D130N, M154L	(6)	J7I0S9
NDM-8	D130G, M154L	(7)	M1VE66
NDM-9	E152K	(8)	T2A6Y2
NDM-10	R32S, G36D, G69S, A74T, G200R	(9)	S5ZIP8
NDM-11	M154V	From UniProt	A0A0B5ECY2
NDM-12	M154L, G222D	(10)	A0A024FRL9
NDM-13	D95N, M154L	(11)	A0A0A8J940
NDM-14	D130G	(12)	A0A0C5H135
NDM-15	M154L, A233V	From UniProt	A0A0F6ZNP0
NDM-16	V88L, M154L, A233V	From UniProt	A0A140IHH4
NDM-17	V88L, M154L, E170K	(13)	A0A1P8C735

Table S2. Primer Sequences Used for Site-Directed Mutagenesis to Generate NDM Variants

Position	Primers for MIC experiment	Primers for protein over-expression
P28A_F	CGGGTGCATGgccGGTGAAATCC	not required
P28A_R	CTCAGCATCAATGCAGCGGC	
D95N_F	CGCCTGGACCaacGACCAGACCG	CGCCTGGACCaatGACCAGACCG
D95N_R	GTATCGACCACCAGCACG	GTATCGACCACCAGCACGC
M154L_F	GCAAGAGGGGctgGTTGCGGCGC	GCAAGAGGGGctgGTTGCGGCGC
M154L_R	GGGGCAAGCTGGTTCGACAAC	GGGGCAAGCTGGTTCGACAAC
M154V_F	GCAAGAGGGGgtgGTTGCGGCGC	GCAAGAGGGGgtcGTTGCGGCGC
M154V_R	GGGGCAAGCTGGTTCGACAACG	GGGGCAAGCTGGTTCGAC
V88L_F	CCGCGTGCTGctgGTCGATACCG	CCGCGTGCTGctgGTCGATACCG
V88L_R	CCGCCATCCCTGACGATC	CCGCCATCCCTGACGATC
A233V_F	CGCCGCGTCAgttCGCGCGTTTG	CGCCGCGTCAgttCGCGCGTTTG
A233V_R	TAGTGCTCAGTGTCGGCATC	TAGTGCTCAGTGTCGGCATC
D130N_F	GGGCGGTATGaatGCGCTGCATG	GGGCGGTATGaacGCGCTGCATG
D130N_R	ATCTTGTCCTGATGCGCGTG	ATCTTGTCCTGATGCGCGTGAGTC
D130G_F	GGGCGGTATGggtGCGCTGCATG	GGGCGGTATGggcGCGCTGCATG
D130G_R	ATCTTGTCCTGATGCGCG	ATCTTGTCCTGATGCGCGTGAGTCACC
E152K_F	TGCCCCGCAAaagGGGATGGTTG	TGCCCCGCAAaagGGGATGGTTG
K152K_R	AGCTGGTTCGACAACGCATTGGC	AGCTGGTTCGACAACGCATTGGC
E170K_F	TGGCTGGGTCaagCCAGCAACCG	TGGCTGGGTCaagCCAGCAACCG
E170K_R	TTGGCGGCGAAAGTCAGG	TTGGCGGCGAAAGTCAGG
G222D_F	CGGCAATCTCgacGATGCCGACAC	CGGCAATCTCgatGATGCCGACA
G222D_R	AGCGACTTGGCCTTGCTG	AGCGACTTGGCCTTGCTG
R32S G36D_F	gattgacCAGCAAATGGAAACTGGC	CTCCGGCTCCgatCAGCAAATGG(for G36D only)
R32S G36D_R	gtcgggctGATTTCACCGGGCATGCA	GCGCCGCCCTGAAAATAC(for G36D only)
G69S A74T_F	gcagtcacgTCCAACGGTTTGATCGTC	gcagtcaccTCCAACGGTTTGATCGTC
G69S A74T_R	cccgaagctCGGCATGTCGAGATAGGA	cccgaagctCGGCATGTCGAGATAGGA
G200R_F	TGGGATCGACcgcACCGACATCG	TGGGATCGACcgcACCGACATCG
G200R_R	ACGGTGATATTGTCACTGGTG	ACGGTGATATTGTCACTGGTG

Table S3. Zinc Content in NDM Variants, As-Purified, Determined by ICP-OES.

Purified NDM Variant	Zinc Content (Equiv)
NDM-1	1.8 ± 0.1
NDM-3	1.9 ± 0.1
NDM-4	1.6 ± 0.1
NDM-5	1.6 ± 0.1
NDM-6	1.8 ± 0.1
NDM-7	1.6 ± 0.1
NDM-8	1.7 ± 0.1
NDM-9	1.6 ± 0.1
NDM-11	1.9 ± 0.1
NDM-12	1.5 ± 0.1
NDM-13	1.4 ± 0.1
NDM-14	2.0 ± 0.1
NDM-15	1.8 ± 0.1
NDM-16	1.9 ± 0.1
NDM-17	1.9 ± 0.1

Table S4 (a). Steady-State Kinetic Parameters of NDM Variants for Chromacef Hydrolysis in the Presence of Excess Zinc (10 μM)

NDM Variant	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
NDM-1	14.0 ± 0.4	0.55 ± 0.07	2.5×10^7
NDM-3	11.6 ± 0.3	1.7 ± 0.1	6.8×10^6
NDM-4	5.4 ± 0.1	0.50 ± 0.04	1.1×10^7
NDM-5	15.6 ± 0.8	2.7 ± 0.3	5.8×10^6
NDM-6	11.3 ± 0.2	1.4 ± 0.1	8.1×10^6
NDM-7	8.1 ± 0.4	2.0 ± 0.3	4.1×10^6
NDM-8	5.3 ± 0.1	1.3 ± 0.1	4.1×10^6
NDM-9	12.5 ± 0.4	1.2 ± 0.1	1.0×10^7
NDM-11	6.2 ± 0.1	0.70 ± 0.05	8.9×10^6
NDM-12	5.7 ± 0.1	0.73 ± 0.07	7.8×10^6
NDM-13	6.1 ± 0.2	1.5 ± 0.1	4.1×10^6
NDM-14	7.5 ± 0.2	0.69 ± 0.06	1.1×10^7
NDM-15	11.0 ± 0.4	1.4 ± 0.1	7.9×10^6
NDM-16	10.0 ± 0.2	0.77 ± 0.06	1.3×10^7
NDM-17	9.5 ± 0.2	0.69 ± 0.06	1.4×10^7

Table S4(b). Steady-State Kinetic Parameters of NDM Variants for Meropenem Hydrolysis in the Presence of Excess Zinc (10 μM)

NDM Variant	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
NDM-1	176 ± 7	58 ± 7	3.0×10^6
NDM-3	87 ± 3	63 ± 5	1.4×10^6
NDM-4	141 ± 3	75 ± 4	1.9×10^6
NDM-5	250 ± 10	130 ± 10	1.9×10^6
NDM-6	69 ± 3	71 ± 7	9.7×10^5
NDM-7	141 ± 3	90 ± 5	1.6×10^6
NDM-8	96 ± 2	84 ± 5	1.1×10^6
NDM-9	90 ± 3	40 ± 4	2.3×10^6
NDM-11	71 ± 3	75 ± 8	9.5×10^5
NDM-12	104 ± 3	78 ± 6	1.3×10^6
NDM-13	133 ± 5	110 ± 10	1.2×10^6
NDM-14	75 ± 3	72 ± 7	1.0×10^6
NDM-15	305 ± 8	113 ± 7	2.7×10^6
NDM-16	240 ± 20	220 ± 30	1.1×10^6
NDM-17	123 ± 4	121 ± 8	1.0×10^6

Table S5. Dissociation Constants for L-Captopril and Dizinc (II) NDM Variants as Determined by ITC.

NDM Variants	K_d (μM)	n
NDM-1	4 ± 1	1.0 ± 0.1
NDM-3	5 ± 2	1.1 ± 0.6
NDM-4	1.9 ± 0.7	1.0 ± 0.1
NDM-5	2.8 ± 0.5	1.0 ± 0.7
NDM-6	3.5 ± 0.7	1.00 ± 0.05
NDM-7	1.6 ± 0.5	1.00 ± 0.05
NDM-8	1.4 ± 0.9	1.02 ± 0.07
NDM-9	1.3 ± 0.9	1.0 ± 0.8
NDM-11	3 ± 2	1.0 ± 0.1
NDM-12	3 ± 2	1.0 ± 0.4
NDM-13	3 ± 0.8	1.0 ± 0.1
NDM-14	2 ± 0.7	1.0 ± 0.1
NDM-15	5 ± 2	1.0 ± 0.2
NDM-16	2 ± 0.6	1.07 ± 0.06
NDM-17	3 ± 1	1.1 ± 0.1

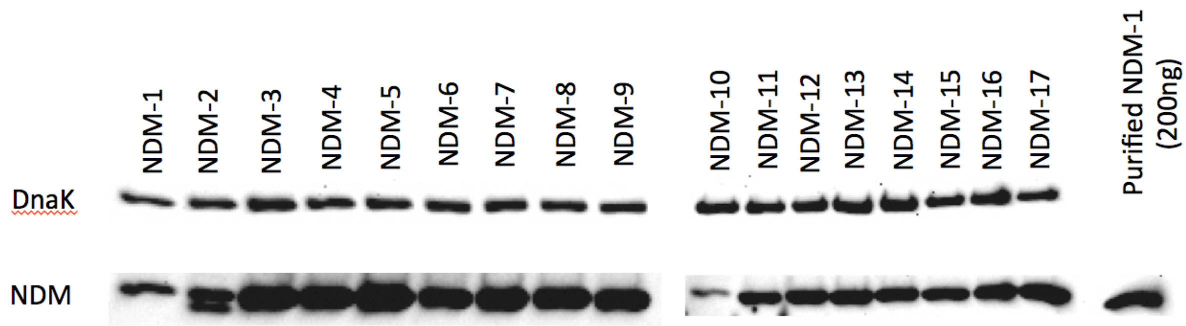


Figure S1. Immunoblotting of NDM Variants To Gauge Protein Levels. Immunoblotting was used to evaluate the expression levels of each NDM (1-17) variant. Five-milliliter cultures of *E. coli* DH10B cells containing pHSG-298 phagemids harboring the various bla_{NDM} variants in Mueller-Hinton (MH) broth containing 50 µg/mL kanamycin were grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.8. Fifty-microliter aliquots of whole cells from these cultures were pelleted and frozen overnight. Pellets were resuspended in 20 µL loading dye, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Novex, Life Technologies, Carlsbad, CA) by electroblotting. After blocking for 1 h with 5 % nonfat dry milk, bla_{NDM} presence on the blot was detected by incubation in 5 % nonfat dry milk with anti-NDM-1 polyclonal antibody mouse serum (1/200 dilution) and 1/10,000 dilution of mouse anti-DnaK monoclonal Ab (Enzo Life Sciences) overnight at 4°C. The membrane was washed four times, 15 min each, in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 and subsequently incubated in 5 % nonfat dry milk with 1/10,000 dilution of HRP-goat anti-mouse Ab conjugate (Santa Cruz Biotechnology). After four additional washes, the membrane was processed for exposure using the ECL kit (GE Healthcare) and FOTO/AnalystVR FX (Fotodyne).

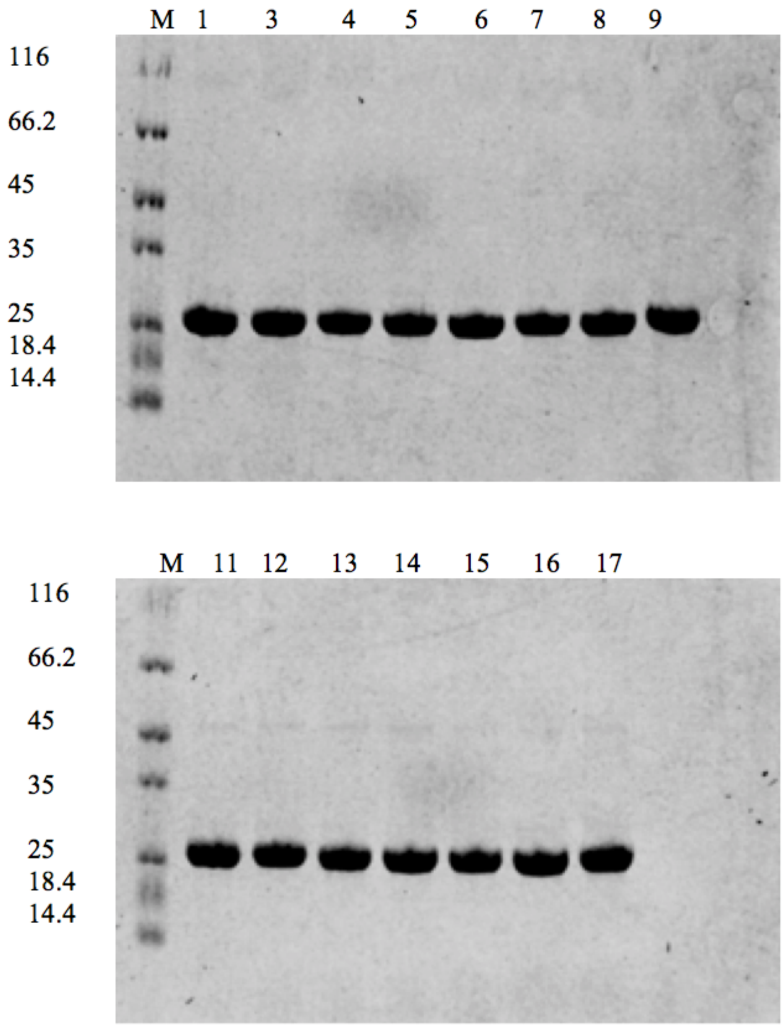


Figure S2. SDS-PAGE analysis (12.5%) of purified NDM variants after removal of His-tags. Each variant is labeled by column. The molecular weight markers (M, mass indicated on left in KDa) are EZ-Run Protein Ladder (Fisher).

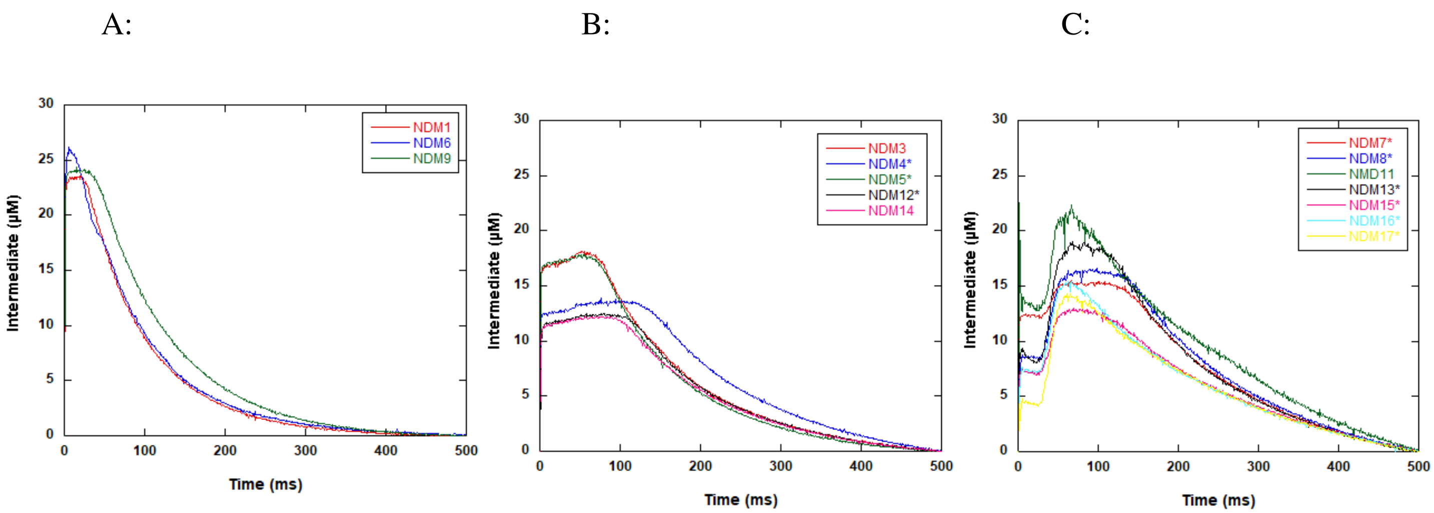
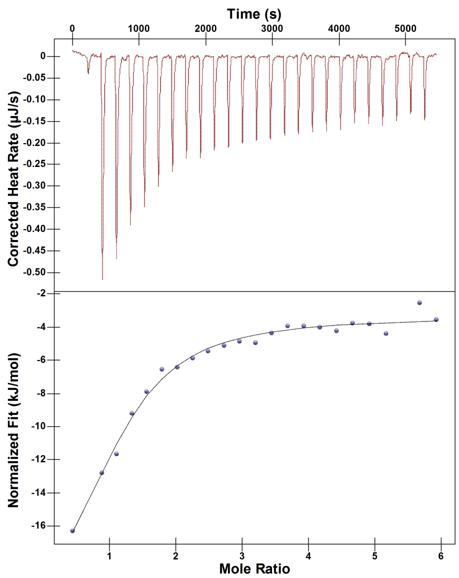


Figure S3. Stopped-Flow Kinetics of Chromacef Turnover by Dizinc (II) NDM Variants. Formation of an anionic reaction intermediate at (575 nm) is followed for each NDM-variant and grouped qualitatively by A) variants that show fast formation of the intermediate followed by a slow decay, B) variants that show fast formation of the intermediate that is maintained for a period before decay, and C) variants that show an initial lag period before formation and subsequent decay of the intermediate. Variants containing the M154L mutation are indicated with an asterisk.

Figure S4 Starts below (a multipage figure)



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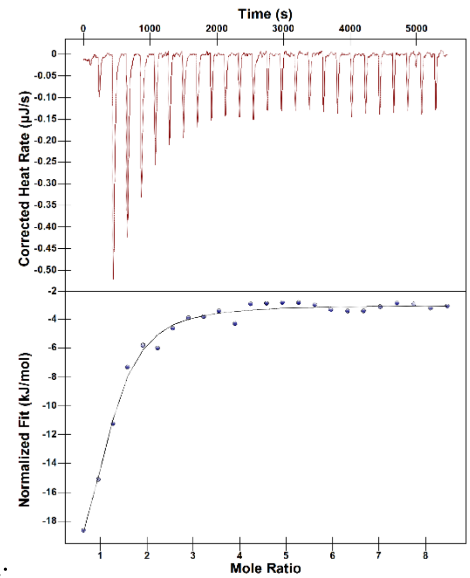

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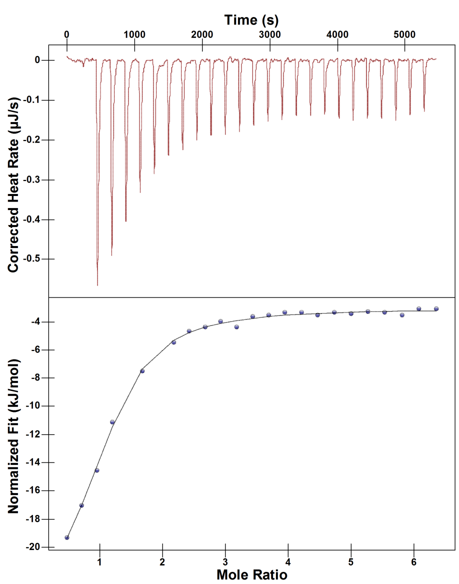
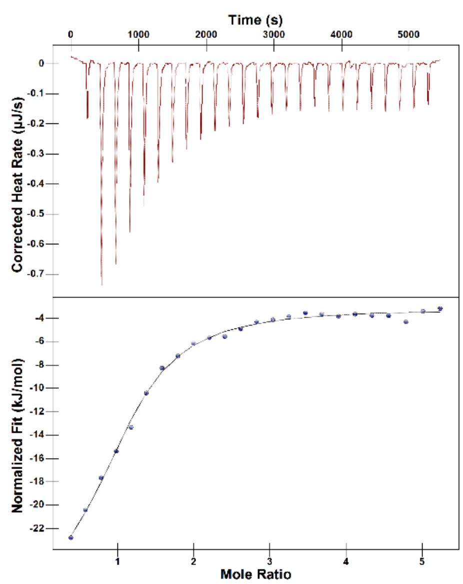
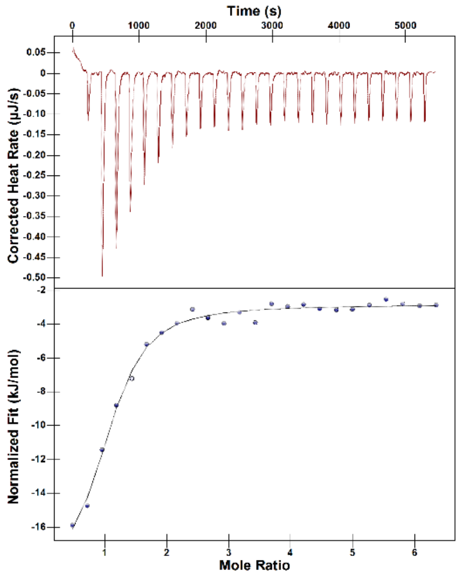


NDM-5:

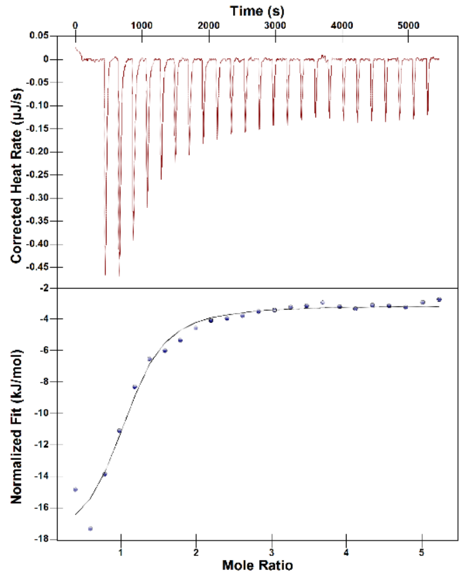


NDM-6:

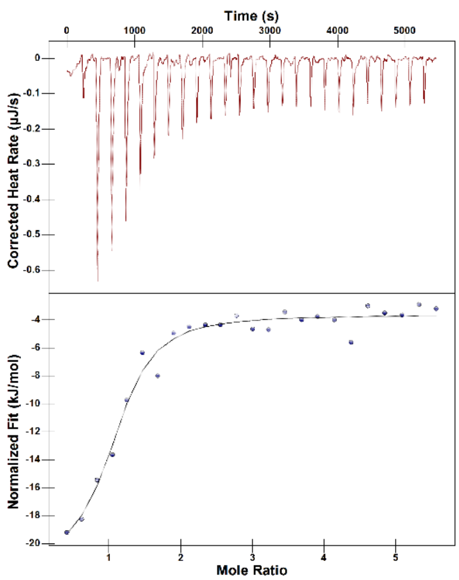
NDM-7:



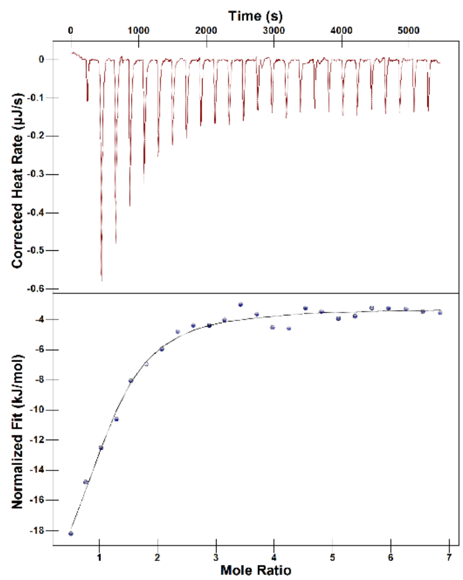
NDM-8:

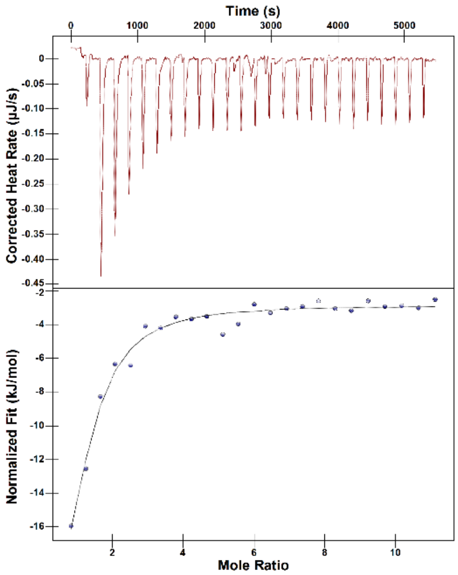


NDM-9:



NDM-11:





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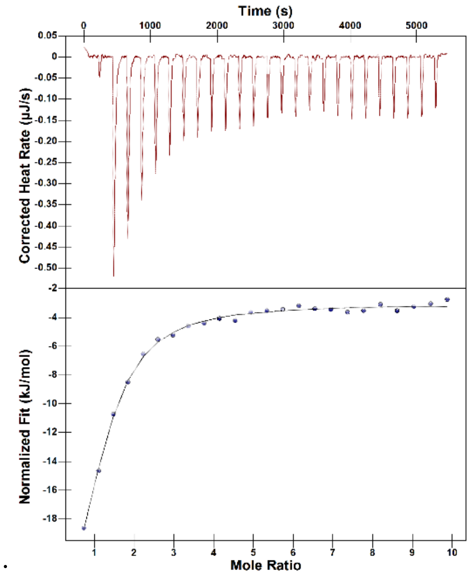

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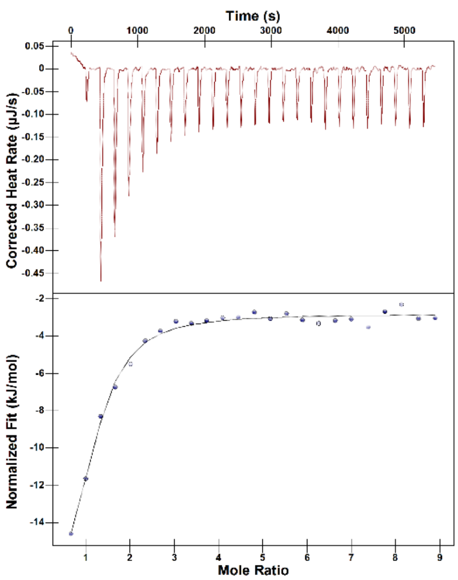
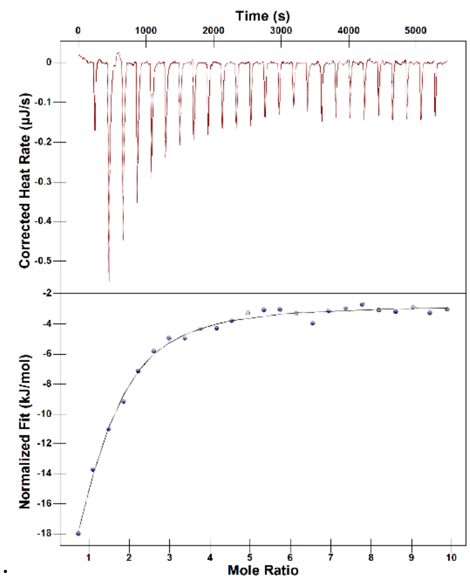
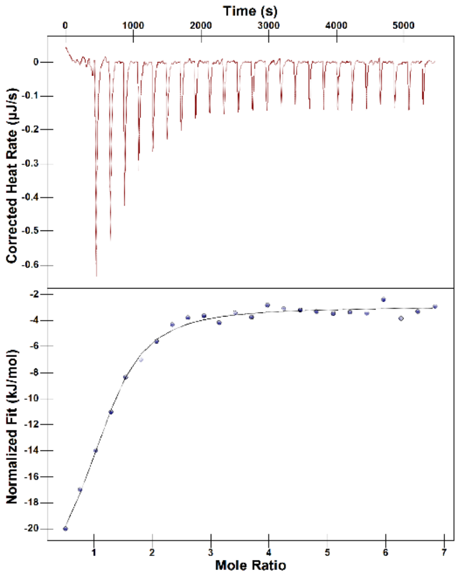


NDM-14:



NDM-15:

NDM-16: 

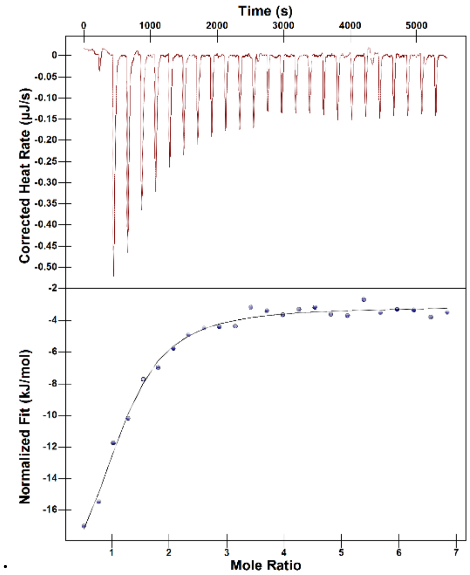
NDM-17: 

Figure S4. Thermograms and Dissociation Constant Fits for L-Captopril and NDM Variants. Upper panes of each are titration thermograms and lower are the data integration with fitted curves.

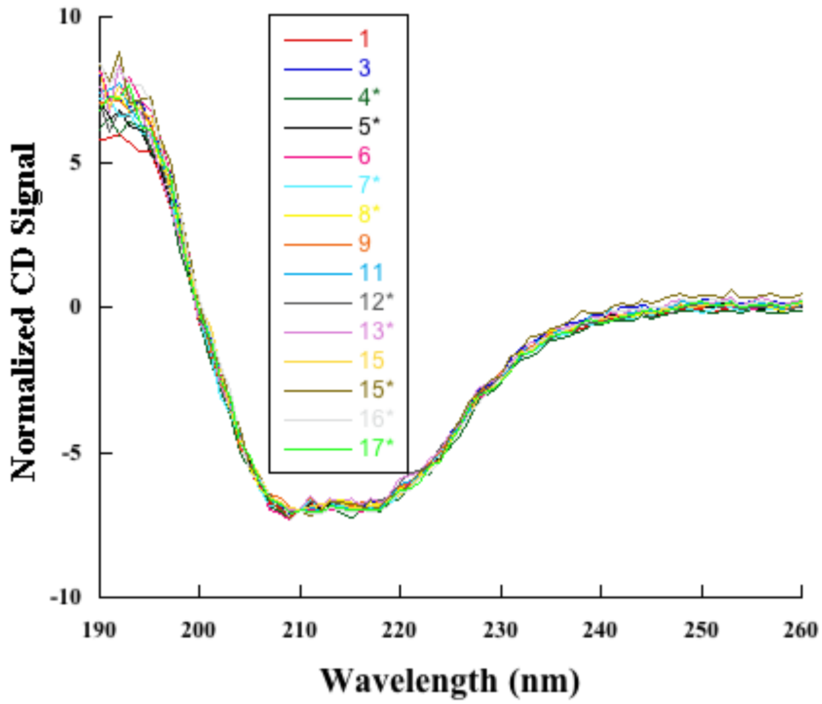


Figure S5. Far-UV CD spectra of purified NDM Variants. Data were obtained for each variant (4 µM) at room temperature (ca. 25 °C) in phosphate buffer (20 mM), at pH 7.

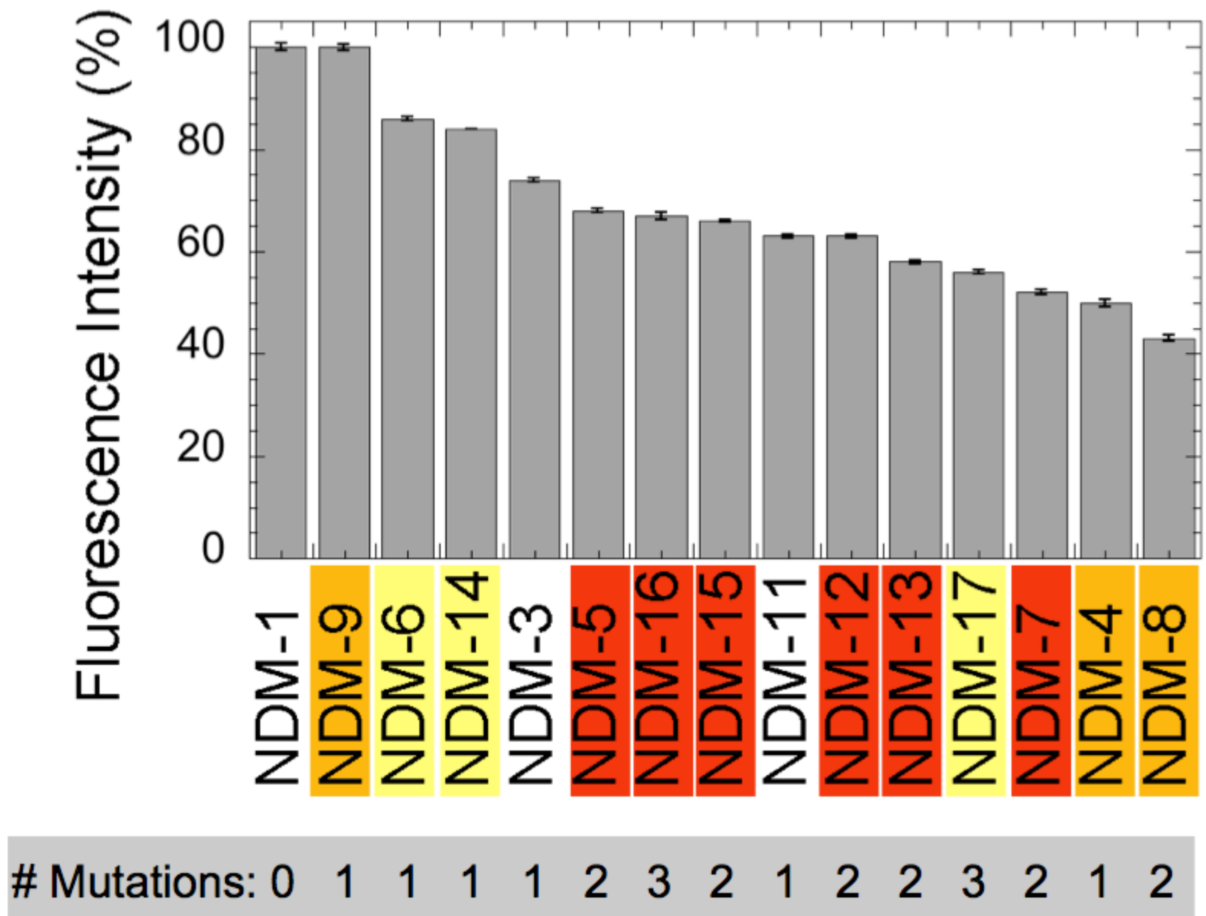


Figure S6. Relative Intrinsic Trp Fluorescence of NDM Variants. Each protein sample (2 μM) was measured in HEPES buffer (50 mM) at pH 7.5, with data representing the mean of triplicate experiments and error bars depicting standard deviation.

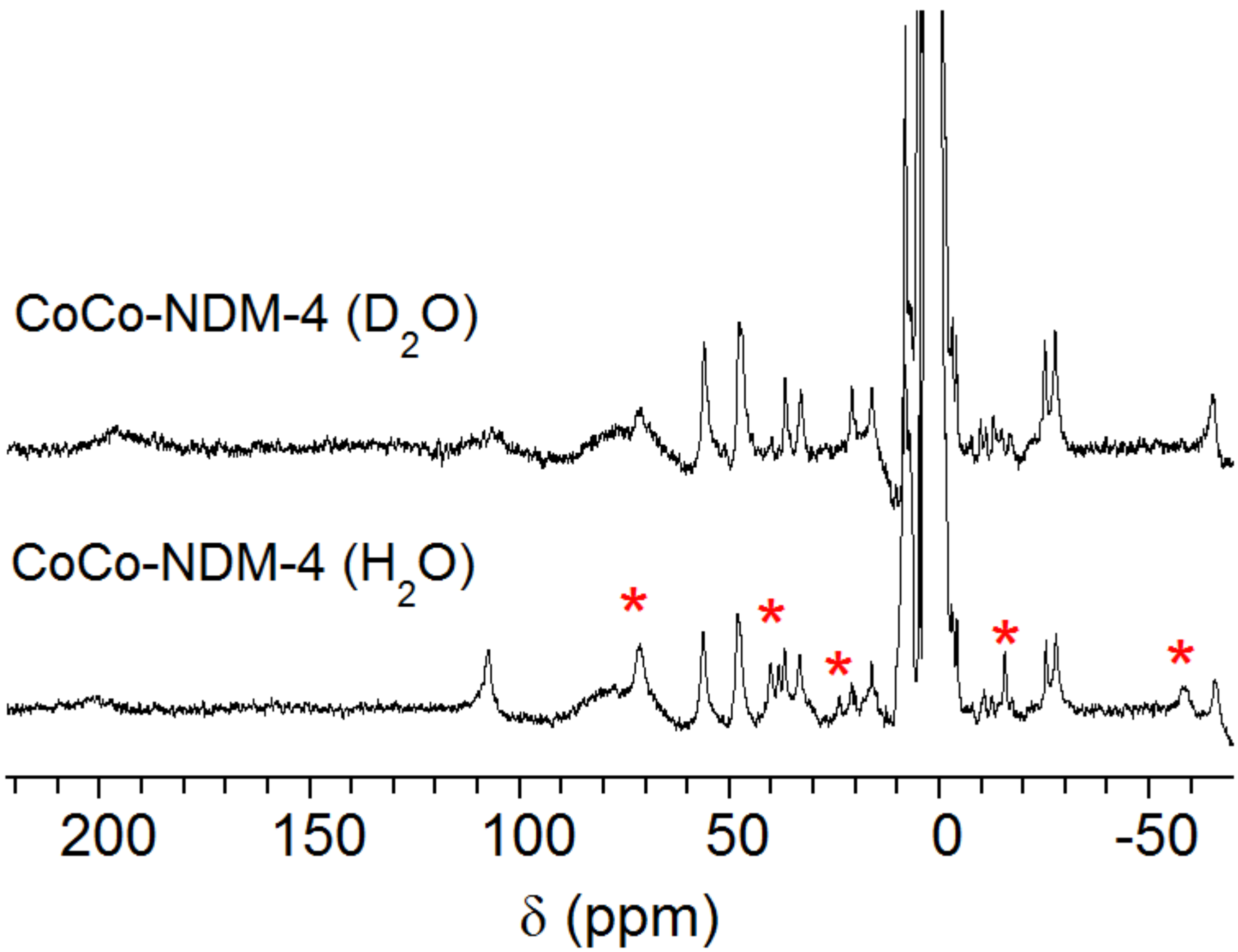
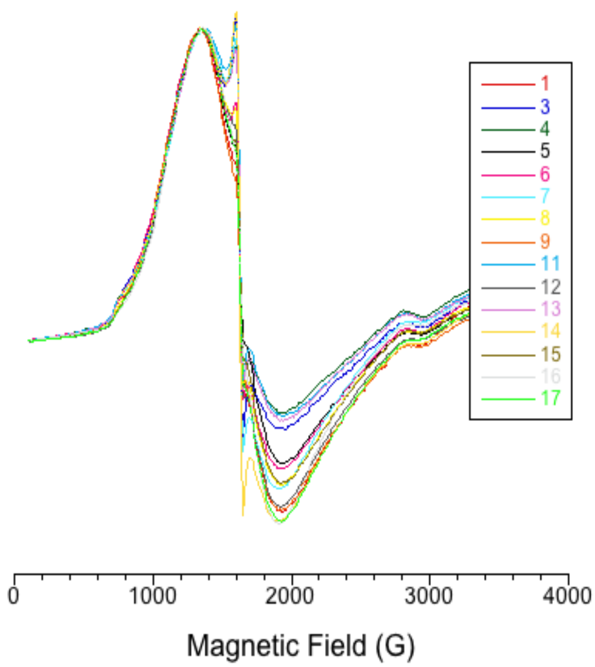


Figure S7. Comparison of 300 MHz [1]HNMR of CoCo-NDM-4 in 100 % D_2O (top) and in 90/10 H_2O/D_2O. Exchangeable resonances are marked with a red asterisk.

A:



B:

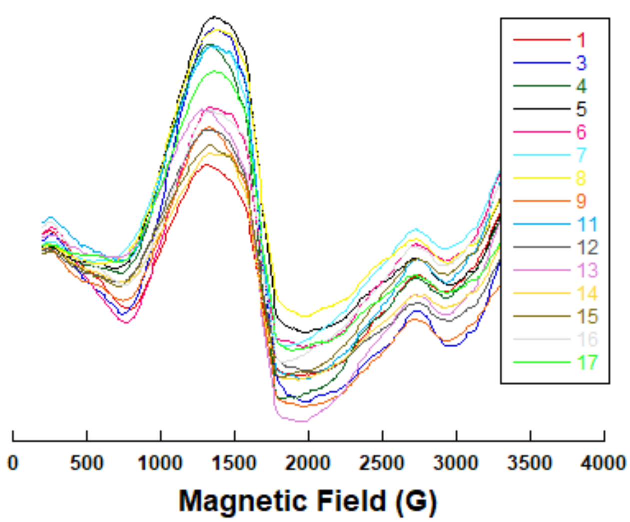
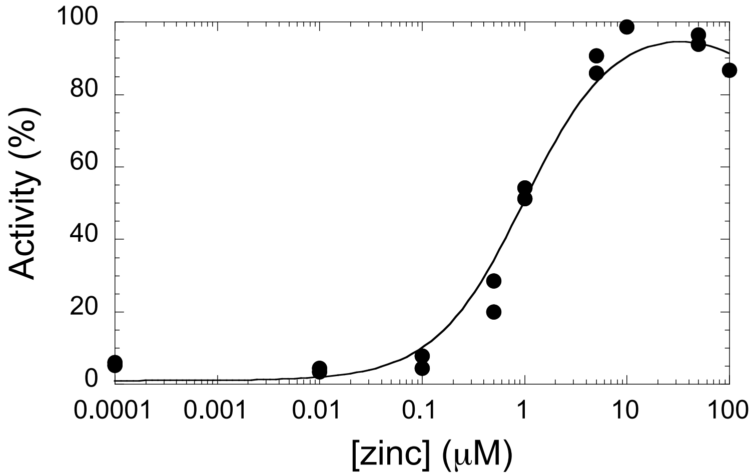


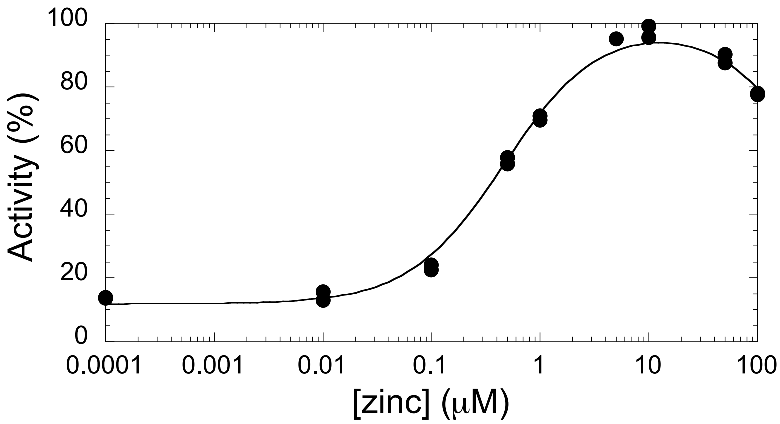
Figure S8. EPR Spectra of dicobalt (II) metalloforms of NDM Variants. A) Perpendicular and B) parallel (spectra smoothed using Kaleidegraph) CW-EPR spectra of the metalloforms (0.5 mM), as labeled. Sharp spikes at 1600 G are due to minor contamination by iron.

Figure S9 Starts below (a multipage figure)

NDM-1:



NDM-3:



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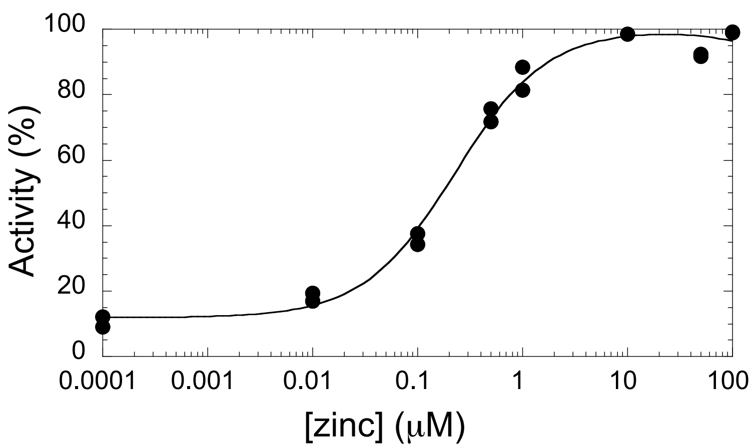

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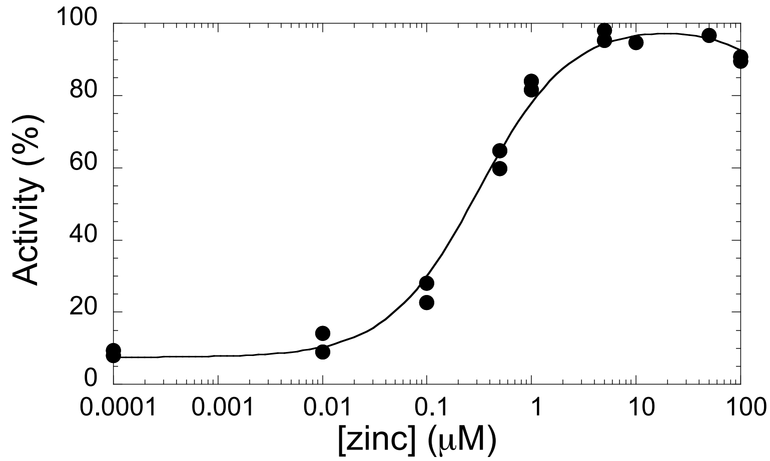

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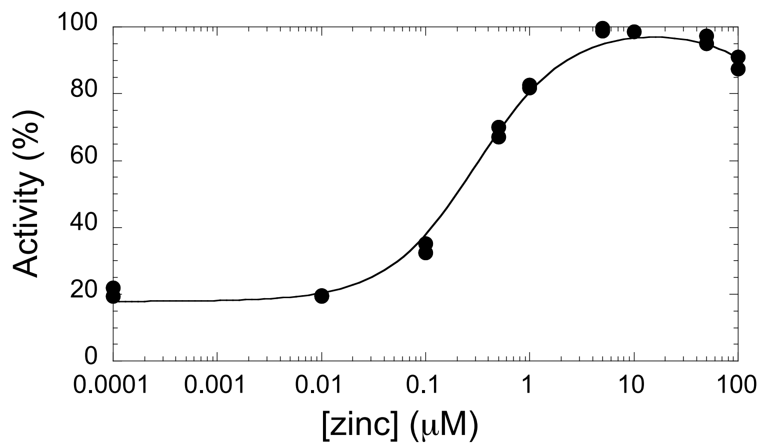

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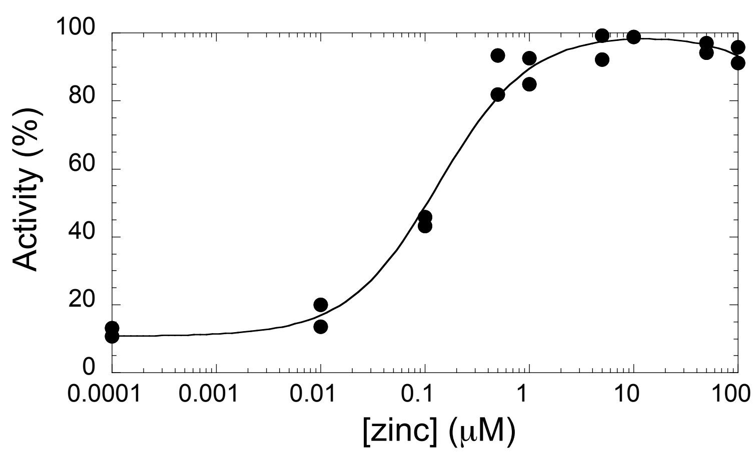

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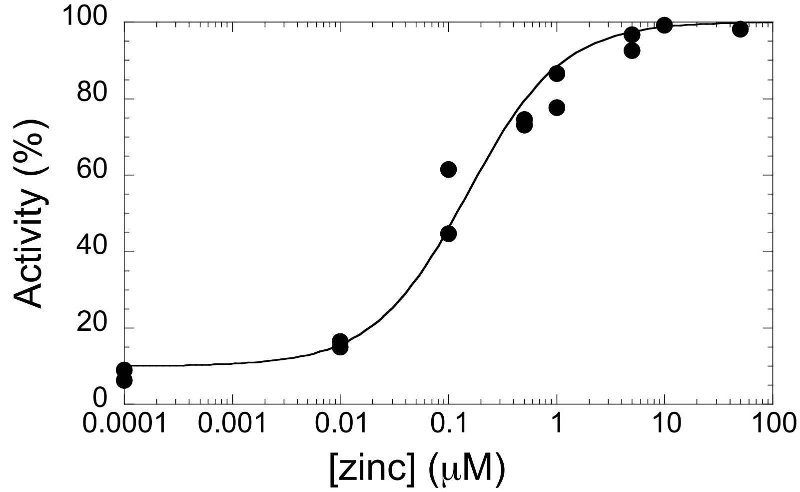

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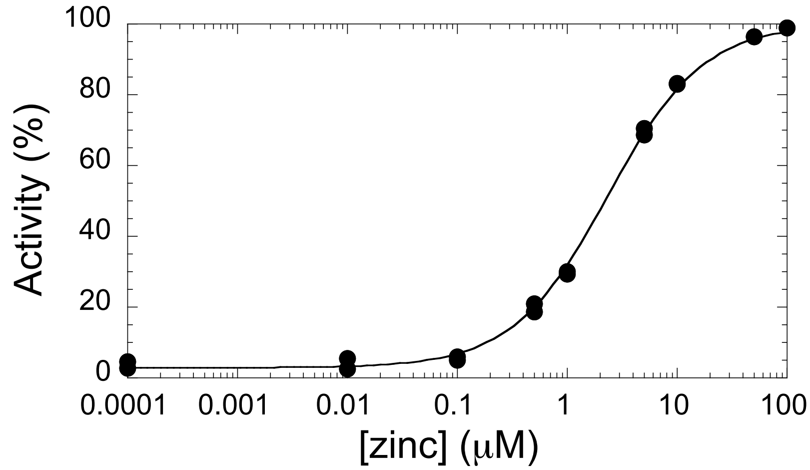

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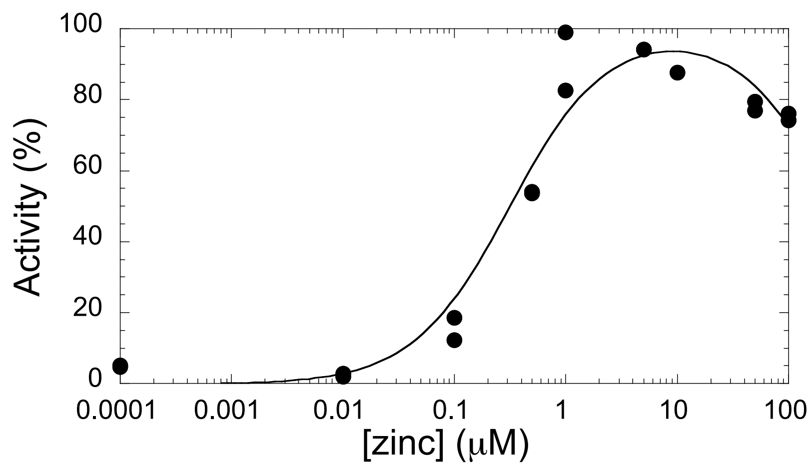

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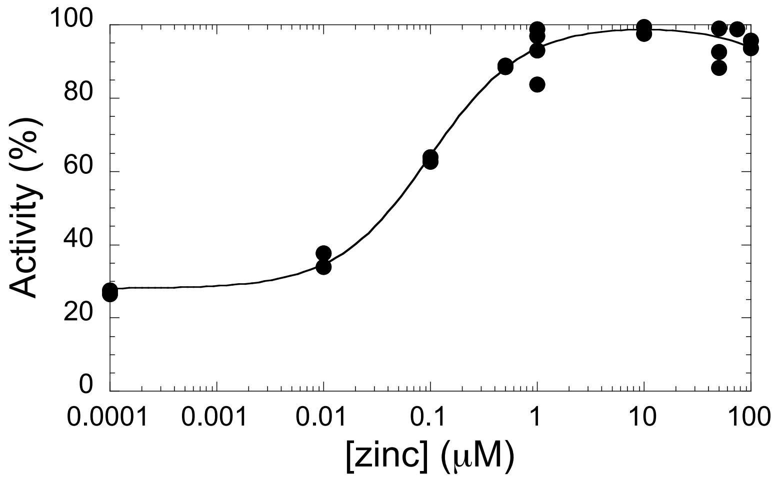

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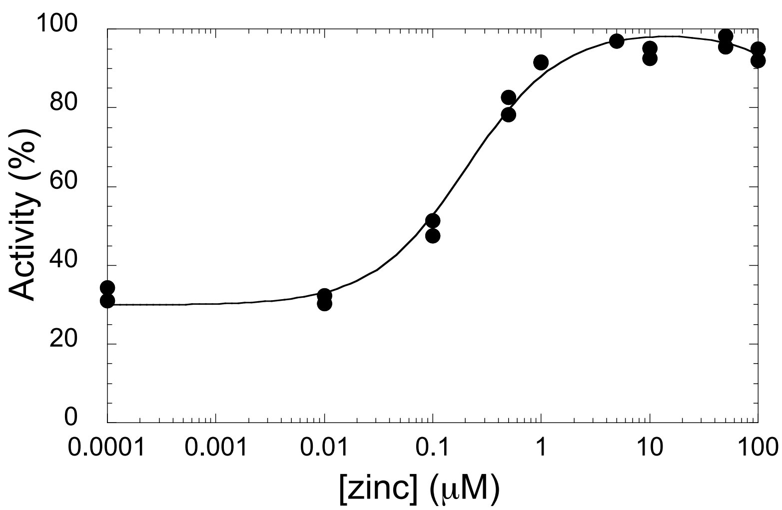

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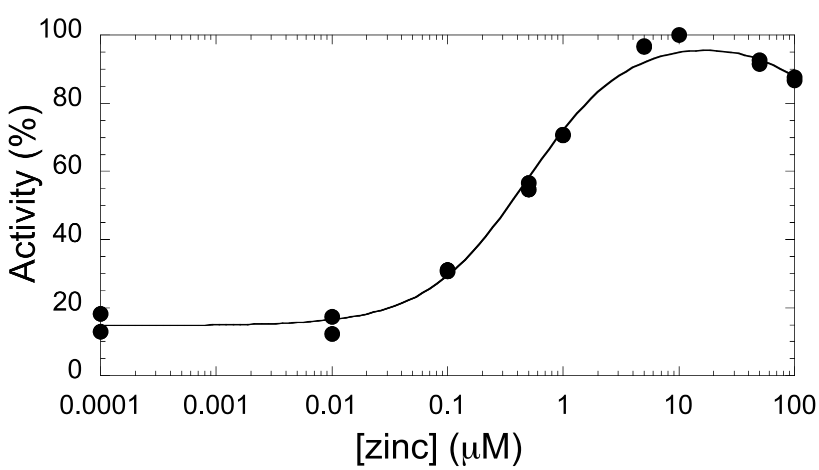

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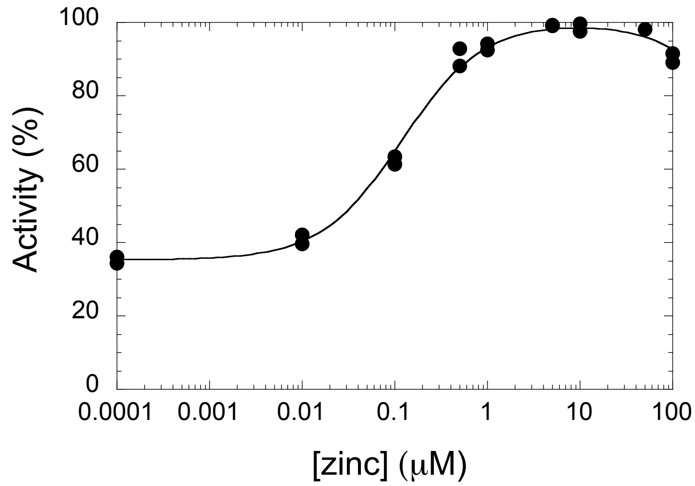

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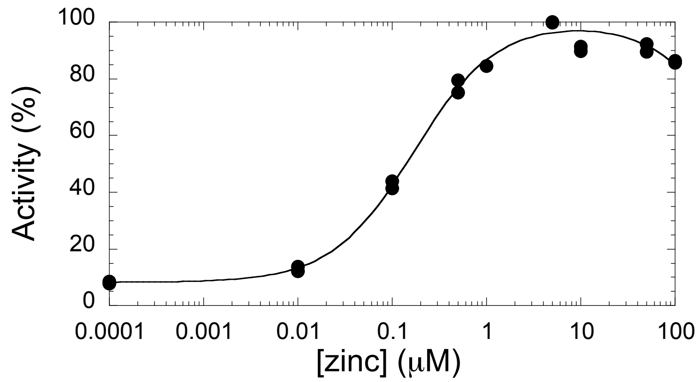

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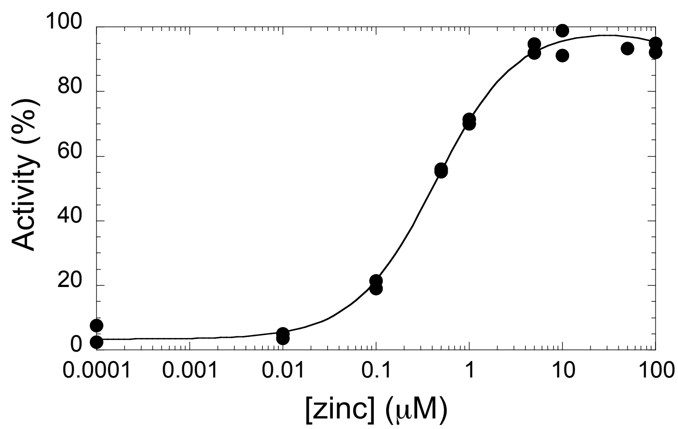

Figure S9: Zinc(II) dependence of NDM variants for hydrolysis of Ampicillin. Fits are as described for Figure 10 and Table 4. Each variant used is labeled in the separate plots above.

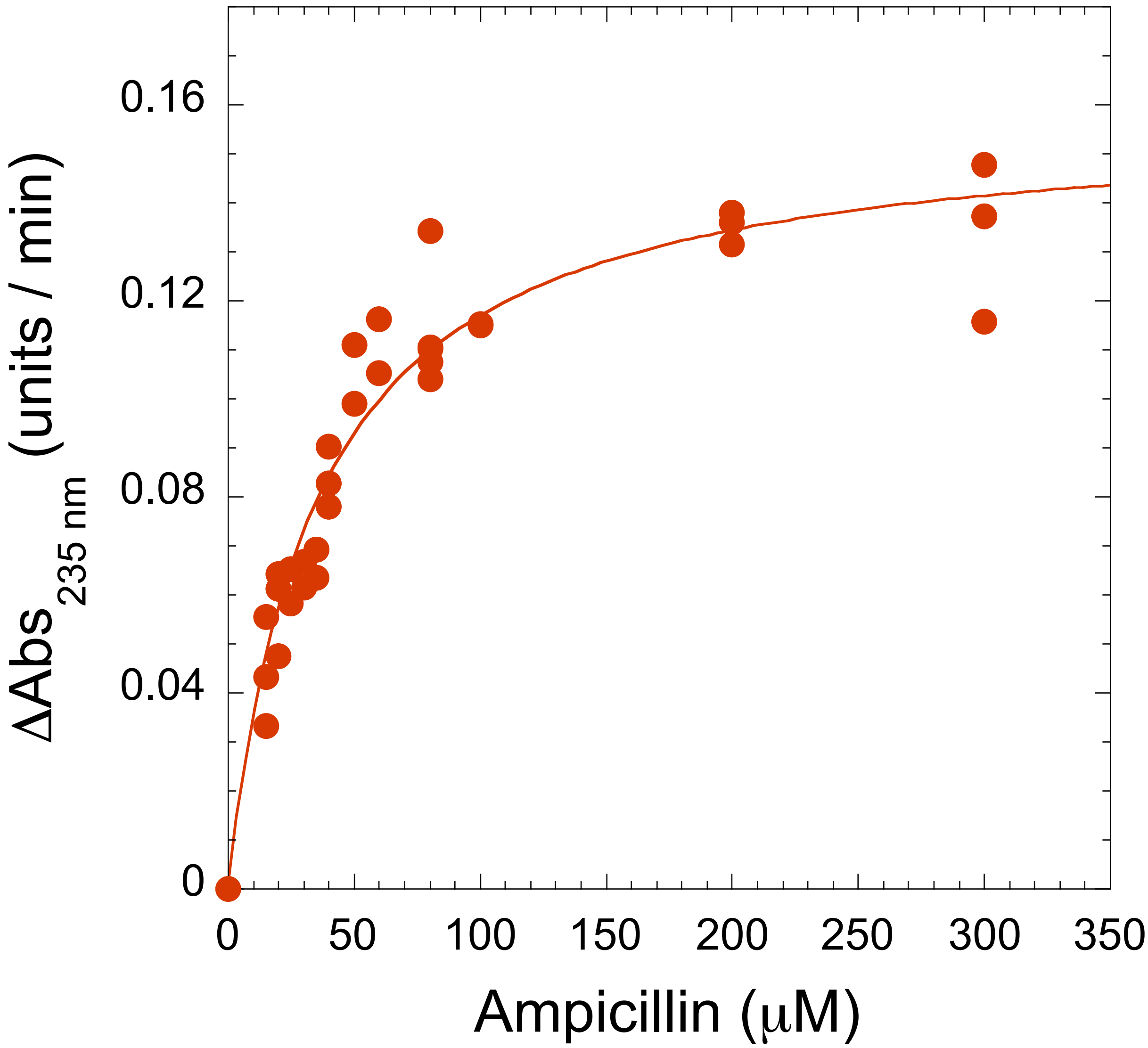

Figure S10. Determination of Kinetic Parameters for Monzinc NDM-15. These experiments were completed using 0.1 nM added $ZnCl_2$ and are near the limit of detection for this assay in our hands. Values for k_{cat} and K_M are given in Table 5.

Supporting Information References

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