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A Kinetic Study of the Replacement by Site Saturation Mutagenesis of Residue 119 in NDM-1 Metallo-β-Lactamase

Francesca Marcoccia,[a] Paola Sandra Mercuri,[b] Moreno Galleni,[b] Giuseppe Celenza,[a] Gianfranco Amicosante,[a] Mariagrazia Perilli[a]

[a]Dipartimento di Scienze Cliniche Applicate e Biotecnologiche, Università degli Studi dell'Aquila, L'Aquila, Italy

[b]Laboratoire de Macromolécules Biologiques, Centre d'Ingénierie des Protéines, Université de Liège, Liège, Belgium

ABSTRACT New Delhi metallo-β-lactamase 1 (NDM-1) is a subclass B1 metallo-β-lactamase that exhibits a broad spectrum of activity against β-lactam antibiotics. Here we report the kinetic study of 6 Q119X variants obtained by site-directed mutagenesis of NDM-1. All Q119X variants were able to hydrolyze carbapenems, penicillins and first-, second-, third-, and fourth-generation cephalosporins very efficiently. In particular, Q119E, Q119Y, Q119V, and Q119K mutants showed improvements in k_{cat}/K_m values for penicillins, compared with NDM-1. The catalytic efficiencies of the Q119K variant for benzylpenicillin and carbenicillin were about 65- and 70-fold higher, respectively, than those of NDM-1. The Q119K and Q119Y enzymes had k_{cat}/K_m values for ceftazidime about 25- and 89-fold higher, respectively, than that of NDM-1.

KEYWORDS NDM, kinetic, metallo-β-lactamase

New Delhi metallo-β-lactamase 1 (NDM-1) plays a key role in antibiotic-resistant bacterial infections. NDM-1-producing bacteria have been identified from both nosocomial and community-acquired infections, including urinary and pulmonary tract infections, peritonitis, septicemia, and osteomyelitis, throughout the world (1–4). The rapid emergence of this enzyme in multiple enteric bacteria is due to the favorable genetic characteristics of the producing plasmids. Multiple antibiotic resistance genes were found to be harbored by plasmids carrying the bla_{NDM-1} determinant (5, 6). Therefore, NDM-1-producing strains showed high levels of resistance to a wide range of β-lactams, except monobactams, and also other antibiotic families (7). Structurally, the NDM-1 enzyme displays the typical αβ/βα sandwich architecture (8–11). The active site of NDM-1 is surrounded by several loops that are responsible for substrate binding and specificity and by two zinc ion binding sites, referred to as sites 1 and 2 (9). The two zinc ions promote substrate catalysis; Zn-1 is tetracoordinated by the imidazole groups of three histidine residues, H116, H118, and H196, and one water molecule, whereas Zn-2 is pentacoordinated by H263, D120, and C221 (BBL numbering [12]) and two water molecules. NDM-1 displays 32% sequence identity to the most common metallo-β-lactamases (MBLs), IMP-1 and VIM-2 (9). Several studies demonstrated that, in MBLs, D120 plays an important role in orienting the active site hydroxide ion, which serves as a general base (13). A recent study of mutagenesis at position 120 in NDM-1 validated the hypothesis that this residue acts as a strong Zn-2 ligand and is involved in the interaction with substrate (13).

In the present study, to better understand the function of residue 119 in the NDM-1 enzyme, site saturation mutagenesis was performed on the bla_{NDM-1} gene (codons 247 to 249 [CAG]) to generate NDM-1 variants, named Q119X. The codon was randomized using overlap-extension PCR, as described previously (14), with the pFM-NDM-1 plasmid as the template (15). Mutations were introduced into a PCR amplicon using the mutagenic primers Q119X_for (5′-CTCACGCGCATNNNGACAAGATG) and Q119X_rev

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Address correspondence to Mariagrazia Perilli, perilli@univaq.it.

TABLE 1 Pattern of β-lactam resistance mediated by NDM-1 and Q119X mutants in *E. coli* XL-1, in comparison with *E. coli* XL-1/pBC-SK

Antibiotic	MIC (μg/ml)							
	NDM-1	Q119C/Q119S	Q119E	Q119K	Q119F/Q119T	Q119Y/Q119I/Q119L	Q119V	pBC-SK
Benzylpenicillin	512	64	64	>512	512	512	512	0.250
Piperacillin	256	>512	>512	>512	>512	>512	>512	0.500
Amoxicillin	128	256	256	256	256	256	256	0.500
Imipenem	1	1	1	8	16	4	8	0.125
Meropenem	2	16	4	16	16	16	16	<0.0625
Cefepime	32	32	8	>128	128	128	128	<0.0625
Cefoxitin	8	64	32	128	64	64	64	0.125
Cefazolin	128	256	256	>256	256	256	>256	<0.0625
Cefotaxime	128	128	256	256	256	256	256	<0.0625
Ceftazidime	256	128	128	>128	>128	>128	>128	<0.0625

(5′-CATCTTGTCNNNATGCGCGTGAG), where N represents any of the four base pairs at the codon position to be randomized. The external primers NDM_for (5′-GGGGG**GGT ACCCATATG**GGTGAAATCCGCCCGA) and NDM_rev (5′-GGGGG**GAGCTCGAG**TCAGCGC AGCTTGTCGGC) (KpnI/NdeI and SacI/XhoI restriction sites are shown in bold) were used to amplify the entire bla_{NDM-1} gene. The amplification product, digested with KpnI and SacI, was ligated into the pBC-SK(+) Bluescript vector, and the ligation product was transformed into *Escherichia coli* XL-1 Bluescript cells. Functional random mutants were selected by growth in the presence of chloramphenicol (30 μg/ml), ampicillin (100 μg/ml), and meropenem (1 μg/ml) and then were sequenced (ABI Prism 3500 eight-capillary automated sequencer; Life Technologies, Monza, Italy) to identify the amino acid substitutions at the randomized position. Among functional clones, 11 NDM-1 mutants were identified, i.e., Q119I, Q119S, Q119L, Q119Y, Q119C, Q119T, Q119F, Q119K, Q119V, Q119E, and Q119M. The phenotypic profiles were characterized by the microdilution method using a bacterial inoculum of 5×10^5 CFU/ml of *E. coli* XL-1/pBC-SK-Q119X, according to Clinical and Laboratory Standards Institute (CLSI) performance standards (16). The antimicrobial susceptibility to penicillins, cephalosporins, and carbapenems was tested for all recombinant strains, including wild-type *E. coli* XL-1/pBC-SK-NDM-1 and *E. coli* XL-1/pBC-SK (Table 1). Like NDM-1, all recombinant mutants exhibited resistance to penicillins, cefazolin, cefotaxime, ceftazidime, and cefepime. Only *E. coli* XL-1/pBC-SK-Q119E showed a MIC value of 8 μg/ml for cefepime. For cefoxitin, all recombinant strains showed MIC values higher than that of *E. coli* XL-1/pBC-SK-NDM-1. For all strains tested, the imipenem and meropenem MIC values ranged from 1 μg/ml to 16 μg/ml (Table 1). The mutants were categorized on the basis of MIC values and, in this study, only the following 6 NDM-1 variants were investigated on the basis of their phenotypic profiles: Q119C, Q119F, Q119E, Q119Y, Q119V, and Q119K. For overexpression of the 6 NDM-1 variants, the bla_{Q119X} mutant genes, without signal peptide, were subcloned into the pET-24a(+) vector and transformed into *E. coli* BL21(DE3). Purification of the different NDM-1 mutants was performed as described previously (15). From 400 ml of an overnight culture in tryptic soya broth (TSB), we obtained about 5 mg of each NDM-1 mutant. Kinetic experiments were performed by following the hydrolysis of each substrate at 25°C in 25 mM sodium phosphate buffer (pH 7). The data were collected with a Perkin-Elmer Lambda 25 spectrophotometer (Perkin-Elmer Italia, Monza, Italy). Steady-state kinetic analyses were performed under initial-rate conditions using the Hanes plot linearization method (16). For K_m values of ≤10 μM, the K_m values were determined as K_i values, using nitrocefin as the reporter substrate (17). Each kinetic value is the mean of five different measurements; errors were <5%. In Table 2, we show the K_m, k_{cat}, and k_{cat}/K_m values for Q119C, Q119F, Q119E, Q119Y, Q119V, and Q119K variants, compared to NDM-1 (data for NDM-1 were taken from reference 15), with some carbapenems, penicillins, and cephalosporins.

Imipenem and meropenem were good substrates for all Q119X variants analyzed, with k_{cat} values higher than that calculated for NDM-1 (except for Q119E with imipenem). The Q119F variant showed a k_{cat} value for meropenem of 1,500 s^{-1}, about

TABLE 2 Kinetic parameters for Q119X mutants, compared with NDM-1, with some β-lactams

Substrate and parameter[a]	NDM-1[b]	Q119C	Q119F	Q119E	Q119Y	Q119V	Q119K
Imipenem							
k_{cat} (s^{-1})	64	69	336	41	79	229	393
K_m (μM)	35	50	157	40	30	65	90
k_{cat}/K_m (μM^{-1} s^{-1})	1.83	1.38	2.14	1.02	2.63	3.52	4.37
Meropenem							
k_{cat} (s^{-1})	75	252	1500	82	561	269	357
K_m (μM)	80	100	252	146	105	20	37
k_{cat}/K_m (μM^{-1} s^{-1})	0.94	2.52	5.95	0.56	5.34	13.45	9.65
Benzylpenicillin							
k_{cat} (s^{-1})	105	100	166	100	34	231	414
K_m (μM)	250	71	89	5[c]	8[c]	45	15
k_{cat}/K_m (μM^{-1} s^{-1})	0.42	1.41	1.86	20.0	4.25	5.10	27.6
Carbenicillin							
k_{cat} (s^{-1})	108	52	158	234	216	267	929
K_m (μM)	285	59	131	18	31	32	35
k_{cat}/K_m (μM^{-1} s^{-1})	0.38	0.88	1.21	13.00	6.97	8.34	26.54
Cefazolin							
k_{cat} (s^{-1})	42	48	169	63	561	269	125
K_m (μM)	20	11	27	4[c]	105	20	32
k_{cat}/K_m (μM^{-1} s^{-1})	2.10	4.36	6.26	15.75	5.34	13.45	3.90
Cefoxitin							
k_{cat} (s^{-1})	23	99	152	64	85	193	58
K_m (μM)	26	23	21	23	17	18	49
k_{cat}/K_m (μM^{-1} s^{-1})	0.88	4.30	7.24	2.78	5.00	10.72	1.18
Cefotaxime							
k_{cat} (s^{-1})	20	43	59	4	7	21	89
K_m (μM)	14	7[c]	6[c]	2[c]	2[c]	10[c]	3[c]
k_{cat}/K_m (μM^{-1} s^{-1})	1.43	6.14	9.83	2.00	3.50	2.00	29.67
Ceftazidime							
k_{cat} (s^{-1})	18.5	74	257	14	68	7	106
K_m (μM)	50	163	403	140	2[c]	3[c]	11
k_{cat}/K_m (μM^{-1} s^{-1})	0.37	0.45	0.64	0.10	34.00	2.33	9.64
Cefepime							
k_{cat} (s^{-1})	13	22	67	18	30	71	60
K_m (μM)	35	30	16	40	4[c]	10[c]	18
k_{cat}/K_m (μM^{-1} s^{-1})	0.37	0.73	4.19	0.45	7.50	7.10	3.33

[a]Each kinetic value is the mean of five different measurements; the error was <5%.
[b]Kinetic data for NDM-1 were from reference 15.
[c]K_m values were determined as K_i values, using nitrocefin as the reporter substrate (19).

20-fold higher than the NDM-1 value. Regarding penicillins, the Q119X variants showed lower K_m values toward both benzylpenicillin and carbenicillin, with values ranging from 5- to 50-fold less than the NDM-1 values. The k_{cat} values for benzylpenicillin were quite similar to the NDM-1 value except for Q119Y, with a k_{cat} value about 3-fold lower than the NDM-1 value, and Q119K, with a k_{cat} value 4-fold higher than the NDM-1 value. Concerning carbenicillin, the Q119X variants (not including Q119C) showed higher k_{cat} values.

All NDM-1 variants hydrolyzed first-, second-, third-, and fourth-generation cephalosporins very efficiently (Table 2). Indeed, the k_{cat} values calculated for cefazolin, cefoxitin, and cefepime were higher than the NDM-1 values. Differences in the k_{cat} values determined with cefotaxime were observed among the Q119X variants. The Q119V variant exhibited the same k_{cat} value as NDM-1, the Q119E and Q119Y variants showed lower k_{cat} values, and the Q119C, Q119F, and Q119K variants showed higher k_{cat} values. Regarding ceftazidime, we observed different behavior among the Q119X variants. For example, the Q119Y showed a k_{cat} of 68 s^{-1}, a K_m of 2 μM, and a k_{cat}/K_m

TABLE 3 Ratios of k_{cat}/K_m values for NDM-1 and mutants

Substrate	k_{cat}/K_m for Q119X variant/k_{cat}/K_m for NDM-1					
	Q119C	Q119F	Q119E	Q119Y	Q119V	Q119K
Benzylpenicillin	3.4	4.4	47.6	10.1	12.1	65.7
Carbenicillin	2.3	3.2	34.2	18.3	21.9	69.8
Imipenem	0.7	1.2	0.6	1.4	1.9	2.4
Meropenem	2.7	6.3	0.6	5.7	14.3	10.3
Cefazolin	2.1	2.9	7.5	2.5	6.4	1.9
Cefoxitin	4.9	8.2	3.2	5.7	12.2	1.3
Cefotaxime	4.3	6.9	1.4	2.4	1.4	20.7
Ceftazidime	1.9	1.7	0.3	89.5	6.1	25.4
Cefepime	2.0	11.3	1.2	20.3	19.2	9.0

value about 10-fold higher than the NDM-1 value. Cefepime, a fourth-generation cephalosporin, was more stable than cefazolin, cefoxitin, cefotaxime, and ceftazidime with all NDM-1 variants. For all Q119X variants (with rare exceptions), increases in the catalytic efficiency (k_{cat}/K_m), compared with NDM-1, were detected with all β-lactams examined (Table 3). In particular, the Q119E, Q119Y, Q119V, and Q119K variants showed significant improvements in k_{cat}/K_m for penicillins, due to lower K_m values. The catalytic efficiencies of the Q119K variant for benzylpenicillin and carbenicillin were about 65- and 70-fold higher, respectively, than those of NDM-1. The Q119K and Q119Y enzymes had k_{cat}/K_m values for ceftazidime about 25- and 89-fold higher, respectively, than that of NDM-1. The Q119E variant showed high catalytic efficiencies with penicillins but lower efficiencies with carbapenems and ceftazidime. The replacement of glutamine (Q) with cysteine (C), phenylalanine (F), glutamic acid (E), tyrosine (Y), valine (V), or lysine (K) considerably increased the activity of NDM-1 for some β-lactams, such as benzylpenicillin, carbenicillin, meropenem (except Q119E), ceftazidime (particularly true for Q119Y and Q119K), and cefepime (particularly true for Q119Y and Q119V). However, no clear correlation between the MIC values and the kinetic data could be observed, mainly because the MIC values for the majority of substrates did not differ substantially between wild-type NDM-1 and the mutants. For cefazolin, cefoxitin, and meropenem, the Q119V variant was always the most efficient enzyme (up to 10-fold greater than NDM-1), followed by Q119F and Q119E (about 2- to 5-fold greater than NDM-1). For benzylpenicillin, carbenicillin, cefotaxime, ceftazidime, and meropenem, Q119K was the most efficient enzyme, with catalytic efficiencies about 66-, 69-, 20-, 25-, and 10-fold higher, respectively, than those of NDM-1.

Residue 119 is positioned in the L5 loop, which includes important catalytic residues such as H116, H118, and D120. Q119 is adjacent to D120 and we presume that it could play an important role in the activities of NDM-1. Structural studies of NDM-1 (18) suggested that Q119 maintains the correct orientation of H118 and D120 in the Zn-1 coordination and therefore plays a major role in the catalytic properties of NDM-1. The study by Chiou et al. demonstrated that Q119D and Q119A mutants showed reduced carbapenem, ampicillin, and cefepime MIC values and Q119D showed reductions in k_{cat} values for the same β-lactams (18); this seems to be a discrepancy with respect to the results obtained with our mutants, for which substitutions at position 119 led to enzymes with major activity, compared to the wild-type NDM-1 enzyme. The results obtained in the present study suggest that the involvement of residue 119 in NDM-1 activity is related to the amino acid structure. Our results highlight this position as a good candidate for the evolution of the NDM MBLs.

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We have no conflicts of interest to declare.

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