THE REACTION MECHANISM OF METALLO-β-LACTAMASES IS TUNED BY THE CONFORMATION OF AN ACTIVE SITE MOBILE LOOP

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Running title: Mobile loop tunes reaction mechanism of MβLs

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ABSTRACT

Carbapenems are “last resort” β-lactam antibiotics, used to treat serious and life-threatening healthcare-associated infections caused by multidrug resistant Gram-negative bacteria. Unfortunately, the worldwide spread of genes coding for carbapenemases among these bacteria is threatening these life-saving drugs. Metallo-β-Lactamases (MβLs) are the largest family of carbapenemases. These are Zn(II)-dependent hydrolases that are active against almost all β-lactam antibiotics. Their catalytic mechanism and the features driving substrate specificity have been matter of intense debate. The active sites of MβLs are flanked by two loops, one of which, loop L3, was shown to adopt different conformations upon substrate or inhibitor binding, and thus being expected to play a role in substrate recognition. However, the sequence heterogeneity observed in this loop in different MβLs has limited the generalizations about its role. Herein we report the engineering of different loops within the scaffold of the clinically relevant carbapenemase NDM-1. We find that the loop sequence dictates its conformation in the unbound form of the enzyme, eliciting different degrees of active site exposure. However, these structural changes have a minor impact on the substrate profile. Instead, we report that the loop conformation determines the protonation rate of key reaction intermediates accumulated during the hydrolysis of different β-lactams in all MβLs. This study demonstrates the existence of a direct link between the conformation of this loop and the mechanistic features of the enzyme, bringing to light an unexplored function of active site loops on MβLs.

INTRODUCTION

β-Lactams are the most frequently prescribed class of clinically available antibiotics, used to treat infections caused by both Gram-negative and Gram-positive bacteria. β-Lactams inhibit bacterial cell wall synthesis by targeting transpeptidases and carboxypeptidases (bacterial cell wall synthesizing enzymes) (1). The main mechanism of bacterial resistance against β-lactams in Gram-negative bacteria is the expression of β-lactamases, enzymes that selectively hydrolyze the β-lactam ring, rendering the antibiotic ineffective (2, 3). Two distinct types of β-lactamases are currently known: serine-β-Lactamases, which employ a Ser residue as the active nucleophile in catalysis, and metallo-β-Lactamases (MβLs), which are metal-dependent hydrolytic enzymes. MβLs are of medical concern given their ability to hydrolyze and confer resistance to virtually all classes of β-lactam antibiotics. Notably, all MβLs show hydrolytic capacities against carbapenems, the most potent β-lactam antibiotics to date, routinely used as ”last resort drugs” (1). Although some compounds have been found to be effective as MβL inhibitors (4-12), none of them are available to treat clinical infections yet, giving rise to a crisis in antimicrobial chemotherapy (13-18).

MβLs are subdivided in 3 subclasses (B1, B2 and B3) based on the identity of active site essential residues, Zn(II) requirements and substrate profile (15, 16, 19-22). B1 MβLs are those of major clinical concern, because they are broad substrate spectrum β-lactamases and are encoded on mobile genetic elements in pathogenic and opportunistic bacteria. The most clinically relevant B1 lactamases belong to the NDM (New Delhi Metallo-β-Lactamase), VIM (Verona Integron-encoded MβL), and IMP (Imipenemase MβL) families, with NDM-1, VIM-2 and IMP-1 being the most widespread allelic variants. In particular, NDM-1 is a membrane-anchored enzyme (23) and is one of the most widespread MβLs

with a potent carbapenemase activity (16, 24, 25).

Two Zn(II) ions are required for the catalytic activity of B1 MβLs (26, 27). Both Zn(II) atoms are bound to a conserved ligand set: one Zn(II) ion is coordinated to three His residues (116, 118 and 196, after the standard BBL numbering (28)) and a hydroxide molecule (3H site), whereas the other is bound to the same (bridging) OH, an extra water molecule and residues Asp120, Cys221 and His263 (DCH site) (29-31). The metal ion at the DCH site has been shown to be essential in stabilizing key reaction intermediates during hydrolysis of chromogenic cephalosporins (32) and carbapenems (33, 34). This active site is located in a shallow and broad groove flanked by two loops: active site loop L10, and active site loop L3. Amino acid substitutions in both loops are associated with changes in the substrate profile in B1 MβLs (35-40). Despite different families of B1 MβLs present a conserved active site and global protein fold, these β-lactamases share very low sequence identity. This diversity has posed additional challenges for the development of an MβL inhibitor. Thus, the identification of common and distinct features is crucial for the understanding of their mechanism and substrate recognition profile.

Loop L3 has been the focus of several studies in MβLs. Crystallographic and NMR studies identified variable conformations of this loop in many B1 MβLs (38, 41-45), and its role in specific interactions with their substrates is well documented (36, 39, 44, 46). Moreover, an increase of the dynamics of this loop was hypothesized with the broadening of the substrate profile in an *in vitro* evolved lactamase (37). The consensus identifies loop L3 as a mobile flap able to adapt its conformation upon small molecule binding in the active site. However, the sequence heterogeneity observed in the loop L3 from different MβLs has limited generalizations about its specific role.

To explore the role of the loop L3 in the scaffold of NDM-1, we designed a series of

variants in which we replaced the native loop by those of IMP-1 or VIM-2, and a third one in which a Pro residue was introduced at the C-terminus of the loop. Herein we show that the substrate spectra and the active site structure display minor perturbations in these chimeric proteins, despite previous expectations. Crystal structures of two of the obtained chimeras show that different loops in the same scaffold adopt quite different conformations, spanning from an open to a close active site in the unbound form of the enzyme. However, the loop conformation cannot correlate to the substrate profile observed for the different variants. Instead, the loop conformation directly impacts on the accumulation of the anionic reaction intermediates, disclosing an auxiliary structural determinant of the mechanism of hydrolysis. These findings suggest a new role of this mobile loop in the catalytic mechanism of MβLs.

RESULTS

Loop L3 engineering gives rise to active and stable NDM variants

A closer look at the primary sequence of the loop L3 of IMP-1, VIM-2 and NDM-1 reveals significant differences (Fig. 1). For instance, the loop L3 of IMP-1 displays the same length as that from NDM-1, but it contains more polar residues. In contrast, the VIM-2 loop L3 shows a similar charge distribution but is one residue shorter and less hydrophobic than NDM-1 loop L3. Also, a proline residue is located at the C-terminus of the loop in all cases, except in NDM-1. To study the function of loop L3 in NDM-1, we designed two chimeric proteins in which the NDM-1 loop was replaced by the loops of IMP-1 and VIM-2 (including the Pro residues). These variants were designated as L3IMP and L3VIM, respectively. In order to assess how an insertion could impact in the function of loop L3, we also engineered an extra MβL in which a Pro residue was inserted at the base of the NDM-1 loop, the L3Pro variant.

We used both *in bacteria* and *in vitro* approaches to analyze the L3 variants. *Escherichia coli* cells expressing the chimeric proteins were used to analyze MβLs properties in a natural background. We evaluated the expression levels in whole cells, spheroplasts and periplasmic extracts by immunoblotting (Fig. 2A). All variants were expressed including the native leader peptide of NDM-1, containing the canonical lipidation sequence LSGC (lipobox), which anchors the protein to the inner leaflet of the outer membrane (23, 43). None of the variants was in the periplasmic extracts, revealing an adequate processing of the leader peptide. The analysis of whole cell extracts and spheroplasts showed that NDM-1 and variants L3IMP and L3Pro showed similar expression levels and that the L3VIM variant was expressed at lower levels, but without compromising protein stability (Fig. 2A). These experiments show that our loop L3 engineering has been successful in eliciting stable proteins.

We next tested the susceptibility of *E. coli* cells expressing the three NDM-1 L3 variants against a broad panel of β-lactam antibiotics. Minimal inhibitory concentration (MIC) analyses (Table 1) reveal that the L3 variants confer somehow lower levels of resistance against all tested substrates. In the case of cefepime, the impact of the mutations in the MIC values is larger. MβLs with reduced Zn(II) binding capabilities provide lower levels of resistance because metal binding takes place in the periplasmic space where Zn(II) availability is limited (26, 47, 48). We tested the effect of Zn(II) deprivation on protein expression levels by treating *E. coli* cells expressing NDM-1 variants with the metal chelator dipicolinic acid (DPA). This chelator strongly affected the expression of L3Pro and L3VIM variants, while the impact was moderate for variant L3IMP and wild type NDM-1 (Fig. 2B). We also tested the ability of our NDM-1 variants to bind Zn(II) within the cell by determining the impact of DPA in the MIC values against cefotaxime. The sensitivity of L3IMP to Zn(II)

deprivation resembled that of NDM-1, whereas it was increased for L3Pro and even more for the L3VIM variant (Fig. 2C).

We then expressed and purified all variants in the soluble form, a truncated version in which the first 38 residues, including the signal peptide and the lipidation site, were removed (Δ38). All variants were obtained by expression in rich (LB) medium with similar yields as the wild type NDM-1, except for L3VIM, for which yields one order of magnitude lower were obtained. Also, the Zn(II) affinity of L3VIM was lower than the other variants (Table S1). This could explain the higher sensitivity of L3VIM when challenged with DPA (Fig. 2C) and the lower metal content (Materials and Methods section).

We measured the stability of the purified variants and their apo-derivatives by thermal shift analysis (Table 2). The L3 variants were slightly less stable than the wild type enzyme, with T_M values spanning a narrow range (4 °C). Instead, the non-metallated forms displayed a more pronounced destabilization effect. The L3VIM variant features the largest gap between the T_Ms of metallated and non-metallated forms, indicating that in this case the Zn(II) ions play a crucial role in stabilizing the MβLs. These results added to the differences on the expression levels under DPA addition, demonstrate that the stability of the non-metallated form determines steady state protein expression levels and confirms that the metal uptake in the periplasm is crucial for MβLs stability.

The activity of the enzymes was then studied by steady-state kinetics using purified MβLs. The determined k_{cat}/K_M values were within the same range of those measured for NDM-1 and all variants showed broad substrate spectra (Table 3). In general, the L3Pro variant displayed the lowest catalytic efficiencies, and L3IMP was the only variant that was catalytically more efficient compared to NDM-1 against some penicillins and cephalosporins. We stress that L3Pro displayed both elevated K_M and k_{cat} values, with

larger increases in K_M resulting in overall lower catalytic performances. The kinetic parameters measured for L3VIM, in contrast, are closer to those of NDM-1. As L3VIM present a lower expression level than NDM-1 (Fig. 2A), it is likely that the MICs values obtained may have been lower due to a decreased expression level or Zn(II) uptake in the periplasm. The hydrolysis parameters for nitrocefin were practically unaltered upon loop replacement. On the other hand, for carbapenem hydrolysis both K_M and k_{cat} values presented differences within the variants: L3IMP parameters were lower than the wild type values, and for L3VIM and L3Pro these values were higher than in the wild type enzyme. Notwithstanding these changes in the substrate preferences, a clear trend in the substrate profile elicited by the loop replacement could not be identified in the chimeric proteins.

Loop L3 conformation models the active site cavity size and accessibility with minor alterations on metal ligands

Although residues in loop L3 do not directly interact with the Zn(II) ions, the different sensitivities to Zn(II) deprivation prompted us to test whether the loop L3 replacements induced changes in the metal binding sites in the variants. For that purpose, we used Co(II) as a spectroscopic probe of the metal site coordination geometry, replacing the spectroscopically silent Zn(II) ion (49). Fig. 3 shows the electronic absorption difference spectra in the UV-Vis range of all Co(II) derivatives. These spectra are characterized by distinctive features in two regions: (1) the Laporte-forbidden *d-d* transitions in the visible range (450−650 nm), which provide information on the metal site geometry (mainly of the 3H site); and (2) a ligand-to-metal charge transfer transition (LMCT), ca. 330 nm, which reports on the Cys-Co(II) interaction at the DCH site (29). The band pattern and intensity of the *d-d* bands were preserved in all variants, revealing that the geometry at the 3H site was conserved (Fig. 3). The Co(II) derivatives displayed subtle changes on the position and

intensity of the LMCT band, which reflects minor changes in the Co(II)-thiolate interactions at the DCH site, especially for the L3Pro and L3VIM variants. These results suggest that loop replacement did not significantly alter the coordination sphere of the metal ion in the active site.

To rationalize the impact of changes in loop L3, we performed X-ray crystallographic studies. The L3IMP and L3Pro variants were crystallized, and both structures were solved at a resolution of 1.65 and 1.80 Å, respectively (Table S2). Attempts to obtain crystals of the L3VIM variant were unsuccessful. The structures of both variants are very similar to the previously reported crystal structure of native NDM-1 (PDB code: 3SPU) (43). The overall structure of the enzymes and their active sites are highly conserved (Fig. 4A), as accounted for the low rmsd values of the core structure without considering the loop L3 (< 0.60 Å over all Cα). The active sites of L3IMP and L3Pro displayed bimetallic occupancy, *i.e.,* with metal ions at the 3H and the DCH sites. The presence of Zn(II) was verified by anomalous diffraction. In the case of L3Pro, peaks of 40-50 rmsd were observed at the 3H site, confirming the presence of Zn(II), while no signal was observed at the DCH site (Fig. S1). Instead, the electron density could be properly accounted for by assuming the presence of Cd(II) (from the crystallization buffer) at this position. This metal substitution with a preserved geometry at the active site has already been reported for NDM-1 (3ZR9) (41).

The structures did not reveal significant changes in the metal binding sites (Fig. 4B). The position of the Zn(II) ion and the three ligands in the 3H site remains unaltered among the L3 variants. In the DCH site, despite the different identity of the metals (Zn(II) in L3IMP and Cd(II) in L3Pro), a variation of only 0.5 Å for the metal ion position was observed, with the ligand residues displaying identical conformations. This observation agrees with the

spectroscopic data on the Co(II)-substituted enzymes that reveal a slightly perturbed DCH site, and a conserved geometry at the 3H site.

The conformation of loop L3, instead, is considerably different among the variants (Fig. 4C). The electron density is well defined in these loops in both variants, with B-factors slightly higher than those in the protein core (around 20 and 30 $Å^2$ higher for L3IMP and L3Pro, respectively) (Fig. S2). In L3Pro, the interactions among the residues shaping the β-sheet at the base of loop L3 are disrupted by the insertion of a Pro residue, giving rise to a more open loop conformation. In the case of L3IMP, the loop is stabilized by hydrophobic interactions of Trp64 with residues His263, Val67 and Val61, which pull Trp64 towards the active site inducing a more closed conformation of the loop (Fig. S3). The angle subtended by Zn1, the Cα atom of Ser69 (located on the base of loop L3) and the Cα atom of Gly63 (located on the tip of the loop), provides a *bona fide* description of the loop 3 conformation (the larger the angle, the more open the loop), varying from 68° (L3IMP), 88° (NDM-1) to 110° (L3Pro) (Fig. 4C). Thus, the L3 sequence dictates the conformation of the loop in the unbound form of this enzyme within the same protein scaffold.

Loop L3 determines the accumulation of catalytic reaction intermediates

The catalytic mechanism of MβLs takes place by accumulation of an anionic intermediate that has been characterized for the hydrolysis of the chromogenic cephalosporin, nitrocefin, and several carbapenems (33, 34, 50, 51). The rate-determining step of the reaction is, in both cases, the protonation of this intermediate, leading to the final product. Moali *et al.* reported that changes in loop L3 altered the accumulation of the intermediate during nitrocefin hydrolysis (39). Thus, we decided to study the hydrolysis of nitrocefin and carbapenems by our variant MβLs under pre-steady-state conditions with a photodiode array (PDA) detector coupled to a stopped-flow mixing device (32, 33).

Three absorption bands are observed during nitrocefin hydrolysis at 390, 485 and 605 nm, which correspond to the substrate, product, and the anionic intermediate of the reaction, respectively (Fig. 5A) (32, 50). When we performed the hydrolysis reaction with all the L3 variants, the three bands were evidenced with similar maximum absorbance intensities (Fig. 5B). An analysis of the time course of the reaction (Fig. 5C) reveals that the time frame for accumulation and decay of the intermediate is similar for NDM-1 and the L3IMP variant, with L3IMP displaying the intermediate with the longest accumulation time. Instead, the intermediate is less stable in the L3VIM and, specially, L3Pro variants. Supporting this line, product formation is evidently faster in L3Pro, followed by L3VIM, NDM-1 and L3IMP; indicating that the decrease on the accumulation of the reaction intermediate is due to an increment on the protonation rate. Substrate consumption is also slower in NDM-1, and L3IMP, which could also contribute to a decrease in the rate of the reaction catalyzed by these enzymes.

Carbapenem hydrolysis by MβLs of the three subclasses takes place by a branched mechanism with two anionic intermediate species (EI^1 and EI^2, Fig. 6A) (33, 34, 52). Both species are productive, but the final product and the nature of the proton donor differ. EI^1 is the first intermediate produced, absorbs at 390-375 nm (in the case of imipenem and meropenem hydrolysis by NDM-1, respectively), and its N-protonation is produced by a water molecule bridging the Zn(II) ions (33). This species could give rise either to product or to a second intermediate species, EI^2. This second intermediate species absorbs at 340-336 nm (for imipenem and meropenem, respectively) and is later protonated by a bulk water molecule (not bound to the metal site) leading to the formation of an EP complex lacking a metal-bound water and, later, to a product stereoselectively protonated at C-2 (33).

As shown in Fig. 6B, the formation of both reaction intermediates was detected

during the hydrolysis of imipenem catalyzed by NDM-1 and the L3IMP variant, being more abundant in the second case. However, in the reaction catalyzed by L3VIM or L3Pro, we could not detect the accumulation of any of those intermediates, indicating that the protonation occurred too fast for the species to accumulate to a detectable amount. As accumulation of EI^1 also depends on the formation and consumption of EI^2, the decrease on the accumulation of the first intermediate species could be due only to an alteration of the protonation rate of EI^2, by a bulk water molecule. For meropenem hydrolysis the formation of both reaction intermediates was detected with NDM-1 and the L3IMP variant, being, again, more abundant in the second case (Fig. 6C). On the contrary, the hydrolysis by L3VIM and L3Pro evidenced accumulation of only one intermediate (EI^1 in L3VIM and EI^2 in L3Pro), and to a minor extent than in the wild type enzyme (Fig. 6C). As these results resemble the pattern observed for imipenem, we conclude that these data evidence a general behavior for the hydrolysis of carbapenems (33). Our observations correlate with the steady state parameters for carbapenems where k_{cat} values in L3IMP were lower than the ones of wild type NDM-1, indicating that the reaction rate is diminished, probably due to a decrease in the protonation rate. L3VIM and L3Pro present higher k_{cat} values for carbapenems, correlating with the increased protonation rate.

DISCUSSION

A series of experimental studies of the loop L3 in B1 MβLs performed herein have provided critical information on its role, mostly regarding substrate recognition and mobility. Substitutions at this loop in IMP-1, IMP-12, IMP-43, IMP-18 (36), IMP-2 (45) and VIM-31 (38) elicited changes in the substrate profile. Moali and coworkers reported changes in the catalytic efficiency of BcII by engineering the loop of IMP-1 (39). However, a definitive

300 structural description of the effect of the alterations in this loop is not available. An
301 increased mobility of loop L3 in BcII variants was correlated to a broadening in the substrate
302 profile (37). DEER spectroscopy studies in NDM-1 have demonstrated that loop L3 closes
303 over the active site during catalysis, returning to its original position after hydrolysis (53, 54).
304 Theoretical calculations have predicted a correlation between the movement of loop L3 and
305 the catalytic efficiency in IMP-1 and IMP-6 (55, 56). NMR studies have described in detail
306 the flexibility of loop L3 in different B1 enzymes (37, 57-59), while crystal structures of
307 enzyme-inhibitor or enzyme-product adducts have pointed out how this loop reacts upon
308 small molecule binding to the active site of these MβLs (5-7, 44, 45, 58-62). Here we show
309 that loop replacement in the scaffold of NDM-1 gives rise to stable, folded proteins, and
310 does not shape the substrate profile of this enzyme. Instead, loop engineering affects the
311 catalytic mechanism, governing the accumulation of key reaction intermediates based on its
312 conformation.

313 Loop replacement induced distinct levels of destabilization in the NDM-1 scaffold.
314 Surprisingly, the largest destabilizing effects *in vitro* were evident in the apo (non-
315 metallated) variants, particularly in L3VIM. The destabilization induced in this variant also
316 correlates with a decreased affinity of the protein toward Zn(II), a higher sensitivity to Zn(II)
317 deprivation in bacteria, and lower expression levels. Some of us have recently reported
318 that, under conditions of Zn(II) deprivation, soluble periplasmic MβLs are degraded in the
319 apo forms (23). Our previous results account for the link between the reduced Zn(II) affinity
320 and the phenotype observed in the case of L3VIM. Overall, these data highlight the role of
321 Zn(II) binding for the stabilization of MβLs.

322 The crystal structures and spectroscopic data point to a structurally conserved metal
323 site, with a minor perturbation in the DCH site. Although crystals for the L3VIM variant could

not be obtained, the good agreement of Co(II)-substitution experiments with the crystal structures allow us to extrapolate the spectroscopic results on this variant with confidence to the native Zn(II) enzyme. We therefore expect the metal site in L3VIM to be less perturbed compared to NDM-1. Under this assumption, the observed changes could be attributed to changes in loop L3, as observed for the other two variants.

Major changes in the reported structures in this analysis are related to the conformation of the engineered loops (Fig. 4). Importantly, our results show that different loops can adopt a wide range of conformations within a given MβL scaffold and reveal details on how the loop L3 sequence defines its conformation. The loop L3 in IMP-1 is closer to the one observed in the engineered L3IMP in the NDM scaffold, while inhibitor binding to IMP-1 does not alter the loop conformation as here reported. The same holds for NDM-1, for which inhibitor binding (bisthiazolidines or captopril) does not affect the loop conformation as much as loop engineering does (5-7, 44, 60). We conclude that the loop L3 sequence (regardless of the MβL scaffold) strongly determines its conformation.

The catalytic performances of the engineered variants do not reveal a substantial change in the substrate profile of NDM-1. Instead, we report a significant impact on the catalytic mechanism as witnessed by changes in the accumulation of reaction intermediates in the hydrolysis of nitrocefin, imipenem and meropenem. The stability of these reaction intermediates depends on electrostatic interactions with the metal ions, particularly with the DCH site (33, 34, 63, 64) and it has been shown that subtle changes on the MβL active site could tune the stability of these species (26, 63, 65-68). However, the structure of the catalytic Zn(II) center is not perturbed by loop replacement, indicating that other structural features modulate the half-life time of the intermediate species.

The conformation of loop L3 directly affects the stability of these intermediates: a more closed loop (as in L3IMP) leads to an enhanced accumulation of the intermediate, while a more open active site (such as in L3Pro) decreases the amount of intermediate (Fig. 6). As the half-life of the anionic reaction intermediates depends on their protonation rates (33, 64), these results suggest that the protonation step could be modulated by the solvent accessibility in the different mutants. This also suggests that it is likely that the presence of residues in loop L3 that favor interactions with active site residues (such as Trp64 in L3IMP) could disfavor water accessibility, making the protonation process less effective. We cannot discard the presence of specific interactions of loop L3 with the intermediate species that may increase its stability. This effect may account for the lower k_{cat} values observed for this variant for carbapenem hydrolysis. Overall, these findings show that loop L3 plays an important role in the mechanism of β-lactam hydrolysis by MβLs by tuning the rate of the rate-limiting step and controlling the accumulation of key reaction intermediates according to its conformation.

MATERIALS AND METHODS

Bacterial Strains and Cloning

Escherichia coli DH5α was used for construction and expression of plasmid pMBLe, as well as for all microbiological and biochemical studies. pET26-*bla*$_{NDM-1}$ was kindly provided by Dr. James Spencer (University of Bristol, UK). The construction of pMBLe*bla*$_{NDM-1}$ has been described previously (23) and the same procedure was used for variants cloning. The full-length *bla*$_{NDM-1}$ gene (including its native peptide leaders) was amplified with addition of a C-terminal Strep-tag sequence (for comparative protein detection and quantification), and subcloned into the pMBLe plasmid. The expression of *bla* was induced by the addition of

100 µM IPTG. Addition of the Strep-tag at the C-terminus does not affect MBL ability to confer resistance (23).

Construction of the L3 variants

bla$_{NVIM}$, *bla*$_{NIMP}$ and *bla*$_{NPro}$ containing the *bla*$_{NDM-1}$ gene with the loop L3 of VIM-2, IMP-1 and a Pro residue introduced at the base of the loop, respectively, were custom synthesized (Celtek Genes). The region exchanged comprised residues between Ser57 and Ala68 of the NDM-1 structure.

MβL Detection

MβL expression was measured by immunoblotting of cell extracts as described previously (23). Briefly, 5 mL cultures of *E. coli* DH5α cells carrying the pMBLe *bla*$_{NDM-1}$, *bla*$_{NVIM}$, *bla*$_{NIMP}$, and *bla*$_{NPRO}$ plasmids were grown aerobically at 37 °C in LB broth with 20 µg/ml gentamicin to log phase (OD_{600nm} = 0.4). MβL expression was then induced with 100 µM IPTG, and cultures were left to grow to an OD_{600} of 1. Cultures were pelleted and cells were washed once with 20 mM Tris, 150 mM NaCl, pH 8.0. An aliquot of cell crude extract were separated on this step. The rest of the washed cells were pelleted again and were resuspended in 20 mM Tris, 0.1 mM EDTA, 20% w/v sucrose, 1 mg/mL lysozyme (from chicken egg white, Sigma-Aldrich, protein ≥90%), 0.5 mM PMSF, pH 8 (resuspension volume was normalized based on OD_{600nm}). The cells were incubated with agitation at 4ºC for 30 min. The cells were pelleted and the periplasmic extract was obtaining in the supernatant. The pellet consisting of spheroplasts was washed in 20 mM Tris, 0.1 mM EDTA, 20% w/v sucrose, pH 8 and resuspended in the same volume of this buffer. A total of 120 µL of the different extracts were mixed with 30 µL loading dye and separated by SDS-PAGE (10 µL of whole cells lysate, 25 µL spheroplasts and 25 µL periplasmic extracts) and transferred to a polyvinylidene difluoride membrane (Novex, Life

395 Technologies, Carlsbad, CA) by electroblotting. Strep-Tag® II monoclonal antibodies (at
396 1:1000 dilution from 200 µg/ml solution, Novagen) and immunoglobulin G-alkaline
397 phosphatase conjugates (at 1:3000 dilution) were used to detect MβL expression. GroEL
398 and MPB antibodies were added as loading controls. Protein band intensities were
399 quantified from PVDF membranes with ImageJ software (69).

400 **Cell-Based Assays**

401 To test the phenotypic effect of the loop L3 substitutions, the minimal inhibitory
402 concentrations (MICs) of piperacillin, ceftazidime, cefotaxime, cefepime, imipenem, and
403 meropenem were determined for each clone in the LB medium using the agar macrodilution
404 method according to CLSI guidelines (70). Protein expression was induced with 100 µM
405 IPTG, except for ceftazidime where induction was performed with 20 µM IPTG. In order to
406 measure the effect of Zn(II) availability on antibiotic resistance, the growth medium was
407 supplemented with varying concentrations of the metal chelator dipicolinic acid (DPA,
408 Merck, >98%). In all cases, plasmid expression was induced with 100 µM IPTG (71). An
409 extra measurement was performed with 500 µM of $ZnSO_4$ to reach the maximum activity of
410 the proteins (100%). DPA or $ZnSO_4$ were added to the LB plate along with gentamicin and
411 IPTG.

412 On the steady-state expression of proteins in cells treated with DPA, after 1 h of
413 induction with 100 µM IPTG, *E. coli* DH5α cells expressing the NDM variants were
414 incubated during 15 min with or without 250 µM DPA. Protein expression was detected from
415 10 µl of whole cell lysates by western blot, as previously described.

416 **Protein Purification**

417 For kinetic studies, mature MβLs (residues 39 to 270) were produced in *E. coli* BL21
418 (DE3) and purified as previously published for NDM-1 (5), with the following two
419 modifications. Firstly, LB supplemented with 50 µg/ml kanamycin was used instead of

minimal media. Secondly, MβL production was induced by addition of 0.25 mM IPTG. Protein concentration was measured spectrophotometrically using ε_{280} = 27960 M^{-1} cm^{-1} for NDM-1, L3VIM, and L3Pro, and ε_{280} = 31970 M^{-1} cm^{-1} for L3IMP. Metal content was measured using the colorimetric reagent 4-(2-pyridylazo) resorcinol (PAR) under denaturing conditions (72). The average metal content of the variants were somehow lower (1.5 for L3VIM, 1.6 for L3Pro and 1.65 for L3IMP) compared to the wild type protein (1.8 equivalents of Zn(II) per enzyme) (33).

Determination of Zn(II) Affinity Constants

Dissociation constants for Zn(II) were estimated by competition with the chromophoric chelator PAR, as previously described (26). Briefly, PAR is a metallochromic compound, whose UV-Visible absorption spectrum is modified upon metal uptake, as reflected by a shift of its maximum absorption wavelength from 414 to 500 nm. Using the previously published molar absorption coefficients of free PAR ($\varepsilon_{PAR414nm}$ = 36868 ± 1843 M^{-1} cm^{-1}; $\varepsilon_{PAR500nm}$ = 1289 ± 65 M^{-1} cm^{-1}), PAR-Zn(II) complex ($\varepsilon_{PAR2Zn414nm}$ = 12788 ± 576 M^{-1} cm^{-1}; $\varepsilon_{PAR2Zn500nm}$ = 80000 ± 4000 M^{-1} cm^{-1}) and the disassociation constant (K_d) for the PAR-Zn(II) complex (2.6 ± 0.2 10^{-12} M), we were able to quantify the amount of PAR at each state in a given sample (73).

Disassociation constants for NDM-1 and all the L3 variants were determined at 25 °C by titrations curves on 40 mM MOPS, 0.1 M NaCl, pH 7.3 (previously treated with Chelex) supplemented with 1.5 µM ZnSO4 and PAR at 3 and 6 µM. Apo enzyme was added at increasing amount at each point, until a final concentration of 3 µM was reached, and the absorption spectra between 300 and 600 nm were recorded. Absorbances at 414 and 500 nm were corrected by subtracting the absorbance at 600 nm, which was taken as baseline. Metal binding to wild-type NDM-1, L3VIM and L3IMP could be described by a two-step binding model. Data were fit with DynaFit 3 (Biokin) (74) to the equilibrium shown in

Scheme 1; were K_{d1} and K_{d2} are the disassociation constants of the enzyme:Zn(II) complex, and K_{dPAR} corresponds to the dissociation of the PAR:Zn(II) complex. For the L3Pro variant a second model with one K_d ($K_{d1,2}$, Table S1) for the enzyme-Zn(II) complex was proposed (Scheme 2).

Scheme 1

$$E{:}Zn(II) \overset{K_{d1}}{\rightleftharpoons} E_{apo} + Zn(II)$$

$$E{:}Zn(II)_2 \overset{K_{d2}}{\rightleftharpoons} E{:}Zn(II) + Zn(II)$$

$$PAR_2{:}Zn(II) \overset{K_{dPAR}}{\rightleftharpoons} 2PAR + Zn(II)$$

Scheme 2

$$E{:}Zn(II)_2 \overset{K_{d1,2}}{\rightleftharpoons} E_{apo} + 2Zn(II)$$

$$PAR_2{:}Zn(II) \overset{K_{dPAR}}{\rightleftharpoons} 2PAR + Zn(II)$$

Steady-state Kinetics

β-Lactamase activity was measured in a JASCO V-670 spectrophotometer at 30 °C in 10 mM HEPES pH 7.5 and 200 mM NaCl supplemented with 20 μM $ZnSO_4$ and 20 μg/mL bovine serum albumin (BSA). Substrates were used in the μM range, whereas the enzymes were used in the nM range in order to ensure pseudo-first-order kinetics. It was only considered the concentration of metallated protein. The following differential extinction coefficients were used: nitrocefin, $\Delta\varepsilon_{482}$ = 17400 M^{-1} cm^{-1}; PenG, $\Delta\varepsilon_{235}$ = − 775 M^{-1} cm^{-1}; piperacillin, $\Delta\varepsilon_{235}$ = − 820 M^{-1} cm^{-1}; ceftazidime, $\Delta\varepsilon_{256}$ = −7600 M^{-1} cm^{-1}; cefepime, $\Delta\varepsilon_{260}$ = −750 M^{-1} cm^{-1}; imipenem, $\Delta\varepsilon_{300}$ = −9000 M^{-1} cm^{-1}; meropenem, $\Delta\varepsilon_{300}$ = − 6,500 M^{-1} cm^{-1}; ertapenem, $\Delta\varepsilon_{299}$ = −9970 M^{-1} cm^{-1}.

Stopped-Flow Experiments

The variations in the visible spectra of NDM-1 and its L3 variants during hydrolysis of nitrocefin, imipenem and meropenem were followed with an Applied Photophysics SX.18-MVR stopped-flow system associated to a photodiode-array detector (Applied Photophysics, U.K.). The measurements were performed in 100 mM HEPES, pH 7.5, 200

mM NaCl and 0.3 mM $ZnSO_4$, at 6 °C. Data were corrected for the instrument dead time (2 ms). In all cases, a 1:1 ratio of metallated enzyme and substrate was used.

Thermal denaturation assays

Protein stability to thermal denaturation was determined on both holo and apoprotein by using the Protein Thermal Shift™ Assay (Applied Biosystems, Carlsbad, CA), following manufacturer's instructions. Data were fit to a two-step model, as previously described (75).

Preparation of Apo and Co(II) substituted enzymes

The non-metallated forms of Apo-NDM-1, apo-L3VIM, apo-L3IMP, and apo-L3Pro, were prepared by successive dialysis of the purified holoproteins against chelators, as described previously (49). All buffer solutions used to prepare the apoenzymes were treated by extensive stirring with Chelex 100 (Sigma). Metal content of the apoprotein preparations was checked using PAR, as described above. Co(II) substituted enzymes were obtained after titration on apo derivatives with $CoSO_4$ (49).

X-ray crystallography

The proteins were purified as described before with an additional size-exclusion chromatography step, in final buffer HEPES 10 mM pH 7.5, NaCl 200 mM, and concentrated to 30 mg/ml. Crystals were grown with the hanging-drop vapor diffusion method at 18 ^{o}C. Drops were set by mixing equal volumes of protein and reservoir solution. L3IMP crystals were grown in 100 mM HEPES pH 7.55, 0.1 M NaCl, 1.35 M $(NH_4)_2SO_4$ applying microseeding. L3Pro crystals were grown in 100 mM HEPES pH 7.0, 500 mM $(NH_4)_2SO_4$, 5 mM $CoCl_2$-$NiCl_2$-$MgCl_2$-$CdCl_2$ and 12-30% (*w/v*) PEG 3350. All crystals were flash-frozen, either in mother liquor supplemented with 35% glycerol or 50% paraffin oil, and stored in liquid nitrogen.

X-ray diffraction data were collected at 100 K at the Proxima 1 beamline (Synchrotron Soleil, Saint-Aubin, France). Data reduction was carried out using XDS (76) and Aimless

from the CCP4 program suite (77). The crystal structures of both proteins were solved by molecular replacement using the programs Molrep (78) or Phaser (79) and a previously determined NDM-1 structure (PDB entry 3SPU, chain C) as search probe. The final crystallographic models were obtained through iterative rounds of refinement with Buster (80) and manual rebuilding with COOT (81). Data collection and refinement statistics are summarized in Table S4. Both crystallographic models were validated with MolProbity (82) and the rmsd calculations were performed with PDBeFold (83). Illustrations were made with PyMOL (Schrödinger, New York, USA).

To ascertain the presence of Zn metal ions in the active site of L3Pro crystals, two complete datasets were collected at the ESRF beamline id23eh1 using X-ray wavelengths immediately above (hr, λ=1.27241 Å) and below (lr, λ=1.28348 Å) the Zn K-edge as determined with a fluorescence scan. Using phases from the refined protein model, double difference anomalous maps (Dano(hr) – Dano(lr)) were produced with the programs SFTOOLS and FFT from the CCP4 suite (77) (Supplementary Figure 4). The presence of Ni(II) ions (from the crystallization buffer) mediating protein-protein interactions in L3Pro was confirmed in a similar way (hr, λ=1.48030 Å; lr, λ=1.48840 Å). In all cases, data processing and reduction were carried out as described before (Table S4).

Accession numbers

Structural data are available in Protein Data Bank database under the accession codes 6C6I (L3IMP) and 6CAC (L3Pro).

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536

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Abbreviations: MβLs, metallo-β-lactamases; NDM, New Delhi metallo-β-lactamase; VIM, Verona integron–encoded metallo-β-lactamase; IMP, Imipenemase metallo-β-lactamase; MIC, minimum inhibitory concentration; DPA, dipicolinic acid; PAR, 4-(2-pyridylazo) resorcinol; LMCT, ligand-to-metal charge-transfer (LMCT); PDA, photodiode array; rmsd, root mean square deviation; DEER, double electron electron resonance, PDA, photodiode array detector.

Keywords: metallo-β-lactamase, antibiotic resistance, New Delhi metallo-β-lactamase, enzyme mechanism, enzyme structure.

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Antimicrobial Agents and Chemotherapy

797 **TABLES**

798 **Table 1.** Antimicrobial susceptibility profiles of E. coli DH5α pMBLe producing NDM-1 and
799 its variants at loop L3. Minimum Inhibitory Concentrations (mg/L).

Variant	Imipenem	Meropenem	Piperacillin	Cefotaxime	Ceftazidime	Cefepime
NDM-1	4	2	128	64	1024	16
L3IMP	2	1	64	16	256	16
L3VIM	1	1	64	32	256	2
L3Pro	1	1	16	64	256	0.5
DH5α pMBLe	0.25	0.03	2	0.03	0.25	0.016

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802 **Table 2.** Melting temperatures (TM) of apo- and holo-enzymes measured by thermal shift
803 assay.

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Variant		T_M (°C)	Δ T_M (°C)
NDM-1	Holo	56.6 ± 0.1	18.3 ± 0.1
	Apo	38.3 ± 0.1	
L3IMP	Holo	55.3 ± 0.1	24.4 ± 0.4
	Apo	30.9 ± 0.3	
L3VIM	Holo	55.2 ± 0.1	31.5 ± 0.1
	Apo	23.7 ± 0.1	
L3Pro	Holo	52.5 ± 0.1	19.0 ± 0.2
	Apo	33.5 ± 0.1	

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806

807 **Table 3.** Steady-state kinetic parameters for the NDM-1 and its L3 variants. Experimental
808 conditions: buffer 10 mM HEPES pH 7.5, 200 mM NaCl, 20 μM ZnSO4, 20 μg/mL bovine
809 serum albumin (BSA); 30 °C. ND, not determined.

Substrate	Variant	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)
Imipenem	NDM-1	150 ± 30	570 ± 30	4 ± 1
	L3IMP	60 ± 10	160 ± 6	2.7 ± 0.5
	L3VIM	136 ± 7	631 ± 9	4.6 ± 0.3
	L3Pro	780 ± 90	1200 ± 60	1.5 ± 0.2
Meropenem	NDM-1	140 ± 20	960 ± 40	7 ± 1
	L3IMP	90 ± 10	39 ± 1	0.4 ± 0.1
	L3VIM	540 ± 50	1810 ± 70	3.4 ± 0.4
	L3Pro	1100 ± 200	2500 ± 200	2.3 ± 0.6
Ertapenem	NDM-1	25 ± 2	420 ± 10	17 ± 2
	L3IMP	29 ± 6	29 ± 1	1.0 ± 0.2
	L3VIM	110 ± 20	750 ± 40	5 ± 1
	L3Pro	270 ± 20	710 ± 20	2.6 ± 0.3
Penicillin G	NDM-1	80 ± 10	690 ± 20	8 ± 1
	L3IMP	13 ± 1	272 ± 4	21 ± 2
	L3VIM	40 ± 5	323 ± 9	8 ± 1
	L3Pro	690 ± 99	755 ± 49	1.1 ± 0.1
Piperacillin	NDM-1	120 ± 10	1190 ± 40	10 ± 1
	L3IMP	67 ± 8	232 ± 6	3.5 ± 0.5
	L3VIM	410 ± 30	1820 ± 70	4.4 ± 0.5
	L3Pro	700 ± 100	630 ± 70	0.9 ± 0.2
Ceftazidime	NDM-1	60 ± 10	620 ± 20	10 ± 2
	L3IMP	60 ± 10	131 ± 5	2.2 ± 0.4
	L3VIM	58 ± 8	250 ± 8	4.1 ± 0.7
	L3Pro	90 ± 20	120 ± 10	1.3 ± 0.4
Cefepime	NDM-1	50 ± 10	300 ± 20	6 ± 2
	L3IMP	6.1 ± 0.6	178 ± 2	28 ± 3
	L3VIM	ND	ND	1.2 ± 0.1
	L3Pro	ND	ND	0.9 ± 0.5

Nitrocefin	NDM-1	1.3 ± 0.3	38 ± 2	29 ± 8
	L3IMP	2.9 ± 0.9	67 ± 7	23 ± 3
	L3VIM	4.0 ± 0.9	48 ± 3	12 ± 3
	L3Pro	2.6 ± 0.5	85 ± 4	32 ± 1

810

FIGURE LEGENDS

Fig. 1. Engineered substitutions of loop L3 in NDM-1. Sequence alignment of the L3 variants, highlighting the differences at the loop L3 region, including the standard BBL numbering (28).

Fig. 2. Expression levels and Zn(II) limitation susceptibility of NDM-1 and the L3 variants. (A) Immunoblot demonstrating steady-state expression in *E. coli* DH5α. Proteins were detected from whole cell lysates (lanes 2-5 and 14), spheroplasts (lanes 6-9) and periplasmic extracts (lanes 10-13). Wild type NDM-1 (W) corresponds to lane 2, 6 and 10; L3IMP (I) lanes 3, 7 and 11; L3VIM (V) lanes 4, 8 and 12; L3Pro (P) lanes 5, 9 and 13, and empty plasmid (E) lane 14. Lane 1 shows protein ladder marker. GroEL molecular weight is 60 kDa and MBP 47 kDa (B) Immunoblot demonstrating steady-state expression of wild type NDM-1 and the L3 variants in *E. coli* DH5α treated with DPA. After induction, cells were incubated with (+) or without (-) DPA and protein expression was detected from of whole cell lysates. Wild type NDM-1 (W) corresponds to lane 2-3, L3IMP (I) lanes 4-5, L3VIM (V) lanes 6-7, L3Pro (P) lanes 8-9 and empty plasmid (E) lanes 10-11. Untreated cells were loaded before treated ones. Lane 1 shows protein ladder marker. (C) Antimicrobial susceptibility profiles of *E. coli* DH5α pMBLe producing β-lactamases against cefotaxime at increasing DPA concentrations. *E. coli* DH5α expressing NDM-1 is shown in blue; L3IMP in green, L3VIM in red; and L3Pro in orange.

Fig. 3. Difference spectrum of Co(II)-substituted wild type NDM-1 and of the L3 variants. The difference spectrum of the Co(II)-substituted MβLs were obtained by subtraction of the

834 spectrum of the non-metallated β-lactamase from the one corresponding to the final bi-
835 Co(II) substituted variant. The difference spectrum of the wild type NDM-1 is shown in blue;
836 L3IMP in green, L3VIM in red and L3Pro in orange.

837

838 **Fig. 4.** X-ray crystal structures of the L3IMP and L3Pro variants compared to NDM-1.
839 Crystal structures of NDM-1 (PDB code: 3SPU, chain D) in blue, L3IMP in green (PDB
840 code: 6C6I, 1.65 Å) and L3Pro in orange (PDB code: 6CAC, 1.80 Å). The images were
841 generated after the complete alignment of NDM-1 and the two L3 variants. (A) The loop L3
842 position is highlighted in a darker color. (B) Relevant conserved amino acids from the active
843 sites of NDM-1 (blue), L3IMP (green), and L3Pro (orange) are represented by sticks; metal
844 ions (Zn(II) in grey and Cd(II) in light orange) and water molecules (red) are represented as
845 spheres. The position and orientation of the metal ligands is conserved among the three
846 structures. The distances between the ions in the two sites are very similar (≈ 3.8 Å) among
847 the different proteins. The position of the ions in the DCH site displays a slight variability
848 among the structures while in the 3H site the position is unchanged. (C) Angle determined
849 by the loop L3 and the plane of the active site of each mutant. The angles were calculated
850 between the Zn1, Cα of Ser69 and Cα of Gly63. The values obtained for each loop L3 were:
851 L3IMP = 68°, NDM-1 = 88°, and L3Pro =110°.

852

853 **Fig. 5.** Photodiode array stopped-flow spectra and traces of nitrocefin hydrolysis by NDM-1
854 and its L3 variants. (A) Reaction mechanism for nitrocefin hydrolysis by NDM-1, adapted
855 from Yang et al (32). (B) Electronic absorption spectra upon the reaction of 10 μM nitrocefin
856 and 10 μM enzyme in 100 mM HEPES, pH 7.5, 0.2 M NaCl and 0.3 mM ZnSO4, at 6 °C.
857 The reaction progresses from black to color: NDM-1 in blue, L3VIM in red, L3IMP in green

858 and L3Pro in orange. Absorption bands with peaks at 390, 485 and 605 nm correspond to
859 maximum absorption of the substrate, product, and the anionic intermediate of the reaction,
860 respectively. (C) Temporary profiles of substrate (390 nm), intermediate (665 nm) and
861 product (485 nm) during the reaction described in (A). Traces from NDM-1 are shown in
862 blue; L3VIM in red; L3IMP in green and L3Pro in yellow.

863

864 **Fig. 6.** Photodiode array stopped-flow spectra of carbapenems hydrolysis by NDM-1 and
865 the L3 variants. (A) General reaction mechanism for carbapenems hydrolysis by MβLs,
866 adapted from Lisa, Palacios et al. (33). The ES complex does not accumulate and is hence
867 depicted in a lighter color (grey). (B) Sequence of difference spectra collected upon the
868 reaction of 100 μM imipenem and 100 μM β-lactamase. The reactions progress from black
869 to color: NDM-1 blue, L3VIM red, L3IMP green and L3Pro orange. The ionic intermediates,
870 EI1 and EI2, were detected as absorption bands with maximum at 390 and 343 nm. The
871 time interval spans up to 0.2 s. (C) Sequence of difference spectra upon the reaction of 100
872 μM meropenem and 100 μM enzyme. The reactions progresses from black to color: NDM-1
873 in blue, L3VIM in red, L3IMP in green and L3Pro in orange. The ionic intermediates, EI1
874 and EI2, are detected as absorption bands with maximum at 390 and 330 nm. The time
875 interval spans up 0.2 s.