Biochemical Characteristics of New Delhi Metallo-β-Lactamase-1 Show Unexpected Difference to Other MBLs

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Abstract

New Delhi metallo-β-lactamase (NDM-1) is a new metallo-β-lactamase (MBL) that has recently emerged as a global threat because it confers bacteria with resistance to almost all clinically used β-lactam antibiotics. To determine the molecular basis of this threat, NDM-1 was purified from Escherichia coli TransB (DE3) carrying cloned blaNDM-1 gene by an anion-exchange chromatography step followed by a gel permeation chromatography step. The purified enzyme was stable even in extremely alkaline buffer (pH 11) and reached its highest activity at a low temperature (15°C), which was different from other MBLs. The 50% inhibition concentration of EDTA against NDM-1 was 412 nM, which showed that NDM-1 was more susceptible to EDTA than other MBLs. The effects of zinc on NDM-1 differed between cephem and carbapenem complexes, but inhibition at high Zn2⁺ concentration was observed for all of tested β-lactam compounds.

Materials and Methods

Plasmids, strains, and reagents

E. coli TransB (DE3) chemically competent cells, E. coli trans5α chemically competent cells, and pEASY-T1–single cloning vector were purchased from Beijing TransGen Biotech (China). Taq DNA polymerase, T4 DNA ligase, and restriction endonucleases were obtained from New England Biolabs (Beijing, China). PCR primers were synthesized by Beijing Sunbio Tech Co., Ltd. (China). Plasmid mini-kits and gel extraction kits were obtained from Beijing Bioned Co., Ltd. (China). Q-Sepharose FF and Superdex 75 columns (5 ml) were purchased from GE Healthcare (Beijing, China). The expression vector pET22b was stored in our laboratory. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Recombinant DNA methodology

ATCC BAA-2146 was used as the bacterial strain for the PCR amplification of the ndm-1 gene. PCR was performed using the primers P1 (5'-CATATGAAGTGTATAATTAAATCTGGG-3') and P2 (5'-CTCGAGTACAGGCAGCTTTGCAGGC-3'). Amplification of the ndm-1 gene by PCR with P1 and P2 was performed using EasyTag polymerase under the following conditions: initial denaturation at 95°C for 3 min; followed by 30 cycles at 94°C for 30 s, 60°C for 30 min, and 72°C for 1 min; and final renaturation at 72 °C for 5 min. The PCR product was subcloned into the pEASY-T1 cloning vector. ndm-1 gene in the recombinant plasmid pEASY-ndm1 was cloned into the expression vector pET22b as an NdeI–Xhol fragment. The sequence of the plasmid pET22b-ndm1 was confirmed by DNA sequencing. The
Expression and purification of NDM-1 protein

E. coli TransB (DE3) harboring the expression plasmid pET22b-ndm1 was grown at 37°C in one liter of Luria–Bertani medium containing 100 µg/ml ampicillin with shaking until the OD₆₀₀ reached 0.4–0.6. Protein expression was induced by adding IPTG to a final concentration of 0.1 mM for 8 h at 25°C with shaking. Cells were harvested by centrifugation, and the supernatant was removed by decantation. The pellet was resuspended in 250 ml of buffer A (20 mM Tris-HCl, pH 7.4), and mild sonication was conducted on ice. After centrifuging the lysate, the supernatant fraction and pelleet fraction were resolved by SDS-PAGE using a 15% acrylamide gel.

The supernatant fraction containing the required proteins was loaded onto a Q-Sepharose FF column on an AKTA Exploria® station (GE Healthcare, USA). After washing the column, the bound proteins were eluted by a linear NaCl gradient (0 M to 1 M) in Tris-HCl buffer (flow rate = 1 ml/min). The fractions containing carabapenemase activity were pooled, dialyzed against 10 mM HEPES buffer (pH 7.5), concentrated 10-fold by ultrafiltration, loaded on an equilibrated Superdex 75 column, and eluted with 10 mM HEPES (pH 7.5). The β-lactamase-containing elution peak was analyzed by SDS-PAGE (15% acrylamide gel) and Bandscan 5.0 software. The protein concentration in solution was assayed with a commercial kit (Bio-Rad protein assay). During the purification procedure, carabapenemase activity was monitored using 100 mM meropenem as a substrate in 50 mM HEPES (pH 7.5) at 30°C.

Kinetic measurements

Purified NDM-1 was used to determine the kinetic parameters k₅₀ and k₉₀. Reactions were performed at 30°C in 0.2 ml of assay buffer [pH 7.5, 50 mM HEPES and 50 µM ZnSO₄]. The assays were performed using a SynergyHT spectrophotometer (Biotek Corp., USA) by observing changes in absorption resulting from the initial hydrolysis of each substrate. The initial hydrolysis rates of each substrate were determined by adding NDM-1 protein to an assay buffer containing varied amounts of ZnSO₄ from 0.2 µM to 200 µM. The final enzyme concentration was 0.5 µg/ml. Averages and standard deviations were reported based on three independent measurements.

Temperature and pH effects

To determine the effects of temperature on enzyme activity, 50 µg/ml purified NDM-1 was incubated for 20 min before assaying with 0.5 mM meropenem in a buffer [pH 7.5, 50 mM HEPES and 50 µM ZnSO₄] at 5°C to 70°C. To determine the effects of pH on enzyme activity, purified NDM-1 was incubated in 50 mM HEPES buffer from pH 5.5 to 11 for 20 min before assaying with 0.5 mM meropenem at 30°C under the aforementioned conditions. Averages and standard deviations were reported based on three independent measurements.

IC₅₀ determination using EDTA

To examine the effects of EDTA on NDM-1, an enzyme assay was performed by determining the IC₅₀, which is defined as the concentration of inhibitor necessary to inhibit 50% enzyme activity. The assay was performed under previously described conditions after incubating the reaction mixture for 10 min with varied concentrations of EDTA from 0.1 µM to 1 µM before initiating the reaction with meropenem.

| substrate class | substrate | nm | K₅₀ (µM) | k₅₀(s⁻¹) | k₅₀/K₉₀ (µM⁻¹ s⁻¹) |
|-----------------|-----------|----|----------|-----------|-------------------|
| penem           | penicillin G | 235 | 103 | 27.5 | 0.27 |
|                 | piperacillin | 230 | 69 | 8.3 | 0.12 |
| cephem          | cefoxitin | 230 | 63 | 38.3 | 0.61 |
|                 | ceftazidime | 260 | 74 | 60.9 | 0.83 |
|                 | biapenem | 294 | 155 | 105.6 | 0.68 |
|                 | meropenem | 297 | 42 | 30.75 | 0.73 |

Figure 1. Coomassie blue stained 12% SDS-PAGE of purified NDM-1. The highly purified and soluble NDM-1 is shown. The molecular weight of NDM-1 is approximately 28 kDa.

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The \( K_m \) values of NDM-1 were similar among different \( \beta \)-lactams, but the \( k_{cat} \) values differed by up to 12-fold. The highest \( k_{cat} \) (105.6 s\(^{-1}\)) was found with biapenem and the lowest (8.3 s\(^{-1}\)) was with piperacillin. Considerable variability was observed within the various \( \beta \)-lactam subfamilies (penam, cephem, and carbapenem compounds). Thus, NDM-1 had no clear preference for any of these \( \beta \)-lactam compounds (Table 1).
Effects of temperature and pH on enzyme activity and stability

NDM-1 had the highest activity between pH 6.5 and 8.0, with the maximum activity at pH 7.0 (Fig. 2A). Most MBLs prefer buffers with pH ranging from 6.5 and 7.0 [9–12]. However, NDM-1 retained >50% of its original activity at pH 11. To determine the buffer effect, NDM-1 activity in 40 mM ethanolamine buffer at pH 10 and 11 was observed. The results were similar to those in HEPES buffer. All these findings suggested that NDM-1 was stable and active at high-pH buffers. NDM-1 had the highest activity at 10 and 25°C, with the maximum activity at 15°C (Fig. 2B). Most MBLs have an optimal temperature of 37°C. However, at 35°C, NDM-1 retained only 60% of its activity at 15°C; at 55°C, less than 10% was retained. Thus, NDM-1 was unstable at high temperature.

IC₅₀ of EDTA against NDM-1

We compared the inhibition effects of EDTA on NDM-1 and on various other MBLs such as DIM-1, VIM-1, and VIM-13, 412 nM (Fig. 2C). Therefore, NDM-1 was more susceptible to EDTA than other MBLs such as DIM-1, VIM-1, and VIM-13, whose IC₅₀ values are 175, 9.3, and 253 μM, respectively. Thus, Zn ions can more strongly bind to NDM-1 than to other MBLs.

Effects of zinc concentration on various substrates

To determine the effect of zinc ion stoichiometry on function, we investigated the NDM-1 ability with increased ZnSO₄ concentration in an assay buffer used to hydrolyze three different classes of substrates (Fig. 3). The hydrolysis rates of cephem (cefazidime and cefepime) were found to be very sensitive to the ZnSO₄ concentration in the assay buffer. However, the hydrolysis rate was only 13%–20% of the maximum activity when no additional ZnSO₄ was added. The rates reached the maximum level when the ZnSO₄ concentration was 100 μM. A subset of these substrates showed increased hydrolysis rates with increased ZnSO₄ concentration. The tested penams showed varied results. Penicillin G showed low sensitivity to the ZnSO₄ concentration, but piperacillin showed effects similar to those of cephem. Carbenapenem (biapenem) showed effects opposite to those of the other substrates, i.e., significantly decreased activity at high ZnSO₄ concentration. When the ZnSO₄ concentration was <4 μM in the assay buffer, the maximum activity was observed with biapenem.

Discussion

Since the first report on NDM-1 in 2009, this enzyme has been extensively studied. However, most of these studies have focused on genetics, genomics, and molecular epidemiology. Only a few papers have been published on the functional properties of NDM-1. So far, four reports on the structural determination of NDM-1 have been published [13–16], with additional coordinates available in the Protein Data Bank. These structural models show that NDM-1 has a typical MBL fold, but these models considerably differ from one another. However, all these reports show that NDM-1 proteins have fusions or tags used for purification (e.g., His tag), which may affect the structure of the active site. In the current study, an engineered E. coli that carried the cloned blaNDM-1 gene on expression plasmid vector pET22b was constructed. The high yield of NDM-1 production (approximately 100 mg L⁻¹ of culture) led to a purified NDM-1 preparation (>98% purity) that was obtained after cation exchange and gel permeation chromatography.

The pH dependence of lactamase is indispensable in elucidating the mechanism of NDM-1. Active sites generally contain acidic or basic groups, and one protonic form of the acid and base is catalytically active. The pH affects the enzyme activity through the group ion state. In this study, we found that NDM-1 was stable and active in an extremely basic environment (even pH > 11.0). However, the enzyme activity in acidic buffer (pH < 6.0) sharply decreased and had no significant activity at pH 5.5. Thus, appropriate acid groups should be considered in the design of NDM-1 inhibitors.

EDTA is a metallo-chelator known to potentiate the activities of some lactam antibiotics against MBL-producing microorganisms because the zinc-containing active site of MBL is inactivated by EDTA [17,18]. In this study, we found that NDM-1 was more susceptible to EDTA than other MBLs, indicating a stronger binding of Zn²⁺ ions in NDM-1. Despite the toxicity of EDTA, which hinders its clinical use, EDTA complexes or analogs are important in finding new NDM-1 inhibitors. A complex of EDTA and calcium ion called calcium disodium EDTA has been prepared as an injectable form of chelator with decreased toxicity. This form has been approved for the treatment of lead intoxication [19,20] and used as an inhibitor of bacterial MBL in a mouse model of Pseudomonas aeruginosa pneumonia [21].

The effects of zinc on NDM-1 varied between cephem and carbenapenem complexes; however, inhibitory effects were observed for both complexes at high Zn²⁺ concentrations. This inhibition can be explained by the nonspecific interactions between the enzyme surface and Zn²⁺ cations, which have also been observed for VIM-2 and other MBLs [22,23]. Low-concentration Zn²⁺ fixation strongly stabilized the enzyme and impaired the active site mobility. However, high-concentration Zn²⁺ destabilized the enzyme and disorganized the active site. Hence, the optimum Zn²⁺ concentration depended on the antibiotic used.

In conclusion, a new and simple method of purifying native (no fusion or tag) NDM-1 was demonstrated. The enzymes were successfully characterized by kinetic measurements as well as analyses on the effects of pH, temperature, Zn, and EDTA. The results showed unexpected differences of NDM-1 from other MBLs.

Author Contributions

Conceived and designed the experiments: TL HW. Performed the experiments: TL FC XL SI HF. Analyzed the data: TL QW DW ZL XH HW. Contributed reagents/materials/analysis tools: HW. Wrote the paper: TL HW.

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