On functional and structural heterogeneity of VIM-type metallo-β-lactamases

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The VIM metallo-β-lactamases are emerging resistance determinants, encoded by mobile genetic elements, that have recently been detected in multidrug-resistant nosocomial isolates of Pseudomonas aeruginosa and other Gram-negative pathogens. In this work a T7-based expression system for overproduction of the VIM-2 enzyme by Escherichia coli was developed, which yielded ~80 mg of protein per litre of culture. The enzyme was mostly released into the medium, from which it was recovered at >99% purity by an initial ammonium sulphate precipitation followed by two chromatography steps, with almost 80% efficiency. Determination of kinetic parameters of VIM-2 under the same experimental conditions previously used for VIM-1 (the first VIM-type enzyme detected in clinical isolates, which is 93% identical to VIM-2) revealed significant differences in Km values and/or turnover rates with several substrates, including penicillins, cephalosporins and carbapenems. Compared with VIM-1, VIM-2 is more susceptible to inactivation by chelators, indicating that the zinc ions of the latter are probably more loosely bound. These data indicated that at least some of the amino acid differences between the two proteins have functional significance. Molecular modelling of the two enzymes identified some amino acid substitutions, including those at positions 223, 224 and 228 (in the BBL numbering), that could be relevant to the changes in catalytic behaviour.

Introduction

Acquired metallo-β-lactamases (molecular class B, group 3 of the functional classification) are emerging resistance determinants in several bacterial species of clinical relevance, including members of the family Enterobacteriaceae, Pseudomonas spp. and other non-fastidious Gram-negative non-fermenters. The ability to hydrolyse most β-lactam compounds efficiently, including carbapenems, the lack of clinically useful inhibitors and the mobile nature of their genetic determinants identify these enzymes as major threats to efficacy of antimicrobial chemotherapy. During the last decade, two types of acquired metallo-β-lactamases have been detected in Gram-negative pathogens, namely the IMP- and VIM-type enzymes. The IMP-type enzymes were detected in the early 1990s and have been the subject of several biochemical and structural investigations. The VIM-type enzymes were identified more recently, and considerably less is known about their functional and structural properties.

The VIM-type enzymes belong to subclass B1 of molecular class B and include at least three variants: VIM-1, VIM-2 and VIM-3. Among them, VIM-2 is apparently the most...
were grown in Luria-Bertani (LB) medium,22 or in Buffered Salt Broth medium (BSB): yeast extract, 20 g/L; tryptone, 35 g/L; NaCl, 5 g/L; buffered with 50 mM sodium phosphate buffer pH 7.0) supplemented with the appropriate antibiotic. Media components were from Difco Laboratories (Detroit, MI, USA).

**Construction of the expression system for overproduction of VIM-2 in E. coli**

The blaVIM-2 gene was amplified by PCR with primers VIM-2-Fwd (5′-GGAATTCATATGTTCAACTTTTGATGTAAG-3′), which added EcoRI (bold) and NdeI (underlined) restriction sites at the 5′-end of the gene, and VIM-2-Rev (5′-CCGGATCCGCTACTAACCAGCTG-3′) which added a BamHI restriction site (bold) after the blaVIM-2 stop codon. Amplification was carried out in 50 µL using 50 pmol of each primer and the Expand PCR system (Roche Biochemicals, Mannheim, Germany), under the conditions recommended by the manufacturer, and the following cycling parameters: initial denaturation at 94°C for 3 min; denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, repeated for 30 cycles; final extension step at 72°C for 10 min. Plasmid pVRP193 (50 ng)21 was used as template. The resulting 0.8 kb amplicon was digested with EcoRI and BamHI and cloned into the plasmid vector pBC-SK (Stratagene) to give recombinant plasmid pJDD-V2. After confirmatory sequencing, to rule out the presence of unwanted mutations introduced by PCR, the 0.8 kb NdeI–BamHI fragment of pJDD-V2, containing the blaVIM-2 gene, was subcloned into the T7-based expression vector pET-9a (Novagen) to obtain plasmid pET-9/VIM-2.

**Purification of VIM-2**

The VIM-2 enzyme produced by *E. coli* BL21(DE3)(pET-9/VIM-2) was purified from the culture supernatants of 200 mL stationary phase cultures grown aerobically at 37°C in BSB medium. Cells were removed by centrifugation (10 000g, for 30 min at 4°C) and solid ammonium sulphate was added to the supernatant to achieve a 50% saturation. After 1 h of gentle stirring at 4°C, the sample was centrifuged (13 000g, for 1 h at 4°C). Solid ammonium sulphate was added to the clarified supernatant to 80% saturation, and the precipitate, collected as described above, was solubilized in 20 mM triethanolamine (pH 7.2) (1/20 of the original volume) and loaded (flow rate 2 mL/min) onto an HR column (5 × 1.6 cm) packed with a linear NaCl gradient (0–1 M in 200 mL), at a flow rate of 2 mL/min. Fractions containing β-lactamase activity were pooled and concentrated ~10-fold using a Centriplus YM10 system (Millipore, Bedford, MA, USA). The concentrated sample was then injected onto a Superdex 75 HR 10/30 column (Amersham–Pharmacia Biotech) equilibrated with HB containing 50 µM ZnCl2 and 0.2 M NaCl, and proteins were eluted in the same buffer at a flow rate of 0.8 mL/min.

**Expression experiments**

*E. coli* BL21(DE3)(pET-9/VIM-2) was grown aerobically at 37°C in 100 mL of BSB medium containing kanamycin, 50 mg/L. When the OD at 600 nm reached a value of ~0.8, the culture was split into two and to one subculture isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration 1 mM) was added. β-Lactamase activity was monitored spectrophotometrically, using 100 µM imipenem as substrate in 10 mM HEPES buffer (pH 7.5) (HB), at 30°C, both in cell extracts and in culture supernatants from samples obtained at different times. Cell extracts were prepared by centrifuging the culture, resuspending the cells in the same volume of HB, and disrupting them by sonication [five cycles, 20 s for each cycle, at 45 W, using a B. Braun Labsonic L sonicator (Melsungen, Germany)]. The amount of enzyme was calculated on the basis of the following kinetic parameters for imipenem: \( k_{cat} = 34/s \) and \( K_m = 9 \mu M \).

**Materials and methods**

**Bacterial strains and culture media**

*E. coli* XL-1 blue (Stratagene Inc., La Jolla, CA, USA) was routinely used for molecular cloning and plasmid propagation. *E. coli* BL21(DE3) (Novagen Inc., Madison, WI, USA) was used for metallo-β-lactamase gene expression. Bacteria were grown in Luria–Bertani (LB) medium,23 or in Buffered Super Broth medium (BSB): yeast extract, 20 g/L; tryptone, 35 g/L; NaCl, 5 g/L; buffered with 50 mM sodium phosphate buffer pH 7.0) supplemented with the appropriate antibiotic. Media components were from Difco Laboratories (Detroit, MI, USA).

**Construction of the expression system for high-level production of the VIM-2 enzyme in *E. coli***

In this work an expression system for high-level production of the VIM-2 enzyme in *Escherichia coli* was developed, and biophysical and biochemical characterizations of the purified VIM-2 enzyme were carried out under conditions identical to those previously adopted for VIM-1.18 Significant differences in the behaviour of the two enzymes with various substrates were confirmed, and molecular modelling was used to investigate the potential correlations between structural differences and the different enzyme kinetics.
Biochemistry of VIM metallo-β-lactamases

Fractions containing β-lactamase activity were pooled and stored at −20°C. During the purification procedure the presence of β-lactamase activity was monitored as described above. Protein concentration in solution was determined with the Bio-Rad Protein assay (Bio-Rad, Richmond, CA, USA), using bovine serum albumin as the standard. The molar extinction coefficient at 280 nm of the purified enzyme was determined as the average of values obtained by the colorimetric protein concentration determination and by theoretical calculation.23

Protein electrophoretic techniques

SDS–PAGE was performed according to Laemmli,24 using final acrylamide concentrations of 12% and 5% (w/v) for the separating and the stacking gels, respectively. After electrophoresis the protein bands were stained with Coomassie Brilliant Blue R-250. Analytical isoelectric focusing (IEF) of the purified protein and detection of enzyme activity were performed as described previously.16

Gel-permeation chromatography

Gel-permeation chromatography to determine the molecular mass of the native VIM-2 enzyme was carried out on a Superdex 75 HR 10/30 column (Amersham–Pharmacia Biotech) equilibrated with HB containing 0.15 M NaCl, to prevent unwanted protein–column matrix interactions. The purified enzyme (100 µL, at a concentration of 0.5 mg/mL) was eluted in the same buffer at a flow rate 0.8 mL/min. The low-range gel filtration calibration kit (Amersham–Pharmacia Biotech) was used for column calibration. Apparent partition coefficients (Kₛ) were calculated as described previously.25

N terminus sequencing and electrospray mass spectrometry

The amino-terminal sequence of the purified VIM-2 protein was determined using a gas-phase sequencer (Procise-492, Applied Biosystems, Foster City, CA, USA), after redissolving the protein (50 pmol) in 0.1% (v/v) trifluoroacetic acid in water and loading the sample onto a PVDF membrane (Millipore Corp.). Electrospray mass spectrometry was carried out using a PE-Sciex API III triple quadrupole mass spectrometer equipped with an ion-spray source (Perkin-Elmer, Rahway, NJ, USA). The sample (150 pmol) was redissolved in 1% (v/v) potassium formate/70% (v/v) acetonitrile in water and injected into the source of the mass spectrometer at a flow rate of 20 µL/min. Source and cone voltages were 5.5 kV and 180 V, respectively. The source temperature was kept at 50°C. Twenty-four scans covering 800–1800 atomic mass units were accumulated and data were analysed with the software delivered with the instrument.

Determination of kinetic parameters

Substrate hydrolysis by the purified enzymes was monitored by following the absorbance variation, at 30°C, using a Cary 100 UV-Vis spectrophotometer (Varian Instruments, Walnut Creek, CA, USA), in a total reaction volume of 500 µL. The sources, wavelengths, changes in extinction coefficients and reaction buffer (HB containing 50 µM ZnCl₂) used in the spectrophotometric assays were the same as described previously.11,18 The final enzyme concentrations ranged from 4.2 to 84 nM. Enzyme dilutions were prepared in the reaction buffer supplemented with 20 mg/L BSA, and were discarded after each working session. The steady-state kinetic parameters (Kₘ and kₐ) were determined under initial-rate conditions using the Hanes–Woolf plot26 and, when standard deviation values exceeded 5%, they were also verified by the analysis of the complete hydrolysis time-courses as described by De Meester et al.27 Kₛ values lower than 10 µM were measured as inhibition constants (Kₛ) with a competitive model, using 100 µM nitrocefin as the reporter substrate, as described previously.18 Purified VIM-1 enzyme for kinetic measurements was prepared as described previously.18

Inactivation by chelating agents

Inactivation time-courses were monitored by following the hydrolysis of 150 µM imipenem in the presence of different concentrations of EDTA, dipicolinic acid or 1,10-o-phenanthroline. Reactions were carried out at 30°C in HB in a final reaction volume of 500 µL. Enzyme dilutions were prepared in the same buffer supplemented with 20 mg/L BSA, and the final enzyme concentration was 2.1 nM. Pseudo-first-order inactivation rate constants (kₛ) were determined and the inactivation efficiencies (kₛ/Kₛ) were calculated according to the proposed model.28

Molecular modelling

VIM-1 and VIM-2 structural models were based on the available X-ray diffraction three-dimensional structure of CcrA from Bacteroides fragilis.5,29 Molecular models were built by knowledge-based modelling using the HOMOLOGY module of the Insight II software (Molecular Simulations, San Diego, CA, USA) running on a Silicon Graphics Indy workstation (Silicon Graphics Inc., Mountain View, CA, USA). Histidines present at the active site were taken as neutral; for the others, the tautomeric form of the imidazole ring was chosen according to the X-ray structure, after geometric analysis of the potential hydrogen bonds. The zinc-coordinated cysteine was in the thiolate form. Other titratable sites were assigned their standard protonation states at pH 7. Hydrogen atoms were added to the structure using the PROTONATE module of the AMBER version 4.1 software.30 Atomic charges of the
AMBER version 4.1 all-atom library were generally used in the calculations, except for the residues of the active site. Atomic point charges for the zinc ions, the zinc coordinating residues and active-site water molecules were those proposed by Banci et al. Docking experiments were carried out with the Insight II software using the benzylpenicillin structure optimized by AM1 semi-empirical methods. The corresponding complex structures were then optimized by molecular mechanics methods using AMBER version 4.1, first by steepest descent energy minimization of atoms with a force >500 kcal/mol/Å and then by conjugate gradient energy minimization until the rms gradient was <0.1 kcal/mol. Graphics were realized with MOLMOL molecular graphics software. The BBL numbering scheme is used throughout this paper.

Results and discussion

High-level expression of the blaVIM-2 gene in E. coli was achieved by cloning the blaVIM-2 coding sequence downstream of the T7 promoter in the expression vector pET-9a, and transforming the T7 RNA polymerase-producing host BL21(DE3) with the construct (pET-9/VIM-2). Under the conditions described in Materials and methods, this strain produced relatively large amounts of the VIM-2 enzyme. The largest amount (~80 mg/L) was obtained when the culture was not induced with IPTG. The protein was initially found in the cell fraction but, at later stages, was mostly released into the medium. Induction with IPTG was detrimental to the efficiency of the expression system (Figure 1). In repeated expression experiments, the production pattern was reproducible and the yield of enzyme in the culture supernatant of uninduced cultures, after 24 h, exhibited a variability <20%. The protein production in the absence of inducer, observed with the above expression system, was not unexpected. In fact, similar T7-based expression systems are known to be leaky unless hosts and vectors are used that provide additional negative regulatory mechanisms.

The VIM-2 enzyme was purified from the supernatant of cultures of E. coli BL21(DE3)(pET-9/VIM-2) by ammonium sulphate precipitation followed by anion-exchange chromatography on a Source 15Q matrix, and a final gel-filtration step through a Superdex 75 column. A typical purification process is summarized in Table 1. Ammonium sulphate precipitation was very effective in concentrating the enzyme, which, after solubilization at pH 7.2, bound directly to the strong anion exchanger without dialysis or buffer exchange. The final gel-filtration step was necessary to remove minor contaminants and supplied the purified enzyme directly in the storage buffer. The yield of purified protein was 62 mg/L of culture, with an overall efficiency of 77%. In repeated purification experiments the protocol exhibited a good reproducibility (the yield variability was within 15%).

In SDS–PAGE the VIM-2 polypeptide migrated with an apparent molecular mass of 25 kDa, and the preparation was estimated to be >99% pure (Figure 2). The isoelectric pH of

Table 1. Summary of a typical purification procedure of the VIM-2 metallo-β-lactamase produced by E. coli BL21(DE3)(pET9/VIM-2)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Sp activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>200</td>
<td>80</td>
<td>1140</td>
<td>14</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate precipitate</td>
<td>10</td>
<td>ND b</td>
<td>1090</td>
<td>ND b</td>
<td>96</td>
<td>ND b</td>
</tr>
<tr>
<td>Source 15Q HR 16/10 eluate</td>
<td>12</td>
<td>14.4</td>
<td>925</td>
<td>64</td>
<td>81</td>
<td>4.6</td>
</tr>
<tr>
<td>Superdex 75 HR 10/30 eluate c</td>
<td>6</td>
<td>12.4</td>
<td>880</td>
<td>71</td>
<td>77</td>
<td>5.1</td>
</tr>
</tbody>
</table>

a One unit of activity is defined as the amount of enzyme hydrolysing 1 µmol of imipenem/min under the conditions described in Materials and methods.

b ND, not determined since ammonium sulphate interfered with protein concentration determination.

c Sum of six chromatographic runs, 200 µL of sample injected per run.
Biochemistry of VIM metallo-β-lactamases

Figure 2. SDS–PAGE analysis of the purified VIM-2 protein (10 μg). Protein size standards are reported in kDa on the right.

purified VIM-2, measured by analytical IEF, was 5.15 (data not shown), in good agreement with the theoretical value (4.9). The molecular mass of the purified enzyme, estimated by gel-filtration, was 25,000 Da, indicating that the native enzyme is monomeric. This value is somewhat smaller than that previously reported (29.7 kDa), but is consistent with results of SDS–PAGE and mass spectrometry (see above and below). The molar extinction coefficient for pure VIM-2 was calculated as 28,500/M-cm. The amino-terminal sequence of the purified protein was determined as NH$_2$-VDSSG, indicating that the mature enzyme is generated by the cleavage of a 26-amino-acid signal peptide and not of a 20-amino-acid signal peptide, as originally predicted. The cleavage position of the signal peptide was identical to that in VIM-1, although the sequences of the two pro-enzymes are different around that position. Electrospray mass spectrometry yielded a value of 25,515 ± 1 Da, in excellent agreement with the calculated molecular mass of the mature protein (25,515.4 Da).

The kinetic parameters of VIM-2 were determined for several substrates under the same conditions as previously used for VIM-1. Moreover, the kinetic parameters of VIM-1 were determined for moxalactam and redetermined for some substrates (benzylpenicillin, ampicillin, meropenem). In the latter cases, the values obtained were always found to be within the confidence limits of those previously reported. Under the experimental conditions employed, VIM-2 hydrolysed all the tested compounds except aztreonam. The individual kinetic parameters ($K_m$ and $k_{cat}$) of VIM-2 with several substrates, and a comparison with those of VIM-1, are reported in Table 2.

With penicillins, the individual kinetic parameters of VIM-2 were quite homogeneous and the hydrolytic efficiencies were high ($k_{cat}/K_m$ ratios were in the range 0.9–4 × 10$^6$/M·s), except for temocillin, which was hydrolysed 50- to 100-fold less efficiently. This lower efficiency was mostly due to a lower turnover rate. Compared with those of VIM-1, the kinetic parameters of VIM-2 were similar for some substrates (carbenicillin, mezlocillin) but remarkably different for others. In some cases these differences resulted in notable differences in hydrolytic efficiency as well (e.g. benzylpenicillin and ampicillin, which were better substrates for VIM-2 than for VIM-1 due to both lower $K_m$ values and higher turnover rates, and azlocillin, which was a better substrate for VIM-1, mostly due to a higher turnover rate). In other cases the differences in individual kinetic parameters balanced out, resulting in similar hydrolytic efficiencies (e.g. piperacillin, ticarcillin and temocillin). Comparison of the kinetic parameters of ticarcillin and temocillin revealed that the presence of a 6-α-methoxy group was detrimental to the activity of both enzymes. Interestingly, however, this effect was much stronger for VIM-2, and the responses of individual kinetic parameters were remarkably different with the two enzymes (e.g. with VIM-1 the $K_m$ was strongly decreased whereas with VIM-2 it was increased by the presence of the 6-α-methoxy group).

Hydrolytic efficiencies of VIM-2 with cephalosporins were highly variable, with values of $k_{cat}/K_m$ ratios ranging from 5 × 10$^4$ to 1 × 10$^7$/M·s. Except for ceftazidine, the $K_m$ values were quite low and usually lower than those of penicillins, whereas the turnover rates exhibited an overall higher variability, which often directly influenced the hydrolytic efficiencies. With ceftazidine, only a pseudo-first-order rate constant was measurable, since the initial velocities remained proportional to substrate concentration up to a value of 400 μM. Compared with VIM-1, both the $k_{cat}$ and $K_m$ values of VIM-2 tended to be lower (with some exceptions for ceftazidine, nitrocefin and moxalactam). With some substrates (e.g. cefaloridine, cefalothin, cefazidime and cefpirome) the differences in individual kinetic parameters again balanced out, resulting in similar hydrolytic efficiencies for the two enzymes, whereas with other substrates (e.g. cefoxitin, cefuroxime, cefotaxime and moxalactam) the $k_{cat}/K_m$ ratios were also different. The largest difference in hydrolytic efficiencies (almost 40-fold higher for VIM-1) was observed with cefazidime, a finding that was consistent with in vitro susceptibility data.

VIM-2 exhibited high hydrolytic efficiencies with all carbapenems ($k_{cat}/K_m$ ratios were in the range 2.5–5.5 × 10$^9$/M·s), resulting from a combination of very low $K_m$ values and relatively low turnover rates. Overall this behaviour was similar to that observed for VIM-1, and differentiates the VIM-type enzymes from all other zinc-β-lactamases that achieve similar hydrolytic efficiencies toward carbapenems as a result of higher turnover rates combined with higher $K_m$ values. Notwithstanding this common trend, differences in individual kinetic constants between VIM-2 and VIM-1 were also apparent with carbapenems, resulting in different hydrolytic efficiencies. In particular, imipenem and meropenem were hydrolysed more efficiently by VIM-2 (30- and 10-fold,
respectively), due to a higher turnover rate and to a lower $K_m$ value, respectively.

Serine-β-lactamase inactivators were hydrolysed by VIM-2, with individual kinetic parameters and hydrolytic efficiencies that are only slightly different from those of VIM-1. The present data are very similar to those previously reported for VIM-2 for some substrates (e.g. cefuroxime), while being significantly different for others (e.g. most cephalosporins). In particular, the $k_{cat}$ values measured in this work tended to be higher than those previously reported, except for cefuroxime and ceftazidime (for the latter substrate the $k_{cat}$ was actually much lower), whereas smaller differences were observed between $K_m$ values. These discrepancies are likely to reflect differences in the experimental conditions (different buffer systems, HEPES versus sodium cacodylate, at pH 7.5 versus 6.5, respectively, were used) and, possibly, a different degree of purity or of specific activity of the enzyme preparation. They also emphasize the importance of carrying out comparative kinetic studies under identical experimental conditions.

VIM-2 was inactivated by EDTA, o-phenanthroline and dipicolinic acid. The inactivation time-courses followed pseudo-first-order kinetics, and the inactivation rates varied proportionally with the chelating agent concentrations within the experimental range, allowing only the measurement of the $k_{cat}/K_m$ ratio (EDTA: 2.5/M·s; o-phenanthroline: 860/M·s; dipicolinic acid: 460/M·s), representing the inactivation efficiency. Although the mode of interaction appeared to follow a mechanism similar to that observed with other metallo-β-lactamases of subclass B1, VIM-2 was more effi-

### Table 2. Kinetic parameters of the purified VIM-2 metallo-β-lactamase, compared with those of VIM-1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VIM-1</td>
<td>VIM-2</td>
<td>VIM-1</td>
</tr>
<tr>
<td>Penicillins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>benzylpenicillin</td>
<td>30</td>
<td>280</td>
<td>840</td>
</tr>
<tr>
<td>ampicillin</td>
<td>35</td>
<td>125</td>
<td>920</td>
</tr>
<tr>
<td>carbenicillin</td>
<td>170</td>
<td>185</td>
<td>75</td>
</tr>
<tr>
<td>piperacillin</td>
<td>1900</td>
<td>300</td>
<td>3500</td>
</tr>
<tr>
<td>azlocillin</td>
<td>1500</td>
<td>200</td>
<td>120</td>
</tr>
<tr>
<td>mezlocillin</td>
<td>260</td>
<td>200</td>
<td>350</td>
</tr>
<tr>
<td>ticarcillin</td>
<td>450</td>
<td>180</td>
<td>1100</td>
</tr>
<tr>
<td>temocillin</td>
<td>0.5</td>
<td>7.7</td>
<td>22</td>
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<tr>
<td>Cephalosporins</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cefaloridine</td>
<td>315</td>
<td>140</td>
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<td>cefalothin</td>
<td>280</td>
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<td>cefoxitin</td>
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<td>cefuroxime</td>
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<td>cefotaxime</td>
<td>170</td>
<td>70</td>
<td>250</td>
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<td>ceftazidime</td>
<td>60</td>
<td>3.6</td>
<td>800</td>
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<td>cefepime</td>
<td>550</td>
<td>&gt;40</td>
<td>150</td>
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<tr>
<td>cefpirome</td>
<td>705</td>
<td>180</td>
<td>290</td>
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<tr>
<td>nitrocefin</td>
<td>95</td>
<td>770</td>
<td>17</td>
</tr>
<tr>
<td>moxalactam</td>
<td>43</td>
<td>90</td>
<td>450</td>
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<tr>
<td>Carbapenems</td>
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<tr>
<td>imipenem</td>
<td>0.2</td>
<td>34</td>
<td>1.5</td>
</tr>
<tr>
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<td>50</td>
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<tr>
<td>biapenem</td>
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<td>8.5</td>
<td>7.5</td>
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<td>Monobactams</td>
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<tr>
<td>aztreonam</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Mechanism-based inactivators</td>
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<tr>
<td>sulbactam</td>
<td>10</td>
<td>23</td>
<td>200</td>
</tr>
<tr>
<td>tazobactam</td>
<td>5.3</td>
<td>28</td>
<td>340</td>
</tr>
</tbody>
</table>

Data for VIM-1 are from Franceschini et al., except for moxalactam (this study). Individual kinetic parameters (or the $k_{cat}/K_m$ ratio, for cefepime) are the means of three measurements. Standard deviations were always <10%.
Biochemistry of VIM metallo-β-lactamases

ciently inactivated than VIM-1\textsuperscript{18} by all three chelators (the $k_{inact}/K_{rat}$ ratios were 4- to 20-fold higher for $o$-phenanthroline and dipicolinic acid, respectively), indicating that zinc ions are probably more tightly bound in the latter enzyme.

At the sequence level VIM-1 and VIM-2 are 93\% identical and can be aligned without the introduction of major gaps with all the subclass B1 β-lactamases,\textsuperscript{33} including those (Bc-II, CcrA and IMP-1) for which three-dimensional structures are available.\textsuperscript{4,5,7,29} Of the latter enzymes, CcrA was chosen as the basis for construction of VIM-1 and VIM-2 structural models, since although Bc-II exhibits a higher similarity, the CcrA structure has better defined coordinates for the loop between strands 3 and 4.\textsuperscript{29} Compared with CcrA, the differences in terms of deletions/insertions for VIM-1 and VIM-2 consist of: (i) the lack of one residue (W64) in the L1 loop between strands 3 and 4; and (ii) the insertion of two residues (L172 and E173) in the L2 loop, between helix 3 and strand 8 (Figure 3).

Of the 17 residues differentiating VIM-2 from VIM-1, most (including those at positions 25, 26, 27, 35, 148, 215, 246, 251, 257, 258, 284, 287, 294 and 299) are located at the protein surface and are distant from the active site, while three (at positions 223, 224 and 228) are located in the neighbourhood of the active site (in loop L3) and could influence substrate binding (Figure 4).

Docking experiments with benzylpenicillin support this view and revealed some interesting structural features that could explain the different affinities of VIM-1 and VIM-2 for this substrate. In both VIM-1 and VIM-2, the phenyl ring of benzylpenicillin could interact with the aromatic bulky side-chains of F61, Y67 and W87 (located in loop L1) by means of aromatic–aromatic interactions, whereas the carbonyl oxygen of the β-lactam fits in the oxyanion hole formed by the zinc atom of the first binding site (Zn1) and the side-chain of N233, which is conserved in most metallo-β-lactamases.\textsuperscript{33,36} A unique feature of VIM-type enzymes is represented by the variability encountered at position 224. In other enzymes of subclass B1 a conserved lysine residue, whose side-chain loosely interacts with the β-lactam carboxylate,\textsuperscript{37} is found here. This could differentially affect the orientation and stabilization of the substrate in the active site. At position 224, VIM-1 has a histidine whose side-chain is shorter and probably neutral in this environment, and would not be expected to create an interaction with the substrate, whereas VIM-2 has a tyrosine whose slightly longer side-chain might positively affect stabilization with some substrates, including those carrying a positively charged C3 group, such as ceftazidime, for which VIM-2 exhibits a higher affinity than VIM-1. Moreover, stabilization of the substrate in the active site could be influenced in different ways by the different residues at positions 228. At this position, VIM-2 has an arginine, whose side-chain protrudes into the active site so that the positively charged guanidium group could interact directly with the benzylpenicillin carboxylate (O–NH$_2$ distance: 3.09 Å), whereas VIM-1 has a serine, whose side-chain is definitely too short to create any interaction with the substrate (Figure 5). The supplementary interaction between R228 and the substrate in VIM-2 could explain the higher affinity for benzylpenicillin observed with the latter enzyme compared with VIM-1.

Conclusions

Comparative biochemical analysis carried out under the same experimental conditions allowed us to confirm that there are notable differences between VIM-1 and VIM-2 in their interactions with several β-lactam substrates and also with metal chelators. This means that at least some of the amino acid differences between the two proteins must have functional significance. For this reason, the two natural VIM variants present a relevant model for understanding the roles of specific residues in the mechanism of zinc-β-lactamases. It should be noted that although the two α/β domains constituting the protein are similar in size, most of the differences...
between the two enzymes (12 of 17) are clustered in the second domain, whereas the first domain is more strongly conserved (it contains only five differences, of which three are located at the N terminus and are not likely to be relevant to the protein fold). Since VIM-1 and VIM-2 are probably derived from a common ancestor, the above observation suggests that the C-terminal domain could evolve at a faster pace to give different specificities to this type of enzyme.

Molecular modelling suggests a possible role for some of the different residues and, on the basis of these indications, we are currently generating specific mutants to investigate this point. Resolution of the three-dimensional structure of the
Biochemistry of VIM metallo-β-lactamas

VIM enzymes is also underway, and will provide an essential contribution to the understanding of the mechanistic properties of these clinically relevant enzymes. The functional diversity encountered among metallo-β-lactamas produced by opportunistic pathogens could be critical to the development of effective ‘broad-spectrum’ inactivators of these enzymes.

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