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Protein Engineering of the Flexible Loops of the IMP and SPM Metallo-ß-lactamases Reveals New Insights into Substrate Binding and Hydrolysis

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ABSTRACT

Background: SPM-1 has a flexible unique 24 amino acid loop close to the active site. Flexible loops of other metallo-ß-lactamases (MßLs) are very different in length and location. To provide an insight into their mechanism of hydrolysis, the IMP-1 loop and SPM-1 loop were substituted.
Methods: The 24 amino acid loop was deleted from SPM-1 and the 10 amino acid loop of IMP-1 inserted (SPM∆IMP) by PCR overlapping primer techniques. The PCR product was over-expressed in *E. coli*, and SPM∆IMP was purified using standard 2-stage protein chromatography. The hydrolytic profile determined for 8 representative ß-lactam antibiotics. Zinc assays were undertaken using atomic absorbance psectroscopy. Circular dichroism determined the structural effect of these changes.
Results: Sequence analysis verified the genetic changes and SDS-PAGE confirmed gene expression and purity. SPM∆IMP gave V_{max}'s for imipenem and meropenem of 0.018 and 0.033 s^{-1} compared to 4 and 3.2 s^{-1} (wt-SPM) respectively. The K_m values for imipenem increased to 107 µM (wt-SPM, 36), and, intriguingly decreased for meropenem from 280 (wt-SPM) to 81 µM. For penicillin G, amipicillin, cefuroxime and ceftazidime, the V_{max}'s (0.045, 0.005, 0.0135, 0.012 s^{-1} respectively) also significantly decreased compared to wt-SPM-1 (2, 2, 6, 5 s^{-1} respectively). The K_m's for penicillin G and ampicillin, increased from 38 and 72 µM (wt-SPM) to 270 and 250 µM, respectively. Interestingly, the K_m's for cefuroxime and ceftazidime decreased from 4 to 3 µm and 46 to 29 µm respectively.
Conclusions: Replacement of the SPM-1 loop with the IMP-1 loop did not consistently result in weaker substrate binding. Lower V_{max} values and, overall, raised K_m values demonstrate that the IMP-1 loop does not compensate for the native SPM-1 loop in substrate binding and hydrolysis and the native loop is central to the hydrolysis of ß-lactams by SPM-1.

INTRODUCTION

The 24 amino acid loop of SPM-1, positioned close to the active site of SPM-1 in both the molecular model and the crystal structure, appears to behave as an active site loop that enhances the binding and hydrolysis of most ß-lactams. All other characterized class B1 metallo-ß-lactamases possess active site loops significantly distinct from that of SPM-1 in being shorter and positioned further upstream within the primary sequence. Sequence alignment of SPM-1with IMP-1 (Figure 1) shows that the residues within the loop region of IMP-1 (residues 21-30) have no identity with the corresponding sequence of SPM-1 (residues 24-33), and among the ten residues constituting the loop only four had a similar function (Figure 2). Accordingly, we exchanged the IMP-1 loop for the corresponding residues of SPM-L-Del (SPM without the loop), to determine whether the active site loop of IMP-1 could act in place of the SPM-1 active site loop and restore an enhanced level of substrate catalysis to the mutant enzyme. This poster describes the generation of S∆I, an SPM-1 protein that lacks the SPM-1 loop, but possesses the IMP-1 loop, and its subsequent analysis.

MATERIALS AND METHODS

The 24 amino acid loop mutant was deleted from SPM-1 and the 10 amino acid loop of IMP-1 inserted (S∆I) by PCR overlapping primer technique. Primers were designed to delete the loop from SPM-1 and insert the IMP-1 loop in the same position as it naturally occurs in IMP-1 (Figures 3 and 4). PCR products were cleaned by commercial kits and sequenced on both strands to check the genetic integrity of the constructs.

The sequenced PCR product was cloned into pET26b and over-expressed in *Escherichia coli*. Induction conditions with IPTG was verified with SDS-PAGE. S∆I was purified from a periplasmic cell preparation using ion-exchange and gel filtration protein chromatography. S∆I was followed through the purification process using nitrocefin hydrolysis. The purity of S∆I was verified using SDS-PAGE (Figure 5). When the protein was > 99% pure, the preparation was used for steady-state kinetic profiling. The hydrolytic profile determined for nine representative ß-lactams: benzylpenicillin, ampicillin, nitrocefin, cefuroxime, ceftazidime, imipenem, meropenem, moxalactam and aztreonam. Hydrolysis was undertaken at the peak absorbance for each of the intact substrates and with the exception of nitrocefin, kinetic values were calculated on substrate depletion. K_{cat} and K_m values were derived from standard Michaelis-Menton kinetics. These K_{cat} and K_m values were compared to SPM-L-Del and to SPM-WT under exactly the same assay conditions.

Zinc assays were undertaken on pure S∆I using atomic absorbance spectroscopy. Circular dichroism was undertaken on pure protein to examine the structural effects of these changes gross changes to S∆I.

RESULTS

- The 4- primer PCR technique functioned successfully creating the hybrid protein S∆I which was ratified by sequencing.
- The over-expressed enzyme did not give particularly high protein yields and it was comparable to that of SPM-L-Del. Subsequent checking of the whole cells to examine whether the protein had been captured in inclusion bodies revealed that this was not the case. The concentration of SPM-L-Del obtained following purification from a 4L culture was typically 4 µM in a volume of 10 mls.
- The molecular mass of S∆I, as determined by ESI mass spectrometry was found to be 24.495 kDa, this is in agreement with the predicted molecular mass of 24.52 kDa, calculated using Expasy and is substantiated by the SDS-PAGE of pure S∆I (Figure 5) which shows a band of approximately 24 kDa.
- In concurrence with wt-SPM, the zinc content of S∆I was found to be 1.5 moles of zinc per enzyme molecule.
- The K_m values differ for each class of ß-lactam assayed (Table 1). For the two penicillin compounds and nitrocefin, the K_m values showed an increase. This was most notable for nitrocefin which showed an 11-fold increase from the K_m shown for wt-SPM, from 4 µM to 44 µM. Ampicillin exhibited the least alteration in K_m with a 3.5-fold rise from that observed for wt-SPM. In contrast, S∆I binded the carbapenems and ceftazidime with greater affinity. A 1.2-fold reduction in K_m was observed for each of these substrates, whilst the K_m for cefuroxime remained unchanged from wt-SPM at 4 µM.
- The rate of substrate turnover k_{cat} (s^{-1}) showed a decrease for S∆I when compared to wt-SPM for all compounds assayed with the exception of nitrocefin (Table 1). The greatest decline was seen for cefuroxime and ceftazidime for which the k_{cat} values reduced by 37- and 28- fold respectively. s^{-1}). The k_{cat} (s^{-1}) values for both the carbapenems assayed decreased by approximately 10-fold. The least reduction was noted for benzylpenicillin and ampicillin which declined by 3.5- and 2.5-fold respectively. In contrast, the k_{cat} for nitrocefin doubled from 0.5 to 1 s^{-1}.

Table 1. Kinetic parameters of S∆I in comparison to SPM-L-Del and SPM-1 wild type.

	S∆I			Wt-SPM			SPM-L-Del		
Antibiotics	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($µM^{-1}$ s^{-1})	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($µM^{-1}$ s^{-1})	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($µM^{-1}$ s^{-1})
Benzylpenicillin	30 + 1	273 + 20	0.1	108 + 4	38 + 1	2.8	62 + 2	226 + 18	0.27
Ampicillin	24 + 2	258 + 34	0.1	117 + 2	72 + 3	1.6	10 + 1	198.5 + 15	0.05
Nitrocefin	1 + 0.05	44 + 5	0.02	0.5 + 0.01	4 + 1	0.12	0.12 + 0.05	15 + 3	0.01
Cefuroxime	1 + 0.2	4 + 0.3	0.25	37 + 3	4 + 1	8.8	5.8 + 3	5.0 + 1.5	1.16
Ceftazidime	1 + 0.05	38.5 + 3	0.02	28 + 2	46 + 2	0.6	20.5 + 5	98 + 3	0.2
Imipenem	2 + 0.1	31 + 16	0.06	33 + 2	37 + 4	1	0.63 + 0.08	9.6 + 2	0.065
Meropenem	6 + 0.2	250 + 30	0.02	63 + 3	281 + 27	0.22	1.4 + 0.05	61 + 11	0.02
Moxalactam	[a]	[a]	[a]	13 + 1	97 + 10	0.13	[a]	[a]	[a]
Aztreonam	[a]	[a]	[a]	<0.01	>1,000	<0.0001	[a]	[a]	[a]

a. Data could not be determined

- As with wt-SPM and SPM-L-Del, hydrolysis of aztreonam and moxalactam by S∆I was not measurable. Increasing the concentration of S∆I or incubation of both compounds with S∆I for one hour did not result in breakdown of the substrate.
- CD spectra was obtained for both wt-SPM and S∆I to facilitate comparison of the mutant protein with the native enzyme (Figure 6). The CD spectra of the S∆I amide region showed that the secondary backbone structure of the mutant protein was intact. When the CD spectra of S∆I was compared to that of wt-SPM, there were no significant differences, indicating that S∆I retained the secondary backbone structure of wt-SPM following the deletion of the loop and the exchange of residues 21-30 of IMP-1 for residues 24-33 in the SPM-1 sequence.

Figure 1: Alignment used to generate the homology model of SPM-1. asterisks = identical residues in IMP-1 and SPM-1; dots = residues of similar function. Residues for the IMP-1 crystal structure of the sequence with pdb identification number 1JJE are numbered in accordance with the Swiss Protein pdb file. The sequence with pdb identification number 2BBK used to model the inserted loop region (SPM-1 residues 113 to 131) was LLVDQRDGWRHKTASRFV (residues 283 to 301) in the pdb file.

```
SPM-1      5        DLP YNLTATKIDS DVFVVTDRDF YSSNVLVAK- ----MLDGTV
1JJE       3        SLP -DLKIEKLDE GVYVHTSFEE VNGWGVVPKH GLVVLVNAEA
                     **  .*    *.*    *.* *  .     ..   .*.*      ....

SPM-1     43  VIVSSPFENL GTQTLMDWVA KTMKPKKVVA INTHFHLDGT GGNEIYKKMG
1JJE      45  YLIDTPFTAK DTEKLVTWFV ERG-YKIKGS ISSHFHSDST GGIEWLNSRS
               .. .**     *. *. *         *    . *..*** *.* ** *    .   .

SPM-1     93  AETWSSDLTK QLRLEENKKD RIKAAEFYKN EDLKRRILSS HPVPADNVFD
1JJE      94  IPTYASELTN ELLKKDGK-- ---------- ---------- --VQATNSFS
                *  .*.**.  .*     .  *                              *  *  *  *

SPM-1    143  LKQGKVFSFS NELVEVSFPG PAHSPDNVVV YFPKKKLLFG GCMIKPKELG
1JJE     120  ---GVNYWLV KNKIEVFYPG PGHTPDNVVV WLPERKILFG GCFIKPYGLG
                 *   . .   .. .** .** *.*.******   .*  .*.*** ** ***   **

SPM-1    193  YLGDANVKAW PDSARRLK-K F-DAKIVIPG HGEWGGPEMV NKTIKVAEKA
1JJE     167  NLGDANIEAW PKSAKLLKSK YGKAKLVVPS HSEVGDASLL KLTLEQAVKG
                *****. ** * **. ** *  .   **.*.*. *.* *  . .. . *.   * *.

SPM-1    241  VGEMRL -
1JJE     217  LNESKK
              . * .
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Figure 2: The homology model of SPM-1 compared with the IMP-1 crystal structure. IMP-1 structure of the sequence with pdb identification number 1JJE is shown in red, the SPM-1 homology model is shown in yellow. SPM-1 zinc binding ligands are shown in magenta.

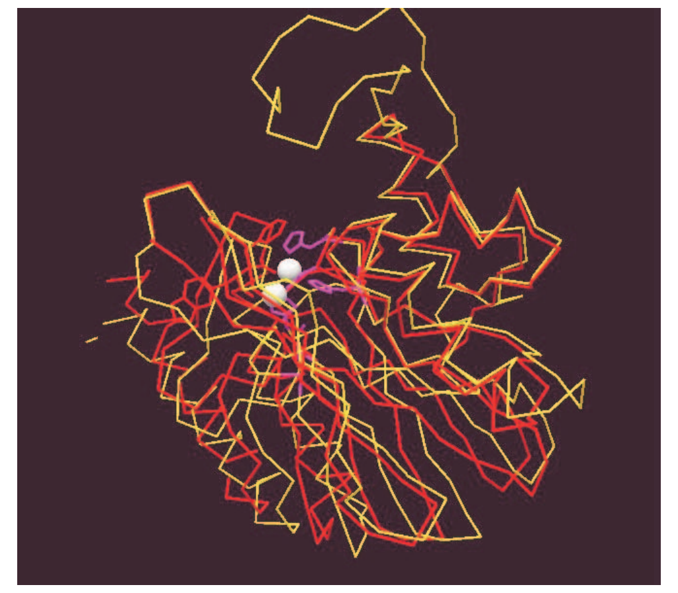


Figure 3: '4-primer' PCR method for site-directed insertion of sequences into genes. Primers 1 and 4 are external primers designed to complement the termini of the gene. Primers 2 and 3 are internal mutagenic 'long primers'.

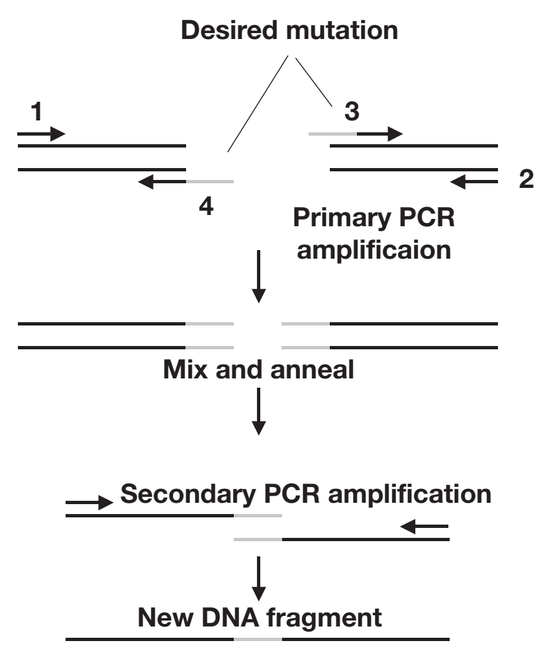


Figure 4. Agarose gel of S∆I and the component amplicons. A = 1kb+ DNA marker, B = full length S∆I (SPM-*Nde*I- and SPM-*Bam*HI primers), C = 5' amplicon (SPM-*Nde*I and IMP-Loop-Ins-R primers), D = 3' amplicon (IMP-Loop-Ins-F and SPM-*Bam*HI primers).

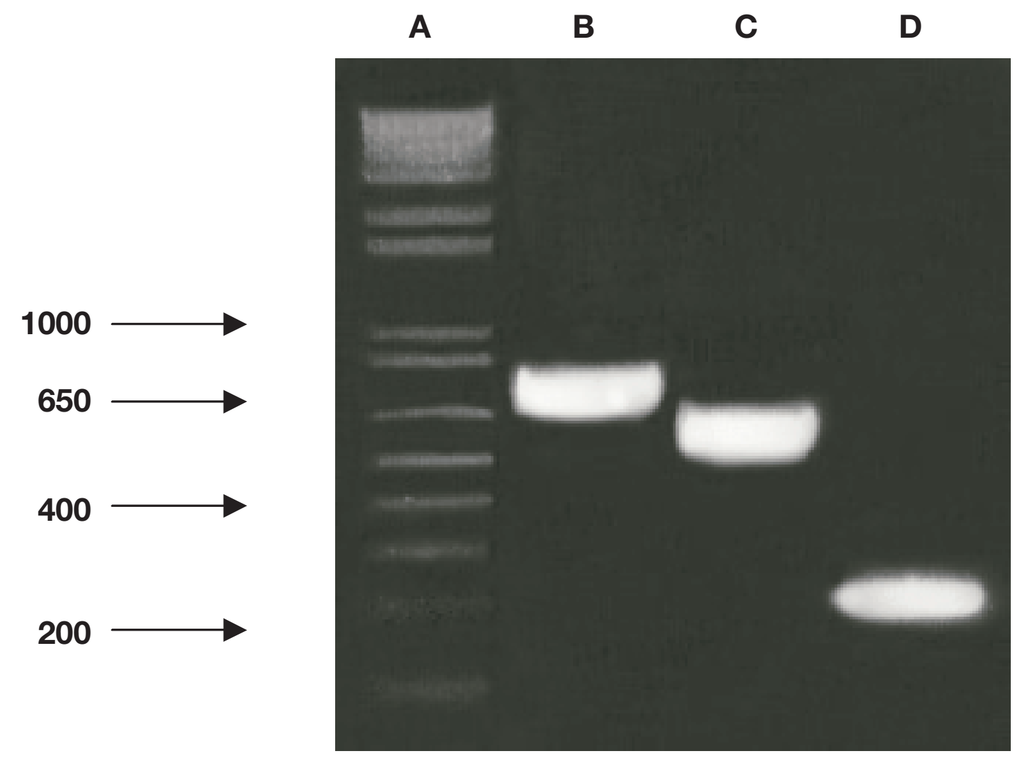


Figure 5. Purity of S∆I eluted from the Superdex-75 gel filtration column. Fractions 56-70 were pooled. (M = Molecular weight marker).

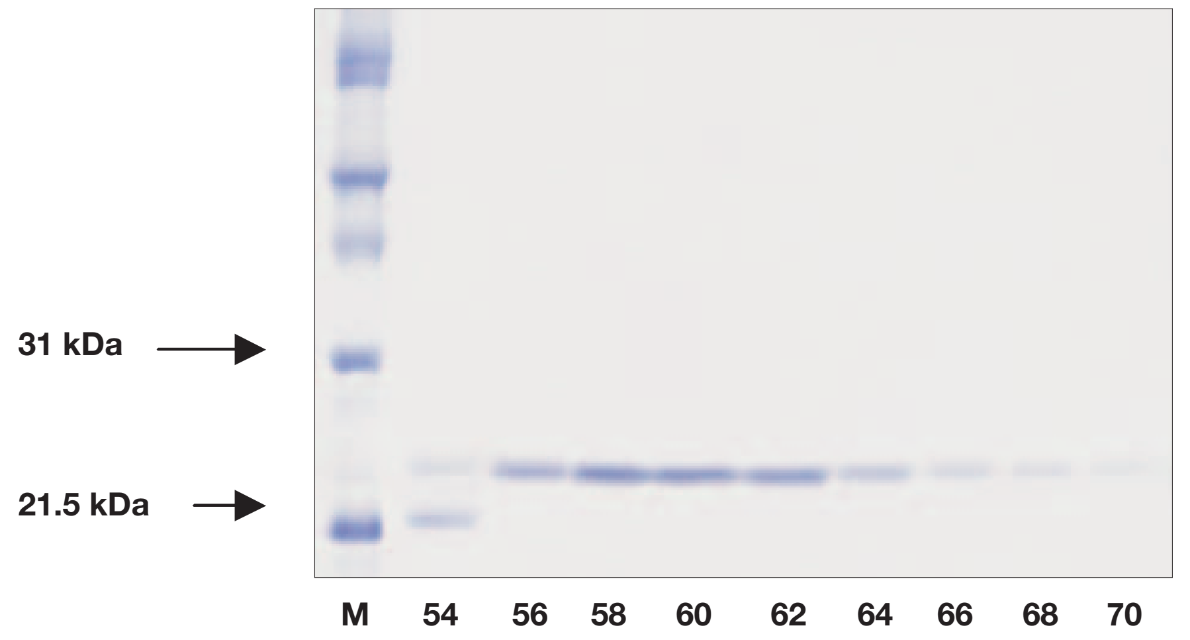
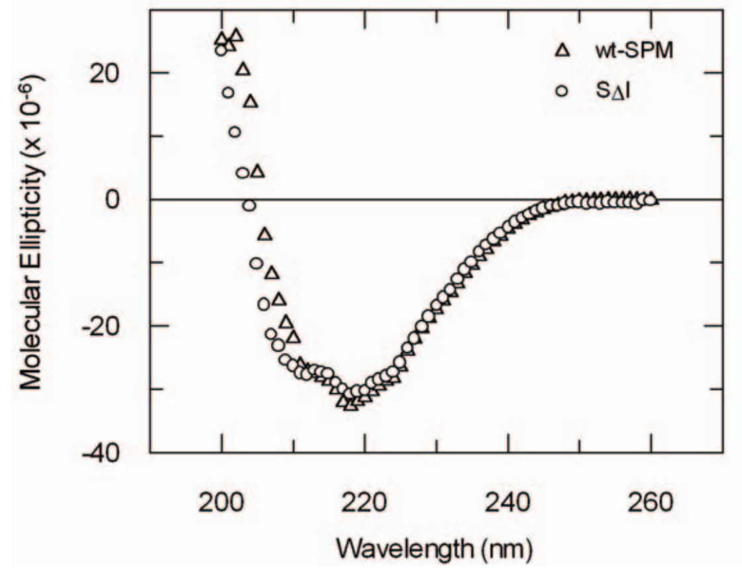


Figure 6. CD spectra for S∆I in comparison with wt-SPM.



CONCLUSIONS

- The findings with respect to S∆I are in contrast to those of Moali *et al* who reported that the BcII∆IMP enzyme is a more effective metallo-ß-lactamase than wt-BCII. The results presented here engender the conclusion that the IMP-1 active site loop does not substitute for the wt-SPM active site loop, and for some substrates is detrimental.
- Although it is impossible to say conclusively from the current data whether the length of the active site loop plays a critical role in substrate hydrolysis, or even if the position of the loop within the structure of the enzyme is important, the loop unequivocally possesses features, absent from the IMP-1 loop which are vital to the function of SPM-1 as a metallo-ß-lactamase.

SELECTED REFERENCES

Haruta S, Yamamoto ET, Eriguchi Y, Sawai T. (2001). Characterization of the active-site residues asparagine 167 and lysine 161 of the IMP-1 metallo-ß-lactamase. *FEMS Microbiology Letter* 197:85-89.

Moali C, Anne C, Lamotte-Brasseur J, Groslambert S, Devreese B, Van Beeumen J, Galleni M, Frere JM. (2003). Analysis of the importance of the metallo-ß-lactamase active site loop in substrate binding and catalysis. *Chemical Biology* 10:319-329.

Murphy TA, Simm AM, Toleman MA, Jones RN, Walsh TR. (2003). Biochemical characterization of the acquired metallo-ß-lactamase SPM-1 from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 47:582-587.

Osano E, Arakawa Y, Wacharotayankun R, Ohta M, Horii T, Ito H, Yoshimura F, Kato N. (1994). Molecular characterization of an enterobacterial metallo-ß-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. *Antimicrobial Agents and Chemotherapy* 38:71-78.

Toleman MA, Simm AM, Murphy TA, Gales AC, Biedenbach DJ, Jones RN, Walsh TR. (2002). Molecular characterization of SPM-1, a novel metallo-ß-lactamase isolated in Latin America: Report from the SENTRY Antimicrobial Surveillance Program. *Journal of Antimicrobial Chemotherapy* 50:673-679.