Title
A Noncanonical Metal Center Drives the Activity of the Sediminispirochaeta smaragdinae Metallo-β-lactamase SPS-1

Permalink
https://escholarship.org/uc/item/0k4109qz

Journal
Biochemistry, 57(35)

ISSN
0006-2960

Authors
Cheng, Z
Vanpelt, J
Bergstrom, A
et al.

Publication Date
2018-09-04

DOI
10.1021/acs.biochem.8b00728

Peer reviewed
A Noncanonical Metal Center Drives the Activity of the Sediminispirochaeta smaragdinae Metallo-β-lactamase SPS-1

Zishuo Cheng,† Jamie Van Pelt,† Alexander Bergstrom,† Christopher Bethel,‡ Andrew Katko, Callie Miller,† Kelly Mason,† Erin Cumming,‡ Huan Zhang,† Robert L. Kimble,† Sarah Fullington,‡ Stacey Lowery Bretz,† Jay C. Nix,‡ Robert A. Bonomo,‡∥§ David L. Tierney,† Richard C. Page,*†§ and Michael W. Crowder*†§

†Department of Chemistry and Biochemistry, Miami University, 651 East High Street, 160 Hughes Laboratories, Oxford, Ohio 45056, United States
‡Research Service, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, Ohio 44106, United States
§Departments of Medicine, Pharmacology, Molecular Biology and Microbiology, Biochemistry, Proteomics, and Bioinformatics and CWRU-Cleveland VAMC Center for Antimicrobial Resistance and Epidemiology, Case Western Reserve University, Cleveland, Ohio 44106, United States
∥Molecular Biology Consortium, Beamline 4.2.2, Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: In an effort to evaluate whether a recently reported putative metallo-β-lactamase (MβL) contains a novel MβL active site, SPS-1 from Sediminispirochaeta smaragdinae was overexpressed, purified, and characterized using spectroscopic and crystallographic studies. Metal analyses demonstrate that recombinant SPS-1 binds nearly 2 equiv of Zn(II), and steady-state kinetic studies show that the enzyme hydrolyzes carbapenems and certain cephalosporins but not β-lactam substrates with bulky substituents at the 6/7 position. Spectroscopic studies of Co(II)-substituted SPS-1 suggest a novel metal center in SPS-1, with a reduced level of spin coupling between the metal ions and a novel Zn1 metal binding site. This site was confirmed with a crystal structure of the enzyme. The structure shows a Zn3 site that is similar to that in NDM-1 and other subclass B1 MβLs; however, the Zn3 metal ion is coordinated by two histidine residues and a water molecule, which is held in position by a hydrogen bond network. The Zn1 metal is displaced nearly 1 Å from the position reported in other MβLs. The structure also shows extended helices above the active site, which create a binding pocket that precludes the binding of substrates with large, bulky substituents at the 6/7 position of β-lactam antibiotics. This study reveals a novel metal binding site in MβLs and suggests that the targeting of metal binding sites in MβLs with inhibitors is now more challenging with the identification of this new MβL.

β-Lactam antibiotics, such as penicillins, cephalosporins, and carbapenems, have long served as treatments for bacterial infections by inactivation of bacterial transpeptidases. These compounds constitute the largest class of clinically available antibiotics. In response to wide use, and overuse, of β-lactam-containing drugs, bacteria have evolved to produce β-lactamases that hydrolyze the β-lactam bond and render the antibiotics inactive.

Of the four classes of β-lactamases (A–D), only class B enzymes require one or two Zn(II) ions, rather than an active site serine, for catalysis and are termed metallo-β-lactamases (MβLs).1–3 MβLs adopt an α/ββα fold, and among the three subclasses (B1–B3), B1 and B3 MβLs generally contain a dinuclear Zn(II) active site, while B2 MβLs are active with mononuclear Zn(II) sites. The B1 MβLs coordinate one Zn(II) (in the Zn1 site) with three histidine residues, while the Zn(II) in the Zn3 site is bound to one cysteine, one aspartate, one histidine, and a terminally bound solvent molecule. The Zn(II) ions are bridged by a solvent molecule, resulting in a Zn–Zn distance of 3.3–3.9 Å.2,4 The B3 MβLs possess the same Zn1 site as the B1 MβLs; however, in the Zn3 site, the cysteine ligand is replaced with an additional His residue. Subclass B1 contains the largest number of clinically relevant MβLs, including VIMs (Verona integron-encoded MβL), IMPs (imipenemase), and NDMs (New Delhi MβL). Most recent studies have focused on MβLs found in pathogenic strains;

Received: July 6, 2018
Revised: August 8, 2018
Published: August 14, 2018
Biochemistry

however, the risk of transfer of antibiotic resistance determinants from a nonpathogenic strain to a more clinically relevant organism necessitates the need to study M\(_{\beta}\)Ls not yet found in known human pathogens.\(^5\)

Recently, with the use of a Markov model and a bioinformatic study, Berglund et al. identified 279 unique, potential subclass B1 M\(_{\beta}\)L genes, and 76 of the corresponding gene products were novel and able to hydrolyze imipenem.\(^7\) One gene, SPS-1, was found in the bacterium *Sediminispirochaeta smaragdinae* (NCBI reference sequence WP_013255389.1). *S. smaragdinae* strain SEBR 4228\(^7\) was discovered in a water sample near a Congo offshore oilfield.\(^7\) SEBR 4228\(^7\) is a Gram-negative, chemoanorganotrophic, and anaerobic bacterium that adopts a spiral, corkscrew-like structure. This organism is halophilic and able to grow in high-salt environments. SPS-1 was predicted to be a M\(_{\beta}\)L; however, SPS-1 lacked the typical H-X-H-X-D motif found in all M\(_{\beta}\)Ls except the B2 M\(_{\beta}\)Ls. In SPS-1, the first consensus histidine residue was substituted with a glycine.\(^3\) In an effort to determine if SPS-1 is indeed a M\(_{\beta}\)L and how the histidine to glycine substitution affected the consensus Zn\(_2\) site, we cloned, overexpressed, and purified the enzyme and characterized the recombinant protein via kinetic, spectroscopic, and crystallographic studies.

## MATERIALS AND METHODS

### Phylogenetic Analysis of SPS-1

The protein sequences of selective class B \(\beta\)-lactamases (M\(_{\beta}\)Ls), as well as SPS-1, were downloaded from the NCBI GenBank and analyzed using Phylogeny.fr (http://www.phylogeny.fr/).\(^5,9\)

### Cloning, Overexpression, and Purification of SPS-1

A codon-optimized SPS-1 sequence (residues 32–276) fused with an N-terminal tobacco etch virus (TEV) cleavage site was synthesized by Genscript Biotech Corp. The synthesized fragment was subcloned into pET28a between the *NdeI* and *XhoI* restriction sites. The resulting overexpression plasmid, pET28a-SPS-1, was transformed into *Escherichia coli* EXPRESS BL21(DE3) Chemically Competent cells (Lucigen). A single colony was transferred into 50 mL of lysogeny broth (LB) containing 50 \(\mu\)g/mL kanamycin, and the culture was shaken overnight at 37 °C. The overnight culture (10 mL) was used to inoculate four 1 L LB cultures containing 50 \(\mu\)g/mL kanamycin, and the culture was shaken at 220 rpm until an OD\(_{600}\) of 0.6 was reached. Protein production was induced by addition of isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) to a final concentration of 500 \(\mu\)M. At the time of induction, cultures were supplemented with Zn(II) with addition of ZnCl\(_2\) to a concentration of 100 \(\mu\)M. Cultures were shaken overnight at 18 °C and then harvested by centrifugation for 10 min at 8000g. Cell pellets were resuspended in 50 mL of 50 mM HEPES (pH 7.5) containing 500 mM NaCl. Cells were lysed by passing the mixture two times through a French press at a pressure between 15000 and 20000 psi. The insoluble components were removed by centrifugation for 1 h at 32000g. The supernatant was loaded onto a HiTrap HP (5 mL, GE) column and washed with 10 volumes of wash buffer containing 50 mM HEPES (pH 7.5), 500 mM NaCl, and 50 mM imidazole. Bound proteins were eluted using elution buffer (wash buffer with 500 mM imidazole). TEV protease was added at a 1:100 (w/w) ratio to SPS-1. The eluted proteins were dialyzed against 1 L of 50 mM HEPES (pH 7.5) containing 500 mM NaCl. After overnight dialysis, the truncated SPS-1 protein was repurified by being passed through a HisTrap column, which removed the His\(_{6}\)-TEV and His\(_{6}\) tag.

Purified recombinant SPS-1 (before and after TEV digestion) was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a Fisher EZ-Gel System (12.5%), a Mini-PROTEAN System (Bio-Rad Laboratories), and Coomassie Blue stain. Protein concentrations were determined by ultraviolet–visible (UV–vis) absorbance at 280 nm using an \(\varepsilon_{280}\) of 35535 M\(^{-1}\) cm\(^{-1}\) for the SPS-1 protein containing the His\(_{6}\) tag and an \(\varepsilon_{280}\) of 3405 M\(^{-1}\) cm\(^{-1}\) for the SPS-1 protein without the His\(_{6}\) tag. These extinction coefficients were calculated using Expasy ProtParam.\(^10\)

### Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry

After dialysis against 10 mM ammonium acetate (pH 7.5), 30 \(\mu\)M tag-free SPS-1 was mixed with a sinapinic acid matrix at a ratio of 1:1. The resulting sample was analyzed using a Bruker AutoFlex MALDI-TOF mass spectrometer.\(^11\)

### Fluorescence Spectroscopy

Fluorescence emission spectra of SPS-1 were recorded on a PerkinElmer luminescence spectrometer (model LS-55) using 2 \(\mu\)M SPS-1 in 50 mM HEPES (pH 7.5). Spectra were acquired using an excitation wavelength of 280 nm and an emission spectrum window from 300 to 500 nm.

### Metal Analyses

The zinc contents of purified SPS-1 samples were determined using a PerkinElmer Optima 7300V inductively coupled plasma spectrometer with optical emission spectroscopy (ICP-OES) detection. Protein samples, used without modification after purification, were diluted to 5 \(\mu\)M with 10 mM ammonium acetate (pH 7.5). Calibration curves were generated using serial dilutions of FisherBrand Zn metal standard ranging from 0 to 16 \(\mu\)M, and the emission line at 202.548 nm was chosen for zinc.

### Steady-State Kinetics

Steady-state kinetic studies were performed using a Synergy HT Multi-Mode Microplate Reader (Biotek) at 37 °C in 50 mM HEPES (pH 7.5) containing 150 mM NaCl and 10 \(\mu\)M ZnCl\(_2\). The changes in molar absorptivity (\(\Delta\varepsilon\), M\(^{-1}\) cm\(^{-1}\)) were used to quantitate products: chromace, \(\Delta\varepsilon_{442} = 18600\); cephalothin, \(\Delta\varepsilon_{365} = -8790\); imipenem, \(\Delta\varepsilon_{300} = -9000\); meropenem, \(\Delta\varepsilon_{293} = -7600\); ampicillin, \(\Delta\varepsilon_{268} = -809\); penicillin G, \(\Delta\varepsilon_{235} = -936\); cefuroxime, \(\Delta\varepsilon_{260} = -5860\); and cephalaxin, \(\Delta\varepsilon_{260} = -7750.12\) Initial rates (within 18 s) versus substrate concentrations were fitted to the Michaelis–Menten equation to determine the steady-kinetic constants \(K_m\) and \(k_{cat}\) using Prism 7 (Graphpad Software).

### Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed using a Nano ITC System (TA Instruments-Waters LLC) with a 500 \(\mu\)L cell. All experiments were performed at 25 °C. SPS-1 and NDM-1 were prepared by diluting the stock solutions with 20 mM HEPES (pH 7.5) containing 150 mM NaCl. \(\Delta\varepsilon_{442}\) was measured in the same buffer. The ITC cell was filled with 50 \(\mu\)M NDM-1 or SPS-1 (300 \(\mu\)L), and the enzyme solutions were titrated with 250 \(\mu\)M captopril. The injection volume was 50 \(\mu\)L, and the time between two injections was 210 s. \(K_m\) values were determined by using NanoAnalyze (TA Instruments-Waters LLC).

### Preparation of Metal-Free Proteins

Metal-free forms of SPS-1 and NDM-1 were prepared through metal chelation. Each purified protein (diluted to 50 \(\mu\)M in 20 mL) was placed in a 10 kDa molecular weight cutoff dialysis bag. These
samples were dialyzed versus dialysis buffers for 4 h at 4 °C before changing to the subsequent dialysis buffer to include a series of five dialysis steps: first, two changes of 50 mM HEPES (pH 6.8) containing 150 mM NaCl with 2 mM EDTA, and, then, three changes of 50 mM HEPES (pH 6.8) containing 150 mM NaCl. The resulting metal-free enzymes were analyzed by using SDS−PAGE and UV−vis and concentrated by using an Amicon Ultra 15 mL centrifugal filter (10K).

UV−vis Spectroscopy. Metal-free SPS-1 and NDM-1 were diluted to 300 μM with 50 mM HEPES (pH 6.8) containing 150 mM NaCl and 1 mM tris(2-carboxyethyl)-phosphine (TCEP). One or two molar equivalents of CoCl2 was added to the protein samples from a 50 mM stock solution. The resulting mono-Co(II)-substituted or di-Co(II)-substituted protein samples were placed in 500 μL quartz cuvettes, and UV−vis spectra were recorded on a PerkinElmer Lambda 750 UV/vis/NIR spectrometer that measured the absorbance between 300 and 700 nm at 25 °C. The blank spectrum of 300 μM apo-SPS-1 or apo-NDM-1 in 50 mM HEPES (pH 6.8) containing 150 mM NaCl and 1 mM TCEP was used to generate difference spectra. All data were normalized to the absorbance at 700 nm.

NMR Spectroscopy. Metal-free SPS-1 was concentrated to ∼0.5 mM with an AmiconUltra-15 centrifugal unit and an Ultracel-10 membrane. To the concentrated protein was added 2 equiv of CoCl2 in the buffer containing 50 mM HEPES (pH 6.8), 150 mM NaCl, and 10% (v/v) D2O. Spectra were recorded at 292 K on a Bruker ASX300 (BBI probe) NMR spectrometer operating at a frequency of 300.16 MHz. Spectra were collected using a frequency switching method, applying a long low-power (270 ms) pulse centered at the water frequency, followed by a high-power 3 μs pulse centered at either 200 or 90 ppm to ensure all resonances were detected. This method allowed for suppression of the water signal with enhancement of severely hyperfine-shifted resonances. Spectra consisted of 120000 transients of 16K data points over a 333 ppm spectral window (τaq ∼51 ms). Signal averaging took ∼12 h per spectrum.

EPR Spectroscopy. EPR samples containing SPS-1 or NDM-1 included approximately 10% (v/v) glycerol as a glassing agent. Samples were loaded into EPR tubes and degassed by repeated evacuation/purging with N2 prior to data collection. Spectra were recorded on a Bruker EMX EPR spectrometer, equipped with an ER4116-DM dual-mode resonator (9.37 GHz, parallel; 9.62 GHz, perpendicular). The data in EPR figures were scaled so that the X-axes matched (perpendicular mode field values were scaled by 9.37/9.62). The temperature was controlled using an Oxford ESR900 cryostat and temperature controller (4.5 K). Other spectral conditions included a microwave power of 0.2 mW, a field modulation of 10 G (100 kHz), a receiver gain of 104, and a time constant/conversion time of 41 ms.

Crystallization, Diffraction Data Collection, and Structural Refinement. SPS-1 was crystallized using 96-well sitting drop vapor diffusion screens prepared using a Phoenix crystallization robot (Art Robbins). The sitting drops contained 400 nL of 1.45 mM SPS-1 or apo-NDM-1 in 50 mM HEPES (pH 6.8) containing 150 mM NaCl and 1 mM TCEP. Six crystallization screens were used: MCSG 1−4 by Microlytic and JCSG and PACT++ by JBScreen. Diffraction quality crystals formed within 2 weeks under a condition consisting of 0.2 M magnesium chloride, 0.1 M sodium cacodylate (pH 6.5), and 50% (v/v) PEG 200. For

Figure 1. Phylogenetic tree of class B metallo-β-lactamase sequences. The protein sequences of SPS-1 and other selective metallo-β-lactamase were downloaded and analyzed by Phylogeny.fr. The sequence include SPS-1 (WP_013255389.1), BcII (M11189), CcrA (M63556), IMP-1 (S71932), VIM-2 (AF191564), BlaB (AF189298), SPM-1 (AY341249), NDM-1 (JN420336), GIM-1 (AJ620678), SIM-1 (AY887066), D1-1 (GU323019), TMB-1 (FR771847), Bla2 (CP001598), KHM-1 (AB364006), CphA (X75102), Sbh-1 (AF197943), ImiS (Y01415), L1 (AB294542), FEZ-1 (Y17886), BJF-1 (NP772870), AIM-1 (AM998375), THIN-B (CAC33832), G0B-1 (AB021417), CAU-1 (CAC87665), CAR-1 (Q6D395), SMB-1 (AB636283), POM-1 (ADC79555), and CRB11 (ACS83724). The line segment with the number 0.6 shows the length of the branch that represents an amount of genetic change of 0.6.
crystal harvesting, MiTeGen MicroLoops were used to harvest crystals that had been cryoprotected with LV CryoOil and then immediately frozen in liquid nitrogen. Crystals were sent to the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory where X-ray diffraction data were collected. Data were integrated and scaled using XDS\textsuperscript{14} and SCALA.\textsuperscript{15} Data resolution cutoffs were determined using a combination of criteria, including completeness and CC\textsubscript{1/2}. Molecular replacement was conducted with PHASER\textsuperscript{16} using a polyalanine search model produced from the structure of SPM-1 [Protein Data Bank (PDB) entry 4BP0].\textsuperscript{17} The SPS-1 amino acid sequence was then docked to the molecular replacement solution. Iterative model building and refinement were conducted using COOT\textsuperscript{18} and PHENIX\textsuperscript{19} using isotropic individual B factors and TLS refinement.\textsuperscript{20} Stereochemical and geometric quality analyses were performed with MolProbity.

<table>
<thead>
<tr>
<th>substrate</th>
<th>SPS-1\textsuperscript{a}</th>
<th>SPM-1\textsuperscript{a,b}</th>
<th>NDM-1\textsuperscript{c,d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromacef</td>
<td>$120 \pm 9$ 2.6 \pm 0.1 0.021</td>
<td>not available</td>
<td>5.1 \pm 0.9 4.2 \pm 0.8 0.8</td>
</tr>
<tr>
<td>ampicillin</td>
<td>not observed</td>
<td>72 \pm 3 120 \pm 2 1.6</td>
<td>22 15 0.66</td>
</tr>
<tr>
<td>penicillin G</td>
<td>not observed</td>
<td>not available</td>
<td>16 11 0.68</td>
</tr>
<tr>
<td>imipenem</td>
<td>$290 \pm 50$ 470 \pm 60 1.63</td>
<td>37 \pm 4 33 \pm 2 1</td>
<td>94 20 0.21</td>
</tr>
<tr>
<td>meropenem</td>
<td>$170 \pm 30$ 520 \pm 50 3</td>
<td>280 \pm 30 63 \pm 3 0.22</td>
<td>49 12 0.25</td>
</tr>
<tr>
<td>cephalothin</td>
<td>$110 \pm 20$ 3.1 \pm 0.1 0.027</td>
<td>4 \pm 1 43 \pm 2 11.7</td>
<td>10 4 0.4</td>
</tr>
<tr>
<td>cefuroxime</td>
<td>not observed</td>
<td>4 \pm 1 37 \pm 3 8.8</td>
<td>8 5 0.61</td>
</tr>
<tr>
<td>cephalexin</td>
<td>not observed</td>
<td>not available</td>
<td>not available</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The buffer consisted of 50 mM HEPES (pH 7.5) containing 150 mM NaCl and 10 \mu M ZnCl\textsubscript{2}.

Table 1. Steady-State Kinetic Parameters for Different $\beta$-Lactams with SPS-1, NDM-1, and SPM-1

![Figure 2. ITC results of l-captopril titrations with NDM-1 and SPS-1. The top panel shows the titration thermograms, and the bottom panel shows the data integration with fitted curves. Data for the NDM-1 protein are colored red, and data for SPS-1 are colored blue.](image-url)
version 4.4. The final model and structure factors were deposited in the Protein Data Bank as entry 6CQS.

RESULTS

Phylogenetic Analysis of SPS-1. M/Ls can be grouped into three subclasses phylogenetically. Subclass B1 M/Ls contain a Zn₁ binding site with three histidine residues (His₁₁₆, His₁₁₈, and His₁₉₆) and a Zn₂ site with three other residues (Asp₁₂₀, Cys₂₂₁, and His₂₆₃). Subclass B2 M/Ls contain a Zn₁ site with one altered residue (Asn₁₁₆, His₁₁₈, or His₁₉₆) and a Zn₂ site identical to that of the subclass B1 M/Ls; the altered Zn₁ site results in B₂ M/Ls exhibiting activity as mononuclear Zn(II) enzymes. The subclass B3 M/Ls have a varied Zn₁ binding site (His/Gln₁₁₆, His₁₁₈, and His₁₉₆) and a distinctive Zn₂ binding site, which does not contain a cysteine residue (Asp₁₂₀, His₁₁₂, and His₂₆₃). To determine the phylogenetic position of SPS-1 in the classification of M/Ls, phylogenetic analysis was conducted using Phylogeny. The analyses showed that enzymes in subclasses B₁ and B₂ descended from a common ancestor and that SPS-1 was grouped in the B₁ class and is highly similar to M/Ls from the SPM-1 family. SPS-1 was grouped in the B₁ class and is highly similar to M/Ls from the SPM-1 family.

Overexpression, Purification, and Characterization of ZnZn-SPS-1. A region of the gene for SPS-1 corresponding to residues 32–276 was synthesized to aid in cloning SPS-1 into a pET28A overexpression vector. The DNA sequence corresponding to the first 31 amino acids was not included as these residues were predicted by Phobius to comprise a signal peptide. Overexpressed SPS-1 was purified with a single-step HisTrap column. TEV protease was used to remove the His₆ tag, and a second HisTrap column was used to separate SPS-1 from the cleaved tag and TEV protease. The purity of the protein was shown to be >95% by SDS–PAGE.

Steady-State Kinetics. Steady-state kinetic studies were conducted at 37 °C using chromacef, ampicillin, penicillin G, imipenem, meropenem, cefalothin, cefuroxime, and cephalalexin as substrates. The resulting kinetic data are listed in Table 1. When using chromacef and cefalothin as the substrate, SPS-1 exhibited $K_m$ values of 120 ± 9 and 110 ± 20 μM and $k_{cat}$ values of 2.6 ± 0.1 and 3.1 ± 0.1 s⁻¹, respectively. When using imipenem and meropenem as substrates, SPS-1 exhibited $K_m$ values of 290 ± 50 and 170 ± 30 μM and $k_{cat}$ values of 470 ± 60 and 520 ± 50 s⁻¹, respectively. When ampicillin, penicillin G, cefuroxime, and cephalalexin were used as substrates, no activity was observed. Compared to those of NDM-1 and SPM-1, the $K_m$ values with different substrates exhibited by SPS-1 are much larger, resulting in lower catalytic efficiencies (Table 1). In contrast to most other M/Ls, SPS-1 exhibits substrate specificities for carbapenems and certain cephalosporins.

Isothermal Titration Calorimetry (ITC). ITC was used to determine if SPS-1 binds to L-captopril, which has been reported to be a competitive inhibitor of several M/Ls. Previous studies have shown that the S₁ atom in L-captopril inserts directly between the metal ions in NDM-1, displacing the bridging water/hydroxide. The crystal structure of the NDM-1/captopril complex showed that L-captopril has two binding faces: a hydrophobic face that interacts with Val₇₃ and Met₆⁷ of the L₁ loop and a hydrophilic face that hydrogen bonds to Asn₂₂₀ on the L₁₀ loop. Under our experimental conditions, L-captopril binds to NDM-1 with a $K_I$ value of 1.95 μM (Figure 2), while there is no binding between SPS-1 and L-captopril at L-captopril concentrations of ≤ 50 μM. This result suggests a different active site configuration in SPS-1 compared to that in NDM-1.

UV–Vis Spectroscopy. To obtain information about the metal binding sites in SPS-1, UV–vis spectra of Co(II)-substituted SPS-1 were recorded, and Co(II)-substituted NDM-1 was used as a control. Metal-free SPS-1 and NDM-1 were prepared as described in Materials and Methods. TCEP was added to metal-free SPS-1, prior to adding Co(II), to prevent Co(II) oxidation. Glycerol (10%) was added to the metal-free SPS-1 before Co(II) addition to prevent protein precipitation.

The resulting spectrum showed a single peak at 330 nm, which was previously attributed to a Cys¬S to Co(II) ligand-to-metal charge transfer transition. The other peaks at 330, 510, and 640 nm (Figure 3) were assigned to ligand field transitions of high-spin Co(II) exhibiting a Jahn–Teller distortion. The extinction coefficients of these ligand field transitions are consistent with one Co(II) having a coordination number of 4 and the other having a coordination number of 5. Conversely, the spectra of Co(II)-substituted SPS-1 revealed a sharp absorption at 346 nm (Figure 3), suggesting a similar Zn₂ site in SPS-1 and NDM-1. However, in the spectra of Co(II)-substituted SPS-1, the ligand field transitions between 500 and 650 nm are broader and lack distinct features. Importantly, the extinction coefficient of this peak is 56 M⁻¹ cm⁻¹ [or ~28 M⁻¹ cm⁻¹ per Co(II)].
sugestng that both Co(II) ions are six-coordinate.\textsuperscript{26} A similar spectrum was previously reported for Co(II)-substituted L1.\textsuperscript{36} 

**NMR Spectroscopy of Co(II)-Substituted SPS-1.** To further probe the metal binding sites in SPS-1, \textsuperscript{1}H NMR spectra of Co(II)-substituted SPS-1 were recorded (Figure 4).

![Image of NMR spectra](image)

Figure 4. \textsuperscript{1}H NMR (300 MHz) spectra of Co(II)-substituted NDM-1 (1 mM) and SPS-1 (0.5 mM).

As with the UV-vis spectra presented above, Co(II)-substituted NDM-1 was used as a control. Previously for Co(II)-substituted NDM-1, we showed that resonances at 64, 72, and 79 ppm are due to solvent-exchangeable protons on Co(II)-substituted NDM-1 was used as a control. Previously for Co(II)-substituted SPS-1 were recorded (Figure 4).

1.27 Co(II)-substituted NDM-1 also showed tightly coupled, active site Co(II) ions in the parallel mode spectra (a deep negative feature near 800 G).\textsuperscript{38} However, no such feature was observed in the spectrum of Co(II)-substituted SPS-1, indicating weaker coupling of active site Co(II) ions in SPS-1. 

**Crystal Structure of SPS-1.** The structure of SPS-1 was determined to 1.70 Å resolution featuring an asymmetric unit containing a single protomer. Details of the crystallographic refinement are listed in Table 2. Two Zn(II) ions are bound in the active site, and a third zinc ion is coordinated by the side chains of the His110 and Asp106 residues of the single protomer within the asymmetric unit and the His110 and

<table>
<thead>
<tr>
<th>Data Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>beamline</td>
</tr>
<tr>
<td>wavelength (Å)</td>
</tr>
<tr>
<td>space group</td>
</tr>
<tr>
<td>α, β, γ (deg)</td>
</tr>
<tr>
<td>resolution range (Å)</td>
</tr>
<tr>
<td>no. of reflections\textsuperscript{a}</td>
</tr>
<tr>
<td>no. of unique reflections\textsuperscript{a}</td>
</tr>
<tr>
<td>completeness (%)</td>
</tr>
<tr>
<td>Wilson B factor (Å\textsuperscript{2})</td>
</tr>
<tr>
<td>CC\textsubscript{1/2}</td>
</tr>
</tbody>
</table>

**Refinement**

| no. of reflections\textsuperscript{a} | 33588 (3290) |
| PDB entry | 6CQS |
| R\textsubscript{work}/R\textsubscript{free} | 0.176/0.194 |
| no. of atoms (protein/water/ligands) | 1939/151/3 |
| average B factor (Å\textsuperscript{2}) (protein/water/ligands) | 24.9/32.9/24.5 |

**Model Quality**

| rmsd for bond lengths (Å) | 0.010 |
| rmsd for bond angles (Å) | 1.073 |
| Ramachandran favored (%) | 98.75 |
| Ramachandran outliers (%) | 0.00 |
| poor rotamers (%) | 0.00 |
| Clash score\textsuperscript{b} | 0.78 |
| Clash percentile\textsuperscript{a} | 99th percentile (N = 819; 1.70 ± 0.25 Å) |
| MolProbity score\textsuperscript{b} | 0.75 |
| MolProbity score percentile\textsuperscript{b} | 100th percentile (N = 9248; 1.70 ± 0.25 Å) |

\textsuperscript{a}Values in parentheses are for the highest-resolution shell. \textsuperscript{b}Values as determined by MolProbity version 4.4 (http://molprobity.biochem.duke.edu/).
Asp106 side chains of the $-X, -Y, -Z + 1/2$ symmetry mate. This third Zn(II) ion assists in the formation of crystal contacts and is unlikely to be physiologically relevant. The SPS-1 structure (Figure 6a,b), generated in part by Pro-origami, indicates the organization of the protein and the contribution of loop residues to coordination of Zn$_1$ and Zn$_2$. (c) An overlay of SPS-1 (green) with SPM-1 (blue, PDB entry 4BP0) highlights the similar overall structure. (d) An overlay of SPS-1 (green) with NDM-1 (pink, PDB entry 4EXS) highlights a shortened SPS-1 $\beta$-hairpin loop between $\beta$-strands $\beta3$ and $\beta4$ and extended $\alpha$-helices $\alpha3$ and $\alpha4$ above the active site for SPS-1 compared to NDM-1. For panels c and d, residues that coordinate zinc ions at the active site are shown as sticks and zinc ions are shown as spheres (SPS-1, gray; SPM-1, navy blue; NDM-1, purple).

$\alpha3$ and $\alpha4$ in NDM-1 expands the active site, the shortened $\beta$-hairpin loop of SPS-1 provides little in the way of expansion of the active site that could be utilized to accommodate substrates with bulky substituents as found in ampicillin, cephalaxin, and cefuroxime.

**SPS-1 Active Site.** The active sites of SPS-1, SPM-1, and NDM-1 (Figure 7a) all feature two Zn(II) ions coordinated by the side chains of histidine, aspartate, or cysteine residues. The Zn$_1$ sites of SPS-1 (Figure 7b), SPM-1 (Figure 7c), and NDM-1 (Figure 7d) are nearly identical, each exhibiting coordination by active site histidine (SPS-1 His245, SPM-1 His258, and NDM-1 His250), aspartate (SPS-1 Asp100, SPM-1 Asp112, and NDM-1 Asp124), and cysteine (SPS-1 Cys207, SPM-1 Cys216, and NDM-1 Cys208) residues. While each protein features at least two histidine residues serving to coordinate Zn$_2$ (SPS-1 His98 and His188, SPM-1 His110 and His197, and NDM-1 His122 and His189), SPS-1 is missing the third coordinating histidine (SPS-1 His108 and NDM-1 His120). In the place of His108/His120, SPS-1 harbors a glycine residue (Gly96). The substitution of a glycine at this position opens up substantial space in the SPS-1 active site below Zn$_1$. As a result, SPS-1 features two water molecules that form a hydrogen bond network (black dashed lines) with Gly96 and Asn192. The active sites of (c) SPM-1 and (d) NDM-1 feature the full complement of expected ligands. Throughout, key residues are shown as sticks and identified by residue name and number and zinc ions are shown as spheres (SPS-1, gray; SPM-1, navy blue; NDM-1, purple).

**Figure 7.** Organization of the *S. smaragdinae* SPS-1 active site. (a) The active sites of SPS-1 (green), SPM-1 (pink, PDB entry 4BP0), and NDM-1 (blue, PDB entry 4EXS) are shown as an overlay. (b) The active site of SPS-1 identifies a water molecule that coordinates to Zn$_1$ in place of a histidine from the canonical HxHxD motif and a second water molecule that completes the hydrogen bond network (black dashed lines) with Gly96 and Asn192. The active sites of (c) SPM-1 and (d) NDM-1 feature the full complement of expected ligands. While additional density in the omit map near Zn$_1$ is likely the result of truncation artifacts in the Fourier series near the zinc ions or anisotropic motion that is not readily fit by isotropic B factors, the omit map density clearly fits the proximal and distal water molecules. The water molecule proximal to Gly96 is held in place by a hydrogen bond to the backbone amide proton of Tyr97 and the active site water molecule proximal to Gly96. This proximal water molecule is further anchored by a

---

**Figure 6.** Overall structure of *S. smaragdinae* SPS-1. (a) The structure of SPS-1 features the canonical $\alpha/\beta$/metallo-$\beta$-lactamase fold with two zinc ions coordinated at the active site. Residues that coordinate zinc ions at the active site are shown as sticks, and zinc ions are shown as spheres (gray). (b) A cartoon schematic, generated in part by Pro-origami, indicates the organization of the protein and the contribution of loop residues to coordination of Zn$_1$ and Zn$_2$. (c) An overlay of SPS-1 (green) with SPM-1 (blue, PDB entry 4BP0) highlights the similar overall structure. (d) An overlay of SPS-1 (green) with NDM-1 (pink, PDB entry 4EXS) highlights a shortened SPS-1 $\beta$-hairpin loop between $\beta$-strands $\beta3$ and $\beta4$ and extended $\alpha$-helices $\alpha3$ and $\alpha4$ above the active site for SPS-1 compared to NDM-1. For panels c and d, residues that coordinate zinc ions at the active site are shown as sticks and zinc ions are shown as spheres (SPS-1, gray; SPM-1, navy blue; NDM-1, purple).

---

DOI: 10.1021/acs.biochem.8b00728

Biochemistry 2018, 57, 5218–5229
hydrogen bond to the side chain NH₂ of Asn192 and coordinates Zn₁. The presence of the distal and proximal water molecules is made possible by a single amino acid substitution outside the primary coordination sphere. SPS-1 Thr187 is a bulky residue in comparison to SPM-1 Ala196 and NDM-1 Gly98, which results in a change in the preferred rotamer for Asn192, thereby setting the stage for the water-mediated hydrogen bond network that situates the Gly96 distal water in position to coordinate Zn₁.

The coordinating residues and the ions in the Zn₂ sites harbor similar positions as the average distance between the Zn₂ site zinc ions across the SPS-1, SPM-1, and NDM-1 harbor similar positions as the average distance between the Zn₁ site zinc ions across the SPS-1, SPM-1, and NDM-1. Throughout, key residues are shown as sticks and water molecules (red) and zinc ions (gray) are shown as small spheres.

**DISCUSSION**

A recent bioinformatics study revealed 279 unique, putative MβL genes, and initial biochemical and microbiological studies confirmed that 76 of the resulting proteins could be MβLs. One member of this group, SPS-1 from *S. smaragdinae*, was particularly interesting because it appeared to lack the consensus HXHXD motif found in all MβLs except those belonging to the B2 subgroup. We were intrigued by the possibility of a novel metal binding site never before observed in the MβLs. Therefore, we endeavored to biochemically and structurally characterize SPS-1.

SPS-1 contains 276 amino acids and shares the highest degree of sequence similarity with SPM-1 from *Pseudomonas aeruginosa* (Figure 1). The overlay of the SPS-1 and SPM-1 structures reveals a 0.92 Å rmsd, indicating broad structural similarity (Figure 6a,c). In addition, both SPS-1 and SPM-1 have a pair of helices located above the active site, and a similarly positioned helix is not found in NDM-1 (Figure 6a,c,d). Even though SPS-1 was sorted into the B1 phylogenetic class, it may also share some similarity with subgroup B2 MβLs, which contain one zinc in the active site (the consensus Zn₂ site) and exhibit a narrow substrate spectrum. While NDM-1, VIM-2, and IMP-1 from the B1 subclass can hydrolyze penems, cephems, and carbapenems, CphA, Sfh-1, and ImiS from the B2 subclass can hydrolyze only carbapenems. Surprisingly, SPS-1 exhibited significant activity against carbapenems, no activity against a penam, and weak and/or selective activity against cephem (Table 1). The most likely explanation for the absence of activity against ampicillin, penicillin G, cefturoxime, and cephalixin is steric clashes with Trp48 and Thr146 located on the extended helices α3 and α4 in SPS-1 (Figures 6, 7, and 9). Overlapping SPS-1 onto structures of NDM-1 in complex with ampicillin, cephalixin, and cefturoxime demonstrates that the elongated α3 and α4 helices of SPS-1 block the binding of...
substrates that contain large substituents at the 6/7 position in the β-lactam (Figure 9). Specifically, the phenyl substituents of ampicillin and cephalixin clash with both Thr146 and Trp148, while the furyl substituent of cefuroxime clashes with Thr146. While it is not surprising that the potential steric clashes (Figure 9b) correlate with the observed absence of activity by SPS-1 against position 6/7-modified penems and cephems, the structures of SPS-1 overlaid with ampicillin, cephalixin, and cefuroxime also identify steric clashes; however, Murphy et al. found that SPM-1 exhibits activity against ampicillin and cefuroxime (cephalexin was not tested). The most likely explanation for the disagreement for the SPM-1 structural and activity data is that the SPM-1 structure was determined in the absence of a substrate and the conformation of the SPM-1 Phe151 side chain is a favored rotamer, 80°, allowed in the absence of any bound substrate. Interestingly, while electron density for the backbone of helix α3 is well-resolved for SPM-1, poor electron density for the Phe151 side chain is observed within the electron density map of 4BP0 (Figure S4). Thus, while the 80° Phe151 rotamer26 modeled into the original SPM-1 crystal structure clashes with ampicillin, cephalixin, and cefuroxime (Figure 9d), altering Phe151 to adopt a different favored rotamer, 45°–30°, resolves the predicted steric clash and would allow SPM-1 to bind ampicillin, cephalixin, and cefuroxime without altering the conformation of the SPM-1 backbone (Figure S4). In contrast, the backbone of SPS-1 helix α3 positions Thr146 such that all possible Thr146 side chain rotamers exhibit predicted steric clashes with ampicillin, cephalixin, and cefuroxime (Figure 9b), consistent with the observed SPS-1 substrate selectivity. Compared to the overlays with cephems and penems, overlays of SPS-1 onto structures of NDM-1 in complex with the carbapenems, imipenem29 and meropenem,29 (Figure 9), which lack a large substituent at the 6/7 position, do not predict any steric clashes between the carbapenems and SPS-1. This is consistent with the observed activity of SPS-1 against imipenem and meropenem.

It is clear that the differences in the relative position of Zn(II) at the Zn1 site do not in general lead to a total loss of hydrolytic activity as SPS-1 is active against antibiotics, such as meropenem, which lack the phenyl or furyl substituents found in ampicillin, cephalixin, and cefuroxime (Figure 9). Although the 1 Å displacement of Zn(II) in the Zn1 site may also contribute to steric clash by forcing the substrate upward, this movement would only exacerbate the clashes predicted for ampicillin, cephalixin, and cefuroxime. In contrast, this 1 Å displacement of the Zn1 site would not preclude binding of imipenem or meropenem. However, the different position and ligand environment of the Zn1 metal ion may explain the relatively larger Km values exhibited by SPS-1, as compared to those exhibited by NDM-1 and SPM-1 (Table 1). SPS-1 has two water molecules that form a hydrogen bond network, which accommodates the loss of a third coordinating histidine found in the Zn1 sites of all other B1 M/Ls (Figure 7). For the dinuclear Zn(II)-containing M/Ls, substrates are thought to bind with the β-lactam carbonyl oxygen interacting with Zn1, and the carboxylate group of the β-lactam interacting with Zn2. Therefore, the different active site geometry in SPS-1 may be the cause of the higher Km values observed for SPS-1 against all tested substrates, reflecting weaker substrate binding to a first approximation. Altered active site geometry leading to weaker binding is also suggested by ITC studies with competitive inhibitor captopril, which show that SPS-1 does not bind captopril. These results suggest that the different position of the Zn1 metal leads to the inability of captopril to bridge the Zn(II) ions in SPS-1. A surprising observation in the steady-state kinetic studies was that despite a different active site structure, higher kcat values were exhibited by SPS-1 toward carbapenems. Given the phylogenetic grouping of SPS-1 near the B2 M/Ls, the positioning of the Zn1 metal above its position in other B1 M/Ls, and the observed steady-state kinetic results, it is interesting to speculate that SPS-1 could be an intermediate enzyme between the dinuclear Zn(II)-containing M/Ls (B1’s) and the mononuclear Zn(II)-containing M/Ls (B2’s).

We have previously reported spectroscopic studies of several Co(II)-substituted M/Ls, including NDM-1,27 VIM-2,4 IMP-13,3 L1,36 Bcl4 CcrA30 Bla2,48 and ImiS. In addition to better understanding the active sites of these enzymes, these studies have allowed us to probe the binding of metal to the M/Ls. Like most reported M/Ls, the Co(II)-substituted analogue of SPS-1 can be prepared by a direct method in which native Zn(II) is removed with a chelator and dialysis and the Co(II) analogue is prepared by adding Co(II) directly to the metal-free enzyme.4,25 The UV-vis spectra of Co(II)-substituted SPS-1 reveal an intense peak at 346 nm, which can be attributed to a cysteine sulfur ligand-to-metal charge transfer band. The intensity of that peak is greatest when SPS-1 has 1 equiv of Co(II), demonstrating, as predicted from the crystal structure, that the first equivalent of metal binds to the Zn2 site. The longer wavelength portion of the SPS-1 spectra is different from those of most M/Ls, which are often dominated by intense ligand field transitions between 450 and 600 nm from four-coordinate Co(II) in the Zn2 site (see the control spectra of NDM-1 in Figure 3). Instead, the spectra of Co(II)-substituted SPS-1 show a broad transition in this part of the spectra, which can also be attributed to ligand field transitions. Similar spectra were reported for M/L L1. The intensity (ε325 = 56 M−1 cm−1) of the ligand field transition(s) in Co(II)-substituted SPS-1 suggests that Co(II) in the Zn2 site is five- or six-coordinate. The small increase in the intensity of this peak upon addition of a second equivalent of Co(II), which populates the Zn1 site, suggests that the coordination number of Co(II) in the Zn1 site is six.

3H NMR and EPR spectra of Co(II)-substituted SPS-1 nicely correlate with the crystal structure of the Zn(II)-containing enzyme. Three solvent-exchangeable peaks can be assigned to histidine residues bound to high-spin Co(II), and other resonances, by comparison to spectra of Co(II)-containing M/Ls, can be assigned to cysteine and aspartate residues. The broader peaks observed in the NMR spectra of Co(II)-substituted SPS-1, as compared to those in the spectra of Co(II)-substituted NDM-1, suggest a slower T1 of the unpaired electrons on Co(II) and Co(II) ions that are at best weakly spin-coupled. The lack of spin coupling is clearly observed in the parallel mode EPR spectra of Co(II)-substituted SPS-1. The positioning of the metal ion in the Zn2 site is most likely the cause of the loss of coupling and nicely explains the lack of binding by captopril.

An intriguing question regarding SPS-1 is why S. maragdinae, specifically strain SEBR 42283 from a water sample near a Congo offshore oilfield, produces an M/L. Spirochetes are spiral-shaped bacteria and are pathogenic, causing yaws, Lyme disease, relapsing fever, and syphilis. One common treatment for some spirochete infections is the administration of β-lactams, and it is possible that another spirochete strain transferred resistance to S. maragdinae strain...
SEBR 42287. SPS-1 from S. smaragdinae has not been identified in any patients, and there are no reports of any human infections caused by S. smaragdinae. According to Hatosy,53 there are four possible mechanisms to explain how bacteria in the ocean gain antibiotic resistance. The first prediction is that antibiotic-resistant bacteria migrate from land to marine environments. The second prediction is that antibiotics enter into the marine environment, causing bacteria to evolve antibiotic-resistant phenotypes. However, both of these mechanisms seem unlikely because of the dilution of the bacteria and antibiotics by the ocean. The third prediction is that antimicrobial compounds are being produced in situ by marine organisms, potentially to give these organisms a survival advantage. The fourth prediction is that the proteins conferring antibiotic resistance phenotypes mutated from a protein that has a different function in vivo. SPS-1, like other MββLs, adopts a characteristic αββα fold. This αββα fold is found in a larger superfamily of metalloenzymes, such as glyoxalase II, arylsulfatase, and RNA-processing enzymes,44,55 with diverse biological functions. It is important to note that the evolution of one of these enzymes into a MββL would likely require the presence of β-lactams to drive evolution.

**CONCLUSIONS**

Despite the question of why this spirochete produces a MββL, SPS-1 is a unique MββL with a novel metal binding site. SPS-1 exhibits substrate specificity similar to that of the B2 MββLs, yet SPS-1 can hydrolyze β-lactams other than carbapenems. The novel ligand set for the SPS-1 active site provides a glimpse at the mechanism of SPS-1 as a possible intermediate between the MββLs, adopts a city similar to that of the B2 MββLs, with broader diversity being possible for this family of enzymes. The uncoupling of the Zn1 and Zn2 metal sites identifies SPS-1 as a possible intermediate between the dinuclear Zn(II)-containing B1 MββLs and mononuclear Zn(II)-containing B2-MββLs. Additional studies are needed to better understand how the different metal center in SPS-1 might affect the mechanism of β-lactam hydrolysis and the interaction with current and future inhibitor scaffolds.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00728.

Four supplemental figures (PDF)

**AUTHOR INFORMATION**

Corresponding Authors

*Department of Chemistry and Biochemistry, Miami University, 651 E. High Street, 160 Hughes Laboratories, Oxford, OH 45056. E-mail: crowderm@MiamiOH.edu. Telephone: (513) 529-2813. Fax: (513) 529-7175.

*E-mail: pagger@MiamiOH.edu.

ORCID

Stacey Lowery Bretz: 0000-0001-5503-8987

Robert A. Bonomo: 0000-0002-3299-894X

Richard C. Page: 0000-0002-3006-3171

Michael W. Crowder: 0000-0002-3138-0283

Author Contributions

Cloning, protein expression and purification, ITC, and fluorescence experiments were conducted by Z.C., C.M., K.M., E.C., R.L.K., and S.F. X-ray crystallography data collection and structure solution were performed by J.V., J.C.N., and R.C.P. M.W.C. and R.C.P. drafted the manuscript with input from their co-authors. All authors approved the final version of the manuscript.

**Funding**

The authors acknowledge funding from the National Institutes of Health (R01 GM111926 to D.L.T., R.A.B., R.C.P., and M.W.C.; R35 GM128595 to R.C.P.; R01AI100560 to R.A.B.; R01AI063517 to R.A.B., and R01AI072219 to R.A.B.). M.W.C. and D.L.T. acknowledge support from the National Science Foundation (CHE-1509285). R.C.P. acknowledges support from Miami University through the Robert H. and Nancy J. Blayney Professorship. The Advanced Light Source is supported by the U.S. Department of Energy under Contract DE-AC03-76SF00098 at Lawrence Berkeley National Laboratory. This study was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, Award 1I01BX001974 (to RAB) from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development and the Geriatric Research Education and Clinical Center VISN 10 (to RAB).

**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS**

EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; MββL, metallo-β-lactamase; NDM, New Delhi MββL; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TEV, tobacco etch virus.

**REFERENCES**


