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1 **CRYSTAL STRUCTURE OF THE METALLO-BETA-LACTAMASE GOB IN THE**
2 **PERIPLASMIC DI-ZINC FORM REVEALS AN UNUSUAL METAL SITE**

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21

22 **ABSTRACT**

23

24 Metallo-beta-lactamases (MBLs) are broad spectrum, Zn(II) dependent lactamases able
25 to confer resistance to virtually every β -lactam antibiotic currently available. The large diversity
26 of active site structures and metal content among MBLs from different sources has limited the
27 design of a pan-MBL inhibitor. GOB-18 is a divergent MBL from subclass B3, expressed by the
28 opportunistic Gram-negative pathogen *Elizabethkingia meningoseptica*. This MBL is atypical
29 since several residues conserved in B3 enzymes (such as a metal ligand His) are substituted in
30 GOB enzymes. Here we report the crystal structure of the periplasmic di-Zn(II) form of GOB-18.
31 This enzyme displays a unique active site structure, with residue Gln116 coordinating the Zn1
32 ion through its terminal amide moiety, replacing a ubiquitous His residue. This situation
33 contrasts with that of B2 MBLs, where an equivalent His116Asn substitution leads to a di-Zn(II)
34 inactive species. Instead, both the mono- and di-Zn(II) forms of GOB-18 are active against
35 penicillins, cephalosporins and carbapenems. *In silico* docking and molecular dynamics
36 simulations indicate that residue Met221 is not involved in substrate binding, in contrast with
37 Ser221, otherwise conserved in most B3 enzymes. These distinctive features are conserved in
38 recently reported GOB orthologues in environmental bacteria. These findings provide valuable
39 information for inhibitor design, and also posit that GOB enzymes might have alternative
40 functions.

41

42 **INTRODUCTION**

43

44 The expression of β -lactamases is the main mechanism of bacterial resistance against β -
45 lactam antibiotics. These enzymes catalyze the hydrolysis of the amide bond in the β -lactam
46 ring characteristic of this family of drugs (1-5). MBLs are metal-dependent hydrolases which
47 generally use Zn(II) as a Lewis acid to activate a water molecule for the nucleophilic attack.
48 These enzymes are refractive to clinically employed lactamase inhibitors (1) and have a
49 particular relevance in the clinical setting as they can hydrolyze a broad spectrum of β -lactam
50 substrates, being able to inactivate carbapenems, the “last resort” antibiotics in antibacterial
51 therapy (6).

52 MBLs have been classified into subclasses B1, B2 and B3, based on sequence identity (7).
53 Crystal structures of MBLs from the three subclasses have revealed that these enzymes present
54 a common $\alpha\beta/\beta\alpha$ sandwich fold, with the active site located within a groove at the interface
55 between these two halves (1-6). The Zn(II)-binding residues vary among different subclasses,
56 giving rise to diverse metal site architectures and metal contents required for activity (1-6). B1
57 and B3 MBLs are broad-spectrum enzymes that hydrolyze penicillins, cephalosporins, and
58 carbapenems with a wide variety of *in vitro* catalytic efficiencies, displaying a broad range of
59 resistance profiles *in vivo* (1-5,8). The di-Zn(II) form of B1 MBLs has been shown to be the active
60 form in the bacterial periplasm, despite contradictory data obtained from *in vitro* studies (8-10).
61 These enzymes display a conserved metal binding motif, where the coordination sphere of the
62 metal ion in the Zn1 site involves three His residues (3-H site), His116, His118 and His196, and a
63 water/hydroxide molecule (the active nucleophile) in a tetrahedral arrangement, while the

64 metal ion in the Zn₂ site adopts a trigonal bipyramidal coordination sphere, with Asp120,
65 Cys221, His263 (DCH site), a water molecule, and the formerly mentioned water/hydroxide
66 molecule as ligands (11-15) (Figure 1). Instead, B2 MBLs are essentially exclusive
67 carbapenemases and are active with one Zn(II) ion bound to the Zn₂ site (superimposable to
68 the B1 DCH site), while binding of a second metal equivalent to the Zn₁ site results in enzyme
69 inhibition (16,17). Substitution His116Asn (present in all B2 enzymes) impairs metal binding to
70 the Zn₁ site by removing a metal ligand. Consequently, nucleophile activation does not involve
71 a metal ion (16,18,19). B3 lactamases, distantly related to the B1 and B2 subclasses, are mostly
72 di-Zn(II) enzymes (20-24). The Zn₁ site of B3 enzymes is a 3-H site similar to that of B1 enzymes,
73 whereas two mutations (Cys221Ser and Arg121His) affect the Zn₂ coordination geometry (20-
74 24), and the metal ion is bonded to Asp120, His121 and His263. Ultimately, Ser221 (equivalent
75 to Cys221 in the DCH site) is no longer a metal ligand, so that the Zn₂ site is a DHH site in B3
76 MBLs. Besides, a third water molecule participates as a fifth ligand, giving rise to a square
77 pyramidal coordination sphere of Zn₂ (20-24). Similarly to B1 enzymes, the Zn₁ ion in B3
78 lactamases is regarded as responsible of nucleophile activation (20-24).

79 The deepest branching member of the MBL B3 subclass, GOB from *Elizabethkingia*
80 *meningoseptica* (formerly *Chryseobacterium meningosepticum*) presents several unusual
81 features. Despite being related to B3 lactamases based on sequence homology, GOB enzymes
82 present a His116Gln substitution that resembles the active site mutation found in B2 enzymes
83 (25). In addition, Ser221 is replaced by a Met residue, suggesting a more divergent metal
84 binding site, or a B2-B3 hybrid enzyme. GOB-type enzymes include 18 allelic variants, all of
85 them expressed by *E. meningoseptica*, a pathogen responsible for neonatal meningitis and

86 opportunistic infections in immunocompromised patients (26-28). So far, two GOB variants
87 have been characterized biochemically: GOB-1 and GOB-18 (29-32), which differ by three
88 residues located far from the active site (29). Expression of GOB-1 in the periplasm of *E. coli* led
89 to a di-Zn(II) enzyme showing a broad substrate profile, similar to most B3 MBLs (30). Instead,
90 cytoplasmic expression of GOB-18 resulted in accumulation of a non-active Fe(III)-substituted
91 form. This variant, when metal depleted and reconstituted with Zn(II), was able to bind only
92 one metal equivalent in the putative DHH site, as confirmed by mutagenesis and spectroscopic
93 studies (29). Besides, mono-Zn(II)-GOB-18 behaved as a fully active, broad spectrum B3
94 enzyme. These data, together with the unusual residues present in positions 116 and 221,
95 suggested that GOB enzymes might have novel structural and functional features.

96 Here we report the crystal structure of the periplasmic form of GOB-18, which shows
97 that GOB enzymes are unique among MBLs. GOB-18 contains a dinuclear Zn(II) site, revealing
98 that replacement of His116 does not preclude these lactamases to bind two metal ions. Indeed,
99 Gln116 is a metal-ligand in the Zn1 site, resulting in a novel HQH site instead of the canonical 3-
100 H site. Met221 (yet another singularity of GOB enzymes) is not a metal ligand and is not
101 involved in substrate binding, further confirmed by docking and molecular dynamics
102 simulations. Thus, GOB enzymes present a divergent metal binding site with novel substrate
103 binding features, which make them unique among B3 MBLs. This observation, together with the
104 finding that the resistance profile of *E. meningoseptica* is elicited mostly by the B1 enzyme BlaB
105 (33), discloses a functional redundancy, and suggests that GOB enzymes may play alternative
106 roles in this microorganism yet to be disclosed.

107

108 **MATERIALS AND METHODS**

109

110 **Chemicals.** Biochemical reagents were purchased from Sigma-Aldrich except when specified.

111 Molecular biology reagents were purchased from Promega, Invitrogen or New England Biolabs.

112 Metal-free buffers were prepared adding Chelex 100 to normal buffers and stirring for 0.5 h.

113

114 **Production of recombinant GOB-18 in the periplasmic space of *E. coli*.** GOB-18 was produced in115 the periplasmic space of *E. coli* C41 (DE3) cells harboring the plasmid pKP-GOB-18 (29,31,32).

116 This vector allows the production of recombinant GOB-18 as a C-terminal fusion to the leader

117 peptide *pelB* (29,31). Secretion via the Sec pathway allows processing of the precursor and118 targeting the mature protein to the periplasmic space of the bacterial host. *E. coli* C41 (DE3)

119 cells harboring plasmid pKP-GOB-18 were grown aerobically at 30°C in LB broth supplemented

120 with kanamycin (50 µg/ml) until reaching 0.6 units of absorbance at 600 nm. Protein production

121 was induced adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1

122 mM, the incubation was continued for 16 h at the same temperature. Mature GOB-18 was

123 extracted from the cellular periplasm by a two-step osmotic shock procedure without lysozyme

124 nor EDTA. Briefly, cells were harvested and resuspended with 1 ml/g (wet weight) of 20 mM

125 Tris-HCl pH 8, 50% w/v sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and incubated for 2 h at

126 4°C under orbital shaking. 10 ml of ice-cold water were added per ml of suspension, and further

127 incubated for 2 h in the same conditions. After centrifugation at 15,000 *g* for 8 min at 4°C, the

128 supernatant (first extract), was collected and stored at 4°C. The pellet was further resuspended

129 in ice-cold water in half the volume used for the first extract, and incubated in the same

130 conditions for 1.5 h at 4°C. The supernatant (second extract) was collected and pooled with the
131 first one rendering the periplasmic fraction.

132

133 **Purification of GOB-18 from the periplasmic fraction of *E. coli*.** Mature GOB-18 was purified to
134 homogeneity with two steps of ionic exchange chromatography (Figure 2). The periplasmic
135 extract was adjusted to pH 7 and loaded onto a Sephadex CM-50 column (Sigma) pre-
136 equilibrated with buffer E (20 mM Tris-HCl, pH 7). The resin was washed with 5 column volumes
137 of buffer E, and eluted with buffer E supplemented with 400 mM NaCl. The elution peak was
138 concentrated, dialyzed against buffer E, and loaded onto a Mono S column (GE Healthcare) pre-
139 equilibrated with buffer E. Elution was achieved with buffer E with added NaCl (gradient 0 to 1
140 M). Samples containing GOB-18 were pooled and concentrated to 20 mg/ml, frozen in liquid
141 nitrogen and stored at -70°C until crystallography assays. An aliquot of the enzyme was stored
142 at 4°C for kinetic analyses. The average purification yields obtained were *ca.* 1 mg of GOB-18
143 per liter of *E. coli* culture, resulting in the expected 31 kDa polypeptide, as estimated by SDS-
144 PAGE.

145

146 **Metal content determination.** The metal content of purified GOB-18 was measured by atomic
147 absorption spectroscopy in a Metrolab 250 instrument operating in the flame mode.

148

149 **Circular dichroism spectroscopy.** The circular dichroism spectrum of periplasmic GOB-18 was
150 obtained using a JASCO J-810 spectropolarimeter at 25°C.

151

152 **Determination of the kinetic parameters.** Antibiotic hydrolysis was monitored by absorbance
153 variation resulting from the scission of the β -lactam ring. Reactions were performed in 15 mM
154 Hepes pH 7.5, 200 mM NaCl at 30°C. The kinetic parameters K_M and k_{cat} were derived from
155 initial rate measurements, as recorded with a Jasco V-550 spectrophotometer, and were
156 estimated by nonlinear data fitting to the integrated form of the Michaelis-Menten equation.

157

158 **Crystallization and data collection.** Crystals grew from a 9.8 mg/ml solution of GOB-18, by
159 adding 0.5 μ l of protein to 1.5 μ l mother liquor (100 mM Tris-HCl pH 8.5, 1.7 M NaCl, 1.7 M
160 $(\text{NH}_4)_2\text{SO}_4$) in a hanging-drop setup with 1 ml mother liquor in the reservoir. These were not
161 single crystals, which were eventually obtained by microseeding fresh drops with identical
162 conditions except for using a slightly modified mother liquor (2 M NaCl/ $(\text{NH}_4)_2\text{SO}_4$ and avoiding
163 Tris-HCl). Single crystals reached a size of *ca.* 150 μ m, and were cryoprotected in mother liquor
164 containing 25% glycerol and flash frozen in liquid nitrogen. X-ray diffraction data were collected
165 in house (Unit of Protein Crystallography, Institut Pasteur de Montevideo, Montevideo,
166 Uruguay), from a single crystal, with a MicroMax-007 HF X-ray source (Rigaku) and a MAR345
167 image plate detector (Mar Research), employing radiation of 1.5418 Å. The diffraction data
168 were processed using XDS (34) and scaled with Aimless (35) from the CCP4 program suite.

169

170 **Structure determination and refinement.** The crystal structure of GOB-18 was solved by
171 molecular replacement using the program Phaser (36) and the model of native FEZ-1 from
172 *Legionella gormanii* (PDB 1K07) as search probe. The asymmetric unit contains two monomers
173 of GOB-18 (chains A and B) with *ca.* 44% of the volume occupied by solvent. SigmaA-weighted

174 Fourier maps (2mFo-DFc) allowed rebuilding the initial model reliably through iterative cycles of
175 manual model building with COOT (37) and refinement with BUSTER (38). To minimize model-
176 bias, initial refinement cycles were carried out including high-temperature (7,500K) slow-
177 cooling simulated annealing procedures in torsional space (as implemented in the program
178 PHENIX (39)). The final model revealed two outlier residues in the Ramachandran plot. Figures
179 were generated and rendered with Pymol 1.5.0.2. (Schrödinger, LLC). Volume calculations for
180 automatically identified cavities were done using the CASTp algorithm (40) and Q-site finder
181 (41), giving similar results.

182 Atomic coordinates and structure factors were deposited in the Protein Data Bank under
183 the accession code 5K0W.

184

185 **Substrate docking and molecular dynamics simulations.** Molecular docking was performed
186 using Autodock 4.2 (42). Deprotonated imipenem was docked setting up a docking box of 40 ×
187 40 × 40 Å (grid-point spacing of 0.375 Å) centered in the enzyme active site. The model of GOB-
188 imipenem was built *in silico* by making a structural alignment with the structure of L1-imipenem
189 using the Multiseq plug-in of VMD (43). Molecular dynamics simulations were performed
190 starting from complexes GOB-18-imipenem and L1-imipenem. Each complex was immersed in a
191 truncated octahedral periodic box with a minimum solute-wall distance of 8 Å, filled with
192 explicit TIP3P water molecules (44). Molecular dynamic simulations were performed with the
193 AMBER14 package (45,46), using ff14SB (47). Particle-mesh Ewald (PME) was implemented for
194 long range interactions with a cutoff distance of 12 Å (48). Temperature and pressure were
195 regulated with the Berendsen thermostat and barostat (49). All bonds involving hydrogen were

196 fixed using the SHAKE algorithm (50). Each initial system was equilibrated at 300K using a
197 conventional protocol, and then subjected to 50 ns of simulation in the NVT ensemble. To
198 maintain the coordination environment around zinc atoms we used harmonic bonds between
199 the metal centers and the residues of the coordination sphere. We applied a restraint to keep
200 the imipenem bound to the active site. Root mean squared deviation (rmsd) and root mean
201 square fluctuations (rmsf) calculations of the molecular dynamics simulations were performed
202 using the cpptraj module of AMBER (51).

203

204 **RESULTS**

205

206 ***Expression and purification of GOB-18 for crystallogenesis***

207 Production of GOB-18 in *E. coli* gives rise to different metallated species depending on
208 the cellular compartment where the protein accumulates. Overexpression of GOB-18 in the
209 cytoplasm results in an inactive Fe(III)-bound form, from which a mononuclear fully-active
210 Zn(II)-variant can be prepared by metal chelation and remetallation *in vitro* (29). Instead, GOB
211 binds exclusively Zn(II) when it is secreted into the bacterial periplasm (29,31).

212 GOB-18 produced in the cytoplasm of *E. coli*, both the Fe(III)-containing variant or the
213 reconstituted mono-Zn(II) form, proved recalcitrant after extensive crystallization trials. An
214 alternative protocol was thus optimized allowing to produce GOB-18 in the bacterial periplasm,
215 which is the physiological cellular compartment for MBLs' expression in Gram-negative species
216 (Figure 2A). This strategy yielded GOB-18 containing exclusively Zn(II), with a maximum metal
217 content of (1.4 ± 0.1) Zn(II) equivalents/ GOB-18 molecule as determined by atomic absorption
218 spectroscopy. This metal-content figure indicates the presence of a dinuclear GOB-18 species,
219 which is consistent with previous reports for GOB-1 (30). Circular dichroism of periplasmic GOB-
220 18 indicates it has similar tertiary structure as the *in vitro* reconstituted Zn(II) form (29) (Figure
221 2B). Periplasmic GOB-18 was able to catalyze the hydrolysis of a broad spectrum of β -lactam
222 substrates with k_{cat} and K_M values similar to those formerly reported for mono-Zn(II) and di-
223 Zn(II)-GOB-1, with no changes in the catalytic efficiencies upon addition of 20 μ M Zn(II) to the
224 reaction medium (Table 1). Nevertheless, in contrast to reconstituted Zn(II)-GOB-18,

225 periplasmic GOB-18 crystallized in different conditions containing high concentrations of NaCl
226 and $(\text{NH}_4)_2\text{SO}_4$ as precipitants.

227

228 ***Crystal structure of periplasmic GOB-18***

229 Periplasmic GOB-18 crystallized in space group $P2_1$, with crystals diffracting X rays to 2.6
230 Å resolution (Table 2). The final refined atomic model contains two protein molecules per
231 asymmetric unit, 4 zinc atoms, 8 chlorides, 77 waters and one glycerol molecule. The two
232 protein chains are very similar, with 0.26 Å root mean squared deviation (rmsd) among 266
233 aligned α -carbons. Chain A includes residues Ser17 to Asp286 (the side chains of residues Asn26
234 and Asp286 were not included in the model due to weak electron density), while monomer B
235 spans residues Val20 to Lys290 (similarly, Val20 side chain was not modeled), with a continuous
236 main chain trace throughout. GOB-18 displays the expected $\alpha\beta/\beta\alpha$ sandwich fold, with two
237 core β -sheets composed of 7 ($\beta 1$ - $\beta 7$) and 5 ($\beta 8$ - $\beta 12$) β -strands, respectively, and 6 α -helices
238 (Figure 3A). Helices $\alpha 1$ - $\alpha 4$ cover the 7-stranded β -sheet, with helix $\alpha 4$ closing one side of the
239 metal-binding groove. On the other hand, helices $\alpha 5$ and $\alpha 6$ wrap around the 5-stranded β -
240 sheet, with the intervening region $\beta 12$ - $\alpha 6$, including three short helical elements, closing up
241 the other side of the catalytic site. As already described for the MBL fold (1-6), the whole
242 domain of GOB-18 can be depicted as a duplication of two structurally similar α/β hemi-
243 domains.

244 GOB-18 is very similar to other B3 lactamases (20-24), despite low sequence identity.
245 Structural alignment of GOB-18 (chain A) with available B3 lactamase models allows for
246 similarity quantification (Figure 3B): FEZ-1 (PDB 1K07, 1.27 Å rmsd for 243 aligned residues);

247 BJP-1 (PDB 3LVZ, 1.50 Å rmsd for 239 residues); and, L1 (PDB 1SML, 1.55 Å rmsd for 236
248 residues). When calculated per residue, the largest local rmsd values are observed for two
249 loops flanking the active site, which are expected to be involved in substrate specificity: loop 1
250 (residues 130-152 in GOB-18, equivalent to 148-172 according to BBL numbering; to facilitate
251 comparative analyses, residue numbering will hereafter be stated for GOB-18 followed by BBL
252 numbering in parenthesis) and loop 2 (198-218(219-239)). Structural differences are also
253 noticeable in the β 12- α 6 loop (residues 239-265(261-287)) as well as in the N-terminus of the
254 protein.

255 All B3 lactamases crystallized so far are characterized by at least one disulfide bridge, in
256 most cases between Cys residues located in the C-terminal helix and the loop linking elements
257 α 5 and β 12 (20-24). GOB-18 is an exception in this regard within B3 enzymes, with only one Cys
258 residue in its primary structure, *i.e.* Cys180(201) (in strand β 10), which is buried within the
259 protein core (Figure 3A) forming hydrogen bonds in the base of the metal binding site (see
260 below).

261

262 ***The active site of di-Zn(II)-GOB-18***

263 The electron density maps revealed the presence of two heavy atoms in the active sites
264 of each GOB-18 monomer in the asymmetric unit (Figure 4A). They occupy the metal binding
265 sites found in other dinuclear MBLs, and can be recognized as the Zn1 and Zn2 ions. The Zn-Zn
266 distance (3.5 and 3.8 Å in chains A and B, respectively) is similar to that found in other dinuclear
267 B3 lactamases (20-24). Zn1 is coordinated by the N δ 1 atom of residue His100(118), His175(196)
268 N ϵ 2, Gln98(116) O ϵ 1 and a Zn-Zn bridging water molecule. The Zn1-O ϵ 1(Gln) distance is 2.0 Å,

269 pinpointing Gln98(116) as a novel metal-binding residue in MBLs. On the other hand, Zn₂ is
270 coordinated to His103(121) and His241(263) N ϵ 2, Asp102(120) O δ 2 and the bridging water
271 molecule, adopting a distorted tetrahedral geometry. However, in all B3 lactamases crystallized
272 so far the coordination sphere in the Zn₂ site adopts a trigonal bipyramidal geometry with two
273 water ligands (20-24). The presence of additional non-protein ligands in the GOB-18 site cannot
274 be ruled out, given our data's resolution limit. In the same line of thought, an axial water
275 molecule with high mobility was modeled in the coordination sphere of the Zn₁ ion in both
276 GOB-18 monomers, but a larger species (such as glycerol from the cryo-protection solution)
277 cannot be excluded.

278 The conformation of the zinc ligands in GOB-18 active site is stabilized by a network of
279 hydrogen bonds (Figure 4B), involving outer sphere ligands. His100(118) N ϵ 2 and His175(196)
280 N δ 1 interact respectively with Asp135(153) and Ser202(223) side chains, and Gln98(116) N ϵ 2
281 establishes contacts with Asn199(220) O δ 1. Additionally, the N δ 1 atom of His103(121) is H-
282 bonded with the main chain N of residues Thr68(85) and Gly69(86) through a bridging water,
283 His241(263) N ϵ 2 interacts with the side chain of Asp51(67), and the carboxylate of Asp102(120)
284 contacts its own amide nitrogen. Most of these outer sphere ligands are conserved in B3
285 enzymes (20-24), despite the divergence introduced by the His116Gln replacement.

286 A novel feature in GOB-18 can be observed at residue Cys180(201): its thiol and amide
287 nitrogen make H-bonds with the backbone carbonyls of Leu96(114) and Thr97(115),
288 respectively (Figure 3A). These residues are located in the β 5- α 3 loop, which contains the
289 metal-binding motif Q₁₁₆XH₁₁₈XD₁₂₀H₁₂₁ that replaces the HXHXDH signature present in the rest
290 of B3 enzymes. Thus, Cys180(201), within the β -strand just downstream from the loop including

291 metal-binding His175(196), connects the bases of two loops that comprise five out of six metal
292 ligands, and all the Zn1-coordinating residues.

293 The residues that define the substrate binding cleft in GOB-18 are mainly found in loops.
294 The catalytic pocket (Figure 5) is delimited by segments 23-32(30-39) (spanning a non-
295 structured coil and a short, kinked helix), 51-52(67-68), 98-103(116-121) (including the QXHXDH
296 motif), 135-144(154-163) (encompassing a short helix), 175-176(196-197) (containing metal-
297 binding His175(196)), 199-204(220-225) (harboring residue Met 200(221)) and 240-244(262-
298 266) (spanning a short helix and including metal-binding His241(263)). This cleft, albeit shallow,
299 is one of the top ranking cavities, comprising 150-200 Å³ with approximate 8 Å x 15 Å on the
300 opening and 10 Å deep. A second, smaller pocket, with residues Ser202(223), Gln244(266),
301 Asn271(293) and Leu275(297) conforming the side limits and Met200(221) and Trp237(259) on
302 the floor, is immediately adjacent to the first one, thus constituting a discontinuous groove.
303 Figure 5 compares the catalytic groove of GOB-18 with those from the B3 lactamases FEZ-1, L1
304 and BJP-1. The wide substrate-binding groove in GOB enzymes correlates with high catalytic
305 efficiencies for a broad spectrum of substrates, in contrast to BJP-1, in which an N-terminal
306 helix partially covers the active site (22).

307

308 ***Substrate docking and molecular dynamics simulations***

309 We attempted to obtain a model for di-Zn(II) GOB-18 complexed with imipenem by *in*
310 *silico* docking calculations. However, none of the resulting models reproduced binding modes
311 consistent with a productive Michaelis complex. Similar docking simulations with L1 were
312 instead successful, yielding a model which reproduced most of the binding features reported in

313 the crystal structure of L1 in complex with hydrolyzed moxalactam (PDB 2AIO) (52) (Figure S1).
314 In our docking model, the carbonyl C7 from imipenem is positioned close to the bridging OH⁻
315 enabling the nucleophilic attack, and the carboxylate C9 interacts with the Zn²⁺ ion, anchoring
316 the substrate, in agreement with the generally accepted productive binding mode of β -lactam
317 compounds to dinuclear MBLs. Based on these results, we generated a model of GOB-18 in
318 complex with imipenem, by structural alignment of GOB-18 onto the L1-imipenem complex.

319 The L1-imipenem and GOB-18-imipenem models were used as starting geometries for
320 molecular dynamics simulations, ran for 50 ns. In the L1-imipenem complex, residues Ser221
321 and Ser223 interact with the carboxylate C9 of imipenem throughout the simulation (Figure
322 6A). Additionally, the OH group C10 of imipenem established a hydrogen bond with the non-
323 chelating oxygen of the Zn²⁺-ligand Asp120. In GOB-18, Ser221 is replaced by a Met residue
324 whose side chain points out of the active site, buried in the hydrophobic core of the protein,
325 and hence unable to interact with the substrate (Figure 6B). This orientation is conserved
326 throughout the dynamics. On the other hand, the imipenem carboxylate contacts residue
327 Ser202(223) through a water molecule, and the interaction of the OH group C10 with
328 Asp102(120) is also preserved in the GOB-18-imipenem complex. Taken together, these data
329 strongly suggest that the Ser221Met substitution may have an impact on substrate binding by
330 GOB enzymes.

331 The mobility of active site loops in MBLs has been related to substrate specificity.
332 Indeed, residues 221 and 223 are located in a loop flanking the active site (Figure S2). We
333 calculated the fluctuations of residues within this loop in GOB-18 and L1, both in the resting
334 state and in the Michaelis complex with imipenem. The rmsf (root mean square fluctuation) of

335 this loop is substantially larger in GOB-18 than in L1 (Figure S2). We propose that the
336 Ser221Met substitution present in GOB enzymes is compensated by a larger flexibility in this
337 loop, which might assist substrate binding within the catalytic groove.
338

339 **DISCUSSION**

340

341 Here we report the crystal structure and biochemical characterization of the periplasmic
342 di-Zn(II) form of GOB-18 from *E. meningoseptica*. The structural data reveal that a Gln residue
343 replaces the ubiquitous His116 in the coordination sphere of the Zn1 ion (Figure 4). Periplasmic
344 GOB-18 is a fully active broad spectrum lactamase in the di-Zn(II) form (Table 1), in agreement
345 with a previous report that studied GOB-1 (30). These results contrast our previous study with
346 recombinant GOB-18 obtained from the cytoplasm of *E. coli* cells (29). In that case,
347 accumulation of the protein in the bacterial cytoplasm resulted in an inactive Fe(III)-bound
348 form, which could be demetallated and subsequently loaded with Zn(II) *in vitro* to obtain an
349 active mono-Zn(II) variant. Indeed, the preferential binding of a given divalent cation over
350 others is influenced by the cellular localization of proteins (53), and mismetallation upon
351 protein overproduction in the cytoplasm of *E. coli* has been documented for a number of
352 systems (54-56). Thus, these separate preparations give us the opportunity to compare mono-
353 and di-Zn(II) variants of GOB-18.

354 We now show that when secreted into the bacterial periplasm, GOB exclusively binds
355 Zn(II), which is relevant given that this is the physiological cellular compartment of MBLs in
356 Gram-negative bacteria (53). Thus, in order to avoid chelation and remetallation steps, we
357 optimized a protocol for the production of GOB-18 in the periplasm of *E. coli* and the
358 preparation of high amounts of pure mature protein in the absence of affinity tags. Indeed,
359 periplasmic GOB-18 preparations containing exclusively Zn(II) were successfully obtained and,
360 while all efforts aimed at crystallizing the cytoplasmic Fe(III)-containing variant or the

361 reconstituted mono-Zn(II) form of GOB-18 were unsuccessful, diffraction quality crystals readily
362 grew from solutions of periplasmic di-Zn(II) GOB-18. These results clearly show that the
363 differences in metal content previously noted between GOB-1 and GOB-18 were due to the
364 procedures employed to produce the recombinant proteins in each case and not due to the few
365 residues differing in their primary structures. Taking all these data into account, it is safe to
366 extrapolate several of the present conclusions to all GOB enzymes.

367 Periplasmic di-Zn(II) GOB-18 catalyzed hydrolysis of β -lactam substrates with k_{cat} and K_M
368 values similar to those reported for mono-Zn(II)-GOB-18 and di-Zn(II)-GOB-1 (Table 1). Besides,
369 previous analyses of mutant GOB-18 Asp120Ser and metal-substituted GOB-18 derivatives,
370 showed that the Zn₂ site is essential for catalysis and for the stabilization of an anionic
371 intermediate in the hydrolysis of nitrocefin (29,57). On the other hand, even though
372 substitution of Gln116 by an isosteric His residue was somewhat detrimental for the resistance
373 profile conferred by GOB-18 (29,31), it had little effect both on metal content as well as on the
374 *in vitro* activity of dinuclear GOB-1 (30) and remetallated mono-Zn(II) GOB-18 (29). Indeed,
375 mutations Gln116Asn and Gln116Ala, which are predicted to impact on the structure of the Zn₂
376 site through perturbations on the second coordination sphere (Figure 4B), reduced the catalytic
377 efficiency of GOB-1 (30). Overall, the available data indicate that the Zn₁ site in GOB enzymes
378 contributes only marginally to activity, whereas it may be critical for the enzyme function *in*
379 *vivo*. This would depend on the zinc availability, which can vary widely depending on
380 environmental conditions (8).

381 The finding of a dinuclear Zn(II) site where Gln116 acts as a metal ligand of the Zn₁ ion
382 contrasts with that found in B2 MBLs, where substitution of His116 by an Asn residue leads to a

383 dinuclear non-active species (18,19). In the case of di-Zn(II)-CphA, residue Asn116 does not act
384 as a metal ligand and the Zn1 ion is coordinated by only two protein residues, adopting a non-
385 productive position (16). Instead, the longer side chain of Gln116 (isosteric to a His residue) in
386 GOB enzymes makes it a good metal ligand, so that the Zn1 ion adopts a position similar to that
387 held in other di-Zn MBLs (Figure 7). The finding of a conserved network of hydrogen bonds
388 among second sphere ligands confirms the requirement of a similar geometric arrangement in
389 the active site. We have also found that a unique Cys residue may help anchoring the two loops
390 that provide protein ligands for the Zn1 ion (Figure 3A). A conservative mutation of this Cys
391 residue to a Ser (29) preserves the enzyme activity, supporting this hypothesis. The role of the
392 Zn1 site in MBLs is to facilitate deprotonation of the bound water to provide an active
393 nucleophile (1). This function cannot be properly fulfilled by the Zn1 ion in B2 enzymes, thus
394 resulting in enzyme inhibition (16), but instead is fully preserved in GOB enzymes. Therefore,
395 the His116Gln mutation is conservative in terms of both structure and function. The role of the
396 Zn2 site is two-fold: to provide an anchoring electrostatic point for substrate binding and to
397 stabilize the development of negative charge in the bridgehead nitrogen. Both functions also
398 depend on the adequate positioning of this metal ion, which is indeed maintained in GOB-18.

399 Position 221 is essential in B1 and B2 MBLs, where a Cys residue acts as a ligand of the
400 Zn2 ion. Instead, in most B3 enzymes a Ser is found in this position. The report of a Met residue
401 in position 221 in GOB enzymes led us to speculate that the thioether moiety could act as a
402 weak metal ligand. However, mutagenesis experiments in GOB-18 showed that Met221 is not
403 involved in metal binding nor in catalysis, but that, instead, has a structural role (31,32). The
404 crystal structure that we are now disclosing, confirms that Met221 is not a metal ligand,

405 actually its side chain does not point toward the substrate-binding site (Figure 6). Besides,
406 docking and molecular dynamics simulations confirm that this residue is not involved in
407 substrate binding, in sharp contrast to the role of the conserved Ser221 in most B3 enzymes.
408 Therefore, these results provide the first available evidence for an MBL where residue 221 is
409 not involved in metal chelation nor in substrate binding, and highlight the diversity of roles
410 fulfilled by this position among enzymes within the family. Additionally, a wide substrate-
411 binding groove in GOB-18 (Figure 5) correlates with a high catalytic efficiency against a diverse
412 range of β -lactam substrates (Table 1) as compared to other B3 MBLs, in agreement with
413 previous observations.

414 The metal binding site of GOB enzymes is unusual not only among MBLs. Indeed,
415 Asn/Gln ligands in protein zinc sites are very rare (0.5 % of the cases, according to Dudev and
416 Lim) (58). Surprisingly, the His116Gln substitution does not have a direct impact on the broad
417 spectrum profile of GOB-18. GOB is also peculiar in that its native organism, *E. meningoseptica*,
418 is the only known bacterium expressing two MBLs: the B1 enzyme BlaB, and the B3 enzyme
419 GOB. We have shown that, even if both genes are actively expressed, the higher levels of BlaB
420 make this enzyme the one responsible for carbapenemase resistance (33). Thus, GOB could be
421 redundant in this organism despite its efficient catalytic performance. GOB-like alleles have also
422 been found in metagenomics studies of a remote Alaskan soil with minimal human-induced
423 selective pressure (59). More recently, new MBLs closely homologous to GOB enzymes have
424 been found in *Pedobacter roseus* (PEDO-1), *Pedobacter borealis* (PEDO-2), *Chrisobacterium*
425 *piscium* (CPS-1) and *Epilithonimonas tenax* (ESP-1) (60,61). All these MBLs feature the
426 characteristic Gln116 residue in the active site, as well as the Cys residue in second sphere

427 position, and a hydrophobic residue in position 221 (Met or Leu), discussed in the present
428 crystal structure, confirming that GOB-like alleles are ubiquitous in environmental bacteria. We
429 can speculate that GOB genes could serve as a rather ancient resistance reservoir, or maybe
430 could give rise to a different, yet unknown function, elicited by the presence of the Gln residue,
431 providing at the same time an independent source of antibiotic resistance. In any case, these
432 findings further highlight the large structural diversity of MBLs in current microorganisms, the
433 details of which constitute valuable information in conceiving better antibiotic design
434 strategies.

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633

634 **FIGURE LEGENDS**

635

636 **FIGURE 1.** *Metallo-beta-lactamase zinc-binding sites.* *B. cereus* BclI (B1, PDB 1BC2, left), *S.*
637 *fonticola* SfhI (B2, PDB 3SD9, center), and *S. maltophilia* L1 (B3, PDB 1SML, right). Zinc atoms
638 are shown as gray spheres, and water molecules (W) are shown as small red spheres.
639 Coordination bonds are indicated with dashed lines.

640

641 **FIGURE 2.** *Purification and characterization of periplasmic GOB-18.* **(A)** SDS-PAGE analysis of
642 fractions along different steps of the purification process. Lanes: 1, total cell extract (T); 2,
643 periplasmic fraction, corresponding to the sample loaded on the CM-Sephadex column (L); 3,
644 CM-Sephadex flow-through (F_{CM}); 4, CM-Sephadex elution, corresponding to the sample loaded
645 on the Mono S column (E); 5, Mono S flow through (F_S); 6-17, Mono S elution fractions (f1 to
646 f12). Molecular weight markers (in kDa) are indicated at the right side. The vertical grey line
647 between lanes 3 and 4 indicates the junction of two different gels prepared and run together,
648 since the Mini PROTEAN III System (BioRad) holds up to 15 lanes. **(B)** Circular dichroism spectra
649 of periplasmic GOB-18 (black line), remetallated Zn(II)-GOB-18 (gray) and apo-GOB-18 (light
650 gray).

651

652 **FIGURE 3.** *Overall structure of periplasmic GOB-18.* **(A)** Cartoon representation of GOB-18
653 (chain A) with helices colored in red, strands in yellow and non-structured loops in green. The
654 inset highlights selected residues (in stick representation). Dashed lines represent atomic
655 interactions. **(B)** Comparison of GOB-18 with the B3 MBLs FEZ-1 (PDB 1K07), BJP-1 (PDB 3LVZ),

656 and L1 (PDB 1SML). The segments of GOB-18 exhibiting the highest rmsd values are highlighted
657 in colors. Zn atoms are shown as gray spheres.

658 **FIGURE 4.** *The active site of periplasmic GOB-18.* **(A)** Upper panel: Comparison of the active site
659 in the two monomers present in the crystal structure of GOB-18. Chain A is colored by atom in
660 yellow (C atoms), blue (N atoms), red (oxygen atoms) and dark gray (zinc atoms). Chain B is
661 shown in light grey throughout. Protein residues are depicted in sticks, zinc atoms are shown as
662 big spheres and water molecules as small spheres. The *2mFo-DFc* sigmaA-weighted electron
663 density, contoured at 1.7σ and represented as a grey mesh, corresponds to chain A. Dashed
664 lines represent atomic interactions and the related distances are of 2.1 Å in all cases except for
665 the interactions Zn1-Gln98 and Zn1-axial water where distances are 2.0 Å and 2.2 Å,
666 respectively. Lower panel: comparison of the active site of GOB-18 (chain A, yellow) with those
667 of the B3 MBLs FEZ-1 (PDB 1K07, pink), BJP-1 (PDB 3LVZ, green), and L1 (PDB 1SML, blue). **(B)**
668 Residues in the second coordination sphere in the active of GOB-18 (chain A) are shown in stick
669 representation with C atoms colored in cyan.

670

671 **FIGURE 5.** *The catalytic groove in GOB-18.* Surface representation of GOB-18 (chain A)
672 highlighting with colors the structural elements that define the catalytic groove. Zinc atoms are
673 shown as gray spheres. For comparison, the region delimited by the rectangle is compared to
674 the equivalent regions of the B3 MBLs FEZ-1 (PDB 1K07), L1 (PDB 1SML) and BJP-1 (PDB 3LVZ)
675 shown at the bottom.

676

677 **FIGURE 6.** *Molecular dynamics simulations calculated using in silico docked imipenem in*
678 *complex with L1 and GOB-18. (A)* Snapshot of the L1-imipenem molecular dynamics simulation
679 showing the interaction between imipenem and residues Asp120, Ser221 and Ser223. **(B)**
680 Snapshot of the GOB-18-imipenem molecular dynamics simulation showing the interaction
681 between imipenem and residue Asp120 and Ser223. Imipenem is shown in stick representation
682 with green carbons, L1 and GOB-18 are displayed in gray and yellow, respectively; zinc atoms
683 and a water molecule are depicted as gray and red spheres, respectively.

684

685 **FIGURE 7.** *Comparison of the active sites of di-Zn(II) GOB-18 and di-Zn(II) CphA.* GOB-18 (chain
686 A) is shown in light gray and CphA (PDB 3F9O) is colored in orange (C atoms), blue (N atoms),
687 red (oxygen atoms) and dark gray (zinc atoms). Protein residues are shown in sticks, zinc atoms
688 are depicted as spheres and dashed lines highlight atomic interactions.

689

690 TABLES

691

692 **Table 1.** Kinetic parameters of hydrolysis of β -lactam substrates by GOB enzymes. Values were
 693 derived from a nonlinear fit of Michaelis-Menten equation to initial rate measurements and
 694 correspond to the average of at least three independent enzyme preparations. k_{cat} values are
 695 expressed in s^{-1} , K_M in μM and k_{cat}/K_M in ($s^{-1} \mu M$).

696

Substrates	Zn(II) added	Periplasmic GOB-18			Cytoplasmatic GOB-18 ^a			Zn(II) added	GOB-1 ^b		
		k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M		k_{cat}	K_M	k_{cat}/K_M
Imipenem	0 μM	37 \pm 1	22 \pm 3	1.7 \pm 0.5	42 \pm 9	26 \pm 2	1.6 \pm 1.5	0 μM	77 \pm 2	18 \pm 0.6	4.2
	20 μM	75 \pm 3	42 \pm 7	1.8 \pm 0.5				50 μM	85 \pm 2	13 \pm 1	6.5
Meropenem	0 μM	96 \pm 4	54.3 \pm 6	1.8 \pm 0.6	72 \pm 0.5	40 \pm 10	1.8 \pm 0.5	0 μM	100 \pm 7	29 \pm 1	3.5
	20 μM	261 \pm 9	170 \pm 20	1.5 \pm 0.7				50 μM	170 \pm 3	22 \pm 1	8.0
Cefaloridine	0 μM	41 \pm 2	100 \pm 10	0.4 \pm 0.1	30 \pm 2	31 \pm 0.5	0.95 \pm 0.08				
	20 μM	59 \pm 2	150 \pm 20	0.4 \pm 0.1							
Cefotaxime	0 μM	80 \pm 3	100 \pm 10	0.8 \pm 0.3	83 \pm 2	88 \pm 6	0.94 \pm 0.09				
	20 μM	106 \pm 5	130 \pm 20	0.8 \pm 0.3							
Penicillin-G	0 μM	1070 \pm 20	450 \pm 20	2.4 \pm 0.9	680 \pm 80	330 \pm 30	2.1 \pm 0.4	0 μM	540 \pm 7	130 \pm 6	4.2
	20 μM	1810 \pm 40	580 \pm 50	3.1 \pm 0.9				50 μM	630 \pm 10	190 \pm 10	3.4

697 ^a Values correspond to mono-Zn(II)-GOB in reference (29).698 ^b Values correspond to reference (30).

699

700

701 **Table 2.** X ray diffraction data collection and refinement statistics.

702

	GOB-18
Space group	P2 ₁
Protein molecules per asymmetric unit	2
Solvent content (%)	43.9
Wavelength (Å)	1.5418
Data Resolution (Å) *	24.33-2.61 (2.75-2.61)
Measured reflections	44417
Multiplicity*	2.8 (2.6)
Completeness (%) *	96.9 (90.5)
R _{meas} (%)**	15.1 (45.3)
<I/σ(I)>*	9.5 (3.3)
a b c (Å)	64.0 48.5 88.9
β (°)	100.1
Refinement resolution (Å)	24.3-2.61
R _{cryst} ‡ (N° refs)	0.192 (15152)
R _{free} ‡ (N° refs)	0.242 (955)
Rms bonds (Å)	0.01
Rms angles (degrees)	1.11
Protein non-hydrogen atoms	4381
Water atoms	77
Zn ⁺² atoms	4
Glycerol atoms	6
Cl ⁻¹ atoms	8
Mean B factor – overall : [chain A / chain B] (Å ²)	28 / 31
Mean B factor - main chain: (chain A / chain B) (Å ²)	25 / 27

Mean B factor – side chains: (chain A / chain B) (Å ²)	32 / 35
Mean B factor – waters (Å ²)	18
Mean B factor – liganded Zn ⁺² (Å ²)	28
Mean B factor – liganded Cl ⁻¹ (Å ²)	47
Mean B factor – liganded glycerol (Å ²)	41
Map vs model correlation coefficient (overall/local) ‡	0.839 / 0.881
N° residues in Ramachandran plot regions § (allowed/favored/outliers)	535/524/2
PDB ID	5K0W

703

704

705 * Values in parentheses apply to the high-resolution shell.

706 † $R_{meas} = \frac{\sum_h \sqrt{N_h(N_h-1)} \sum_i |I_i - \langle I \rangle|}{\sum_h \sum_i I_{\pm}}$; N_h , multiplicity for each reflection; I_i , the intensity of the i^{th}

707 observation of reflection h ; $\langle I \rangle$, the mean of the intensity of all observations of reflection h ,708 with $I_{\pm} = \frac{1}{N_h} \sum_i (I(-) \text{ or } I(+))$; \sum_h is taken over all reflections; \sum_i is taken over all

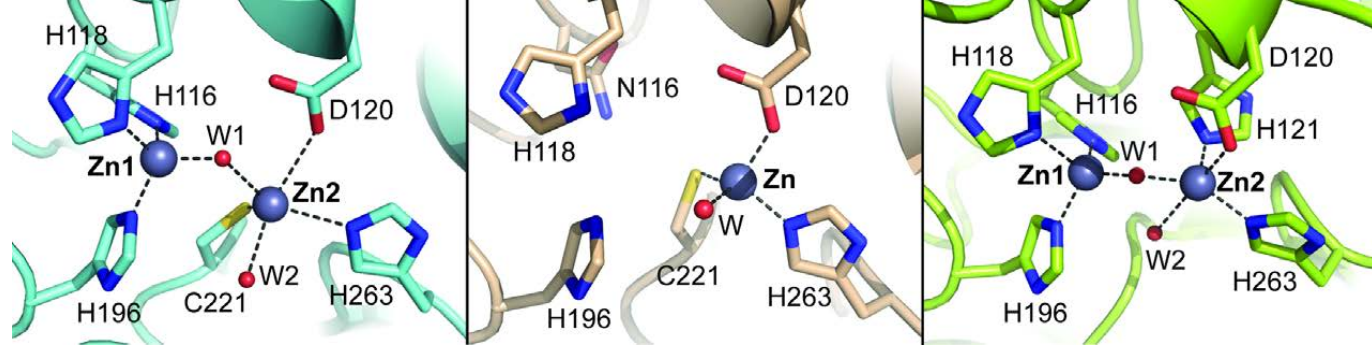
709 observations of each reflection.

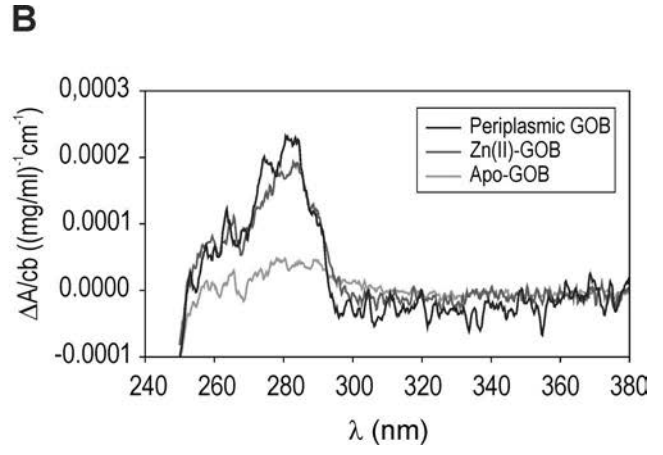
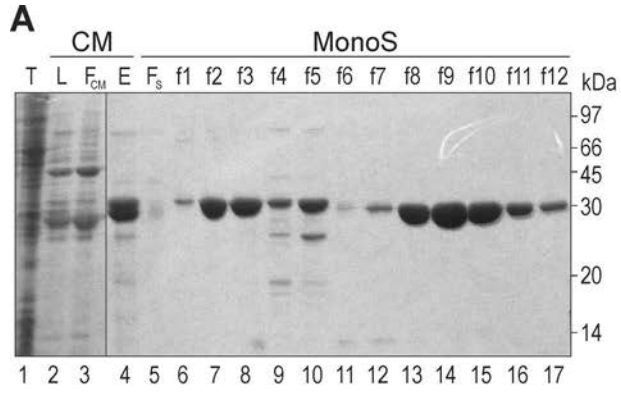
710 ‡ $R = \frac{\sum_h |F(h)_{obs} - F(h)_{calc}|}{\sum_h |F(h)_{obs}|}$; R_{cryst} and R_{free} were calculated using the

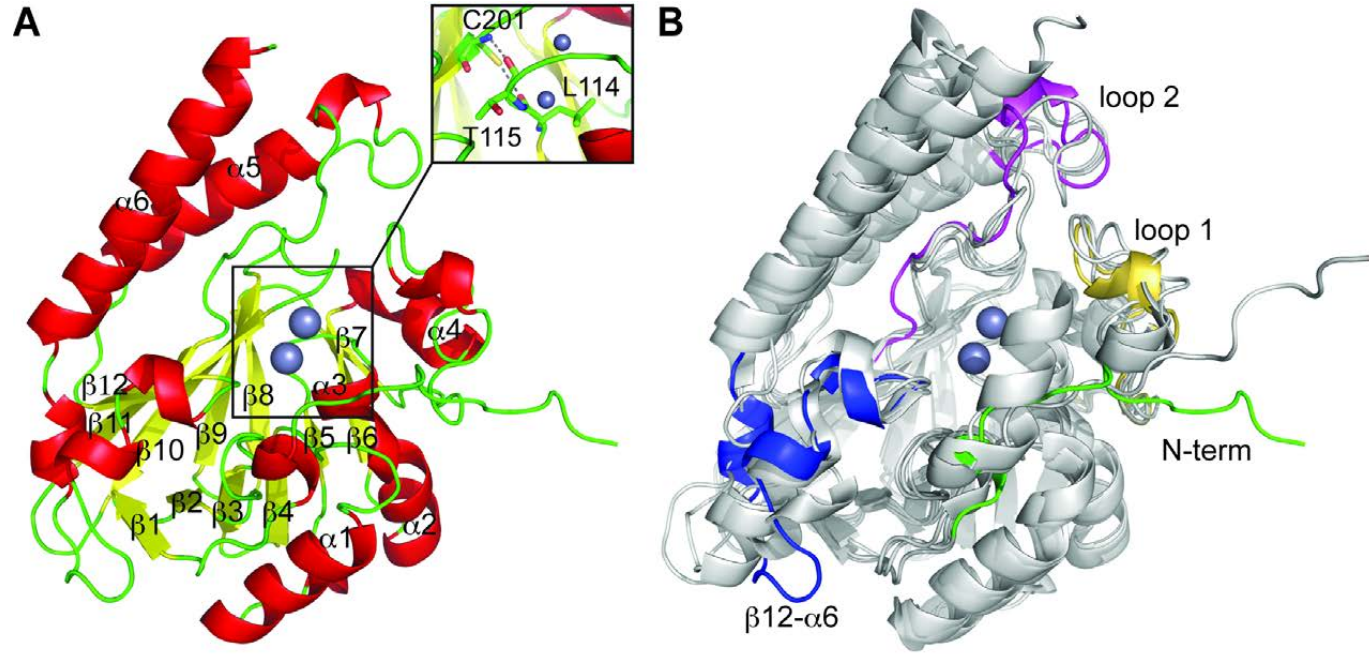
711 working and test hkl reflection sets, respectively.

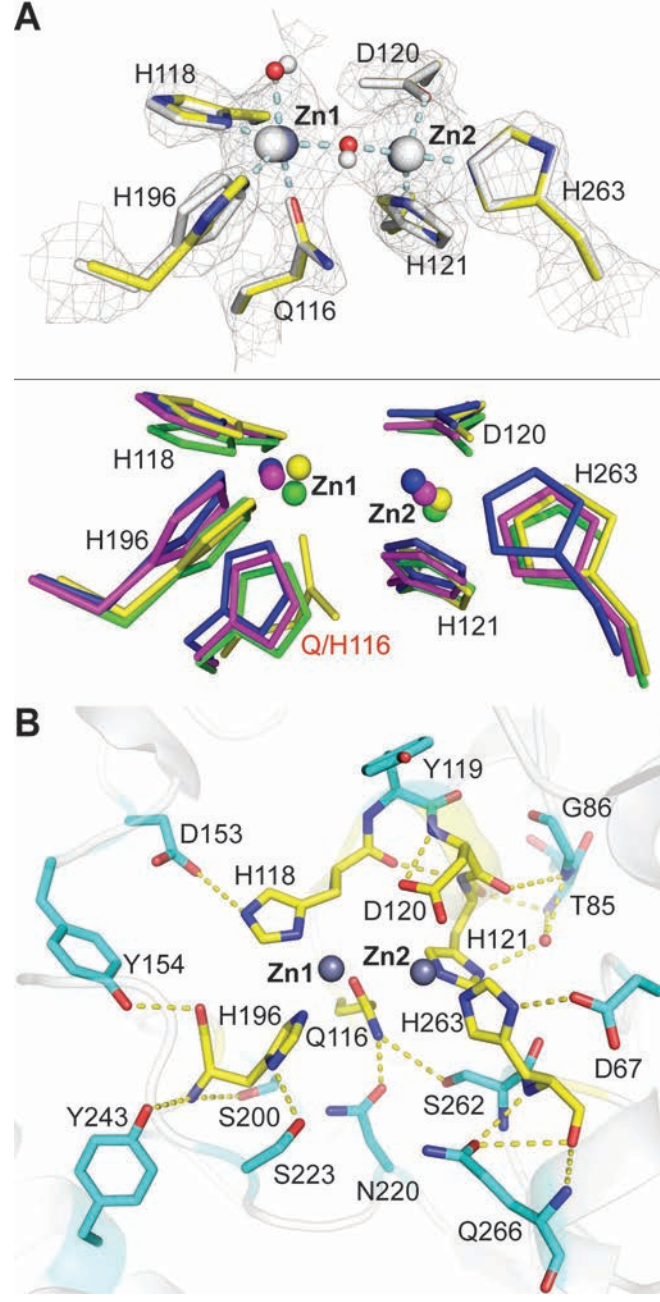
712 †† Calculated with Phenix get_cc_mtz_pdb (39).

713 § Calculated with Mol Probit (62).









GOB-18

