

1 **Localising individual atoms of tryptophan side chains in the metallo- $\beta$ -lactamase IMP-1**  
2 **by pseudocontact shifts from paramagnetic lanthanoid tags at multiple sites**

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21  
22 **Abstract**

23 The metallo- $\beta$ -lactamase IMP-1 features a flexible loop near the active site that assumes  
24 different conformations in single crystal structures, which may assist in substrate binding and  
25 enzymatic activity. To probe the position of this loop, we labelled the tryptophan residues of  
26 IMP-1 with 7-<sup>13</sup>C-indole and the protein with lanthanoid tags at three different sites. The  
27 magnetic susceptibility anisotropy ( $\Delta\chi$ ) tensors were determined by measuring pseudocontact  
28 shifts (PCS) of backbone amide protons. The  $\Delta\chi$  tensors were subsequently used to identify  
29 the atomic coordinates of the tryptophan side chains in the protein. The PCSs were sufficient  
30 to determine the location of Trp28, which is located in the active site loop targeted by our  
31 experiments, with high accuracy. Its average atomic coordinates showed barely significant  
32 changes in response to the inhibitor captopril. It was found that localisation spaces could be  
33 defined with better accuracy by including only the PCSs of a single paramagnetic lanthanoid  
34 ion for each tag and tagging site. The effect was attributed to the shallow angle with which

35 PCS isosurfaces tend to intersect if generated by tags and tagging sites that are identical except  
36 for the paramagnetic lanthanoid ion.

37

## 38 **1 Introduction**

39 The metallo- $\beta$ -lactamase IMP-1 is an enzyme that hydrolyses  $\beta$ -lactams, thus conferring  
40 penicillin resistance to bacteria. First identified 30 years ago in the Gram-negative bacteria in  
41 early 1990s from *Pseudomonas aeruginosa* and *Serratia marcescens* (Bush 2013), IMP-1 has  
42 become a serious clinical problem due to horizontal gene transfer by a highly mobile gene  
43 (*bla*<sub>IMP-1</sub>) located on an integron (Arakawa et al., 1995), as the *bla*<sub>IMP-1</sub> gene has been detected  
44 in isolates of *Klebsiella pneumoniae*, *Pseudomonas putida*, *Alcaligenes xylosoxidans*,  
45 *Acinetobacter junii*, *Providencia rettgeri*, *Acinetobacter baumannii* and *Enterobacter*  
46 *aerogenes* (Ito et al., 1995; Laraki et al., 1999a; Watanabe et al., 1991). Critically, IMP-1  
47 confers resistance also to recent generations of carbapenems and extended-spectrum  
48 cephalosporins (Laraki et al., 199b; Bush et al., 2010; van Duin et al., 2013).

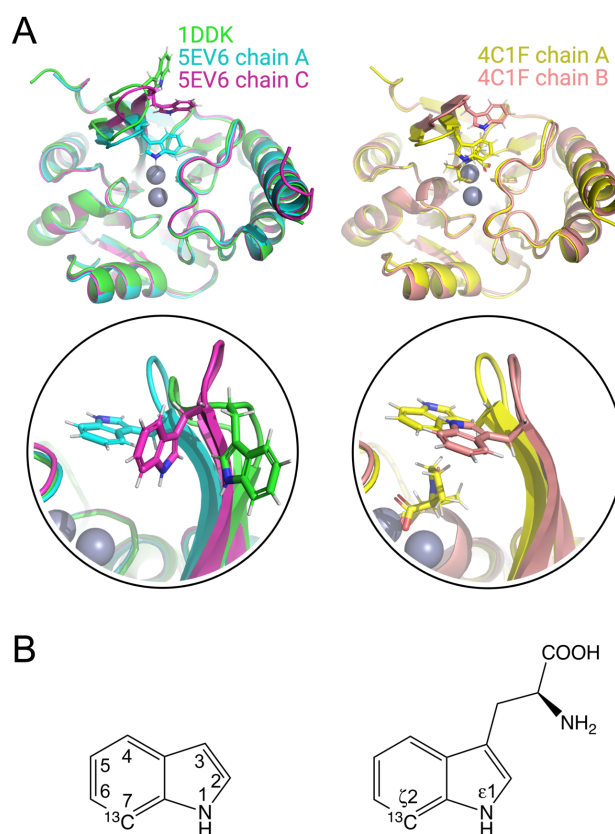
49 Multiple crystal structures have been solved of IMP-1, free and in complex with various  
50 inhibitors (Concha et al., 2000; Toney et al., 2001; Moali et al., 2003; Hiraiwa et al., 2014;  
51 Brem et al., 2016; Hinchliffe et al., 2016; 2018; Wachino et al., 2019; Rossi et al., 2021). IMP-  
52 1 belongs to the subclass B1 of metallo- $\beta$ -lactamases, which contain two zinc ions bridged by  
53 the sulfur atom of a cysteine residue in the active site (Concha, 2000). One of Zn<sup>2+</sup> ions can  
54 readily be replaced by a Fe<sup>3+</sup> ion (Carruthers et al., 2014). The active site is flanked by a loop  
55 (referred to as L3 loop) that contains a highly solvent-exposed tryptophan residue surrounded  
56 by glycine residues on either side. Both the loop and the tryptophan residue (Trp28 in the IMP-  
57 1-specific numbering used by Concha et al. (2000) and Trp64 in the universal numbering  
58 scheme by Galleni et al. (2001)) assume different conformations in different crystal structures,  
59 suggesting that the loop acts as a mobile flap to cover bound substrate (Fig. 1A). The L3 loop  
60 and the functional implication of its flexibility has been studied extensively for different  
61 metallo- $\beta$ -lactamases containing the Gly-Trp-Gly motif in the loop (Huntley et al., 2000; 2003;  
62 Moali et al., 2003; Yamaguchi et al., 2015; Palacios et al., 2019; Gianquinto et al., 2020; Softley  
63 et al., 2020). Flexibility of the L3 loop is a general feature also of many metallo- $\beta$ -lactamases  
64 without the Gly-Trp-Gly motif and is thought to contribute to the wide range of  $\beta$ -lactam  
65 substrates that can be hydrolyzed by the enzymes (González et al., 2016; Linciano et al., 2019;  
66 Salimraj et al., 2018). In the case of the metallo- $\beta$ -lactamase from *B. fragilis*, which is closely  
67 related to IMP-1, electron density could be detected for the Gly-Trp-Gly motif in the crystal

68 structure of the protein in the presence (Payne et al., 2003) but not absence of an inhibitor  
69 (Concha et al., 1996), and an NMR relaxation study in solution confirmed the increased  
70 flexibility of both the L3 loop and, in particular, the sidechain of the tryptophan residue  
71 (Huntley et al., 2000). A similar situation prevails in the case of the IMP-1 variant IMP-13,  
72 where different crystal structures of the ligand-free protein show the L3 loop in very different  
73 conformations, sometimes lacking electron density, while NMR relaxation measurements  
74 confirmed the increased flexibility of the loop (Softley et al., 2020).

75 Due to the rigidity of their sidechains, tryptophan residues frequently contribute to the  
76 structural stability of three-dimensional protein folds and it is unusual to observe tryptophan  
77 sidechains fully solvent-exposed as in the Gly-Trp-Gly motif of substrate-free IMP-1. The  
78 functional role of Trp28 in IMP-1 was assessed in an early mutation study by mutating Trp28  
79 to alanine and, in a different experiment, eliminating the L3 loop altogether. Enzymatic activity  
80 measurements revealed an increase in the Michaelis constant  $K_m$  and a decrease in  $k_{cat}/K_m$  ratios  
81 for all  $\beta$ -lactams tested, illustrating the importance of the Trp28 sidechain for catalytic activity.  
82 Complete removal of the L3 loop reduced the  $k_{cat}/K_m$  ratios even further, but without  
83 completely abolishing the enzymatic activity (Moali et al., 2003).

84

85



86

87 **Figure 1.** Crystal structures of IMP-1 with different conformations of the loop L3 and chemical  
88 structures of indole and tryptophan with atom names. (A) Superimposition of crystal structures  
89 of IMP-1 highlighting structural variations of Trp28 and the associated loop L3. The structures  
90 shown are of the  $Zn^{2+}/Zn^{2+}$  complex without inhibitor (green, PDB ID 1DDK, Concha et al.,  
91 2000; cyan for chain A and magenta for chain C, PDB ID 5EV6, Hinchliffe et al., 2016), with  
92 bound L-captopril (yellow for chain A and salmon for chain B, PDB ID 4CIF, Brem et al.,  
93 2016).  $Zn^{2+}$  ions are represented by grey spheres and bound captopril is shown in the structure  
94 4CIF chain A. (B) Chemical structures of indole and tryptophan with selected ring positions  
95 labelled according to IUPAC conventions. The present work used indole synthesised with a  
96  $^{13}C$ - $^1H$  group in position 7 and deuterium in the ring positions 2, 3, 4, 5 and 6 (Maleckis et al.,  
97 2021).

98  
99 In the crystalline state, the conformation of a solvent-exposed loop is easily impacted  
100 by crystal packing forces. Therefore, it is unclear what the actual conformation of the L3 loop  
101 is in solution. To address this question, we used solution NMR spectroscopy to assess the  
102 location of Trp28 in IMP-1 both in the absence and presence of the inhibitor L-captopril, which  
103 inhibits metallo- $\beta$ -lactamases by binding to the active-site zinc ions (Brem et al., 2016). The  
104 analysis was hindered by incomplete backbone resonance assignments of IMP-1 attributed to  
105 conformational exchange processes in parts of the protein (Carruthers et al., 2014). As it is  
106 difficult to accurately position the atoms of a solvent-exposed polypeptide loop in solution by  
107 nuclear Overhauser effects (NOE), we used pseudocontact shifts (PCS) generated by  
108 lanthanoid ions attached at different sites of IMP-1 to determine the location of Trp28 relative  
109 to the core of the protein. PCSs generated by multiple different paramagnetic metal ions or the  
110 same metal ion attached at different sites of a protein have previously been shown to allow  
111 localising atoms at remote sites of interest, such as in specific amino acid side chains (Pearce  
112 et al., 2017; Lescanne et al., 2018), bound ligand molecules (Guan et al., 2013; Chen et al.,  
113 2016) or proteins (Pintacuda et al., 2006; Keizers et al., 2010; de la Cruz et al., 2011;  
114 Kobashigawa et al., 2012; Brewer et al., 2015) or for 3D structure determinations of proteins  
115 (Yagi et al., 2013; Crick et al., 2015; Pilla et al., 2017).

116 IMP-1 contains six tryptophan residues, each containing several aromatic hydrogens  
117 with similar chemical shifts. To increase the spectral resolution in the 2D NMR spectra  
118 recorded for PCS measurements, we labelled each tryptophan sidechain with a single  $^{13}C$  atom  
119 by expressing the protein in the presence of 7- $^{13}C$ -indole (Fig. 1B; Maleckis et al., 2021). The

120 results show that the localisation spaces defined by the tryptophan PCSs fully agree with  
121 previously determined crystal structures of IMP-1 for all tryptophan residues. They suggest  
122 little change in the average conformation of the L3 loop upon binding of captopril. The results  
123 illustrate the accuracy with which the positions of individual atoms can be determined by PCSs  
124 from lanthanoid tags even in proteins of limited stability.

125

## 126 **2 Experimental procedures**

### 127 **2.1 Production, purification and tagging of proteins**

#### 128 **2.1.1 Plasmid constructs and <sup>13</sup>C-labelled indole**

129 Three different cysteine mutations (A53C, N172C and S204C) were introduced into the *bla*<sub>IMP1</sub>  
130 gene in the pET-47b(+) plasmid using a modified QuikChange protocol (Qi and Otting, 2019).  
131 Deuterated 7-<sup>13</sup>C-indole was synthesized as described with deuteration in all positions other  
132 than position 7 (Maleckis et al., 2021). The amino acid sequence of the protein was that  
133 reported in the crystal structure 4UAM (Carruthers et al., 2014), except that the N-terminal  
134 alanine residue was substituted by a methionine to avoid heterogeneity by incomplete  
135 processing by amino peptidase.

136

#### 137 **2.1.2 Protein production**

138 Uniformly <sup>15</sup>N-labelled samples of the cysteine mutants of IMP-1 were expressed in *E. coli*  
139 BL21(DE3) cells. The cells were grown at 37 °C in Luria–Bertani (LB) medium containing 50  
140 mgL<sup>-1</sup> kanamycin until the OD<sub>600</sub> reached 0.6–0.8 and were then transferred to 300 mL of M9  
141 medium (6 gL<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 3 gL<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 gL<sup>-1</sup> NaCl, pH 7.2) supplemented with 1 gL<sup>-1</sup>  
142 of <sup>15</sup>NH<sub>4</sub>Cl. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG, final  
143 concentration 1 mM), the cells were incubated at room temperature for 16 hours. Following  
144 centrifugation, the cells were resuspended in buffer A (50 mM HEPES, pH 7.5, 100 μM ZnSO<sub>4</sub>)  
145 for lysis by a homogeniser (Avestin Emulsiflex C5). The supernatant of the centrifuged cell  
146 lysate was loaded onto a 5 mL SP column, the column was washed with 20 column volumes  
147 buffer B (same as buffer A but with 50 mM NaCl) and the protein was eluted with a gradient  
148 of buffer C (same as buffer A but with 1 M NaCl).

149 IMP-1 samples containing 7-<sup>13</sup>C-tryptophan were produced by continuous exchange  
150 cell-free protein synthesis (CFPS) from PCR-amplified DNA with eight-nucleotide single-  
151 stranded overhangs as described (Wu et al., 2007), using 7-<sup>13</sup>C-indole as a precursor for the *in*  
152 *vitro* production of tryptophan (Maleckis et al., 2021). The CFPS reactions were conducted at  
153 30 °C for 16 h using 1 mL inner reaction mixture and 10 mL outer buffer. Tryptophan was

154 omitted from the mixture of amino acids provided and deuterated 7-<sup>13</sup>C-indole was added from  
155 a stock solution in 50 % DMSO/50 % H<sub>2</sub>O to the inner and outer buffers at a final concentration  
156 of 0.75 mM. The protein samples were purified as described above. About 5 mg of the indole  
157 was required for preparing each NMR sample.

158

### 159 **2.1.3 Ligation with C2-Ln<sup>3+</sup> tag**

160 To ensure the reduced state of cysteine thiol groups, the protein samples were treated with 2  
161 mM dithiothreitol (DTT) for 1 hour. Subsequently, the DTT was removed using an Amicon  
162 ultrafiltration centrifugal tube with a molecular weight cut-off of 10 kDa, concentrating the  
163 protein samples to 50 μM in buffer A. The samples were incubated overnight at room  
164 temperature with shaking in the presence of five-fold molar excess of C2 tag (Graham et al.,  
165 2011; de la Cruz et al., 2011) loaded with either Y<sup>3+</sup>, Tb<sup>3+</sup> or Tm<sup>3+</sup>. Following the tagging  
166 reaction, the samples were washed using an Amicon centrifugal filter unit to remove unbound  
167 tag and the buffer was exchanged to NMR buffer (20 mM MES, pH 6.5, 100 mM NaCl).

168

### 169 **2.1.4 Ligation with C12-Ln<sup>3+</sup> tag**

170 The ligation reaction of IMP-1 N172C with the C12-Ln<sup>3+</sup> tag loaded with either Y<sup>3+</sup>, Tb<sup>3+</sup> or  
171 Tm<sup>3+</sup> (Herath et al., 2021) was conducted in the same way as with the C2-Ln<sup>3+</sup> tags, except that  
172 the reactions were carried out in buffer A with the pH adjusted to 7.0.

173

## 174 **2.2 NMR spectroscopy**

175 All NMR data were acquired at 37 °C on Bruker 600 and 800 MHz NMR spectrometers  
176 equipped with TCI cryoprobes designed for 5 mm NMR tubes, but only 3 mm NMR tubes were  
177 used in this project. Protein concentrations were 0.6 mM and 0.2 mM for <sup>15</sup>N-HSQC spectra  
178 of samples labelled with the C2 and C12 tag, respectively. The protein concentrations were 0.4  
179 mM for <sup>13</sup>C-HSQC and NOE-relayed <sup>13</sup>C-HSQC spectra. <sup>15</sup>N-HSQC spectra were recorded at  
180 a <sup>1</sup>H-NMR frequency of 800 MHz with  $t_{1\max} = 40$  ms,  $t_{2\max} = 170$  ms, using a total recording  
181 time of 3 h per spectrum. <sup>13</sup>C-HSQC spectra were recorded with a S<sup>3</sup>E filter to select the low-  
182 field doublet component due to the <sup>1</sup>J<sub>HC</sub> coupling of the <sup>13</sup>C-labelled tryptophan side chains.  
183 The pulse sequence is shown in Fig. S9 and the spectra were recorded at a <sup>1</sup>H-NMR frequency  
184 of 600 MHz using  $t_{1\max} = 20$ –50 ms,  $t_{2\max} = 106$  ms and total recording times of 2 h per  
185 spectrum. <sup>13</sup>C-HSQC spectra with NOE relay were recorded without decoupling in the <sup>13</sup>C-  
186 dimension, relying on relaxation and <sup>13</sup>C equilibrium magnetisation to emphasize the narrow

187 doublet component. The NOE mixing time was 150 ms and the total recording time 3 h per  
188 spectrum. The pulse sequence is shown in Fig. S10.

189 To account for uncertainties in concentration measurements, samples with <sub>L</sub>-captopril  
190 were prepared with a nominal ratio of captopril to protein of 1.5:1. In the case of samples  
191 tagged with the C2 tag, however, this lead to gradual release of some of the tag, as captopril  
192 contains a free thiol group and the disulfide linkage of the C2 tag is sensitive to chemical  
193 reduction. To limit this mode of sample degradation, the NOE-relayed [<sup>13</sup>C,<sup>1</sup>H]-HSQC spectra  
194 were recorded with a smaller excess of captopril.

195

### 196 2.3 $\Delta\chi$ -tensor fits

197 The experimental PCSs ( $\Delta\delta^{\text{PCS}}$ ) were measured in ppm as the amide proton chemical shift  
198 observed in NMR spectra recorded for the IMP-1 mutants A53C, N172C and S204C tagged  
199 with Tm<sup>3+</sup> or Tb<sup>3+</sup> tags minus the corresponding chemical shift measured of samples made with  
200 Y<sup>3+</sup> tags. The resonance assignments of the wild-type Zn<sub>2</sub> enzyme (BMRB entry 25063) were  
201 used to assign the <sup>15</sup>N-HSQC cross-peaks in the diamagnetic state. The program Paramagpy  
202 (Orton et al., 2020) was used to fit magnetic susceptibility anisotropy ( $\Delta\chi$ ) tensors to crystal  
203 structures of IMP-1 solved in the absence and presence of the inhibitor captopril.

204

## 205 3 Results

### 206 3.1 Protein production

207 Three cysteine mutants of uniformly <sup>15</sup>N-labelled IMP-1 were produced *in vivo*, where cysteine  
208 residues replaced Ala53, Asn172 and Ser204, respectively. The purified proteins were tagged  
209 with C2 tags containing Tb<sup>3+</sup> or Tm<sup>3+</sup> as the paramagnetic ions and Y<sup>3+</sup> as the diamagnetic  
210 reference. Samples of the uniformly <sup>15</sup>N-labelled mutant N172C were also ligated with C12  
211 tags containing the same set of metal ions. The chemical structures of the tags are depicted in  
212 Fig. S1. To record <sup>13</sup>C-<sup>1</sup>H correlation spectra of the tryptophan side chains with minimal  
213 spectral overlap, additional samples of the cysteine mutants were produced with selectively  
214 <sup>13</sup>C-labelled tryptophan residues. These samples were produced by cell-free protein synthesis  
215 in the presence of 7-<sup>13</sup>C indole, deuterated except at the 7 position, with the omission of  
216 tryptophan, using a recently established protocol (Maleckis et al., 2021). The residual activity  
217 of tryptophan synthase in the cell-free extract was sufficient to produce tryptophan from the  
218 added <sup>13</sup>C-labelled indole. The resulting tryptophan residues contained a <sup>13</sup>C-<sup>1</sup>H group in  
219 position 7 (<sup>13</sup>C<sup>ζ2</sup> and <sup>1</sup>H<sup>ζ2</sup> in IUPAC nomenclature; Markley et al., 1998) and deuterons at all

220 other hydrogen positions of the indole ring except for the H<sup>N</sup> atom (H<sup>ε1</sup> in IUPAC  
221 nomenclature). The cell-free expression yielded about 2 mg of purified protein per millilitre of  
222 inner cell-free reaction mixture. Mass spectrometry indicated that the tryptophan residues of  
223 IMP-1 were <sup>13</sup>C/<sup>2</sup>H-labelled with about 80 % labelling efficiency at each of the six tryptophan  
224 positions (Fig. S2). The purified proteins were ligated with C2-Ln<sup>3+</sup> tags containing either Tb<sup>3+</sup>,  
225 Tm<sup>3+</sup> or Y<sup>3+</sup> as in the case of the <sup>15</sup>N-labelled samples. Ligation yields with the C2 tags were  
226 practically complete as indicated by mass spectrometry (Fig. S2). The ligation yield of the  
227 N172C mutant with C12 tags was about 90 % (Herath et al., 2021).

228

### 229 **3.2 NMR experiments and resonance assignments**

230 [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectra were measured of the tagged proteins in the free state and in the  
231 presence of L-captopril (Fig. S3–S8). <sup>1</sup>H PCSs of backbone amide protons measured in these  
232 spectra were used to establish the Δχ tensors relative to the protein. The resonance assignment  
233 of the [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectra in the presence of inhibitor was transferred from the  
234 corresponding spectra recorded in the absence of inhibitor. As no resonance assignments could  
235 reliably be made in this way in areas of spectral overlap, fewer resonance assignments were  
236 available in the presence than absence of inhibitor. Furthermore, due to captopril releasing  
237 some of the C2 tags from the protein by breaking the disulfide bridge of the tag attachment,  
238 spectra recorded in the presence of captopril contained additional cross-peaks from  
239 diamagnetic protein.

240 To obtain tagged protein that is inert against chemical reduction, we also attached the  
241 C12 tag to the mutant N172C. This tag, however, caused the appearance of additional peaks in  
242 the [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectra (Fig. S7). The additional peaks appeared in different sample  
243 preparations, indicating sample degradation or perturbation of the local protein structure by the  
244 tag. We therefore based the rest of the work mainly on the PCSs obtained with the C2 tags.  
245 Tables S1 and S2 list the PCSs of the backbone amides measured in the absence and presence  
246 of captopril.

247 <sup>1</sup>H PCSs of the tryptophan H<sup>ε2</sup> protons were measured in [<sup>13</sup>C,<sup>1</sup>H]-HSQC spectra  
248 recorded with S<sup>3</sup>E spin-state selection element (Meissner et al., 1997) in the <sup>13</sup>C dimension to  
249 select the slowly relaxing components of the doublets split by <sup>1</sup>J<sub>HC</sub> couplings. Cross-peaks were  
250 observed for all six tryptophan residues except for the mutant N172C, which displayed cross-  
251 peaks of only five tryptophan indoles (Fig. 2). The missing signal was attributed to Trp176  
252 because of its close proximity to the tagging site. The indole H<sup>ε1</sup> proton is located within 2.9 Å



253 of the H<sup>ε2</sup> proton and the NOE between both protons was readily observed in a [<sup>13</sup>C,<sup>1</sup>H]-HSQC  
254 experiment with NOE relay (Fig. 2). The H<sup>ε1</sup> chemical shifts afforded better spectral resolution  
255 than the H<sup>ε2</sup> resonances. Comparison of the predicted and observed PCSs yielded resonance  
256 assignments of all tryptophan H<sup>ε1</sup> cross-peaks with particular clarity in the NOE-relayed  
257 [<sup>13</sup>C,<sup>1</sup>H]-HSQC spectrum (Fig. 2). In addition, the assignment was supported by paramagnetic  
258 relaxation enhancements (for example, Trp88 is near residue 53 and therefore its cross-peaks  
259 were strongly attenuated in the paramagnetic samples of the A53C mutant). Different PCSs  
260 were observed for all six tryptophan sidechains and different PCSs were observed for the H<sup>ε2</sup>  
261 and H<sup>ε1</sup> protons within the same indole sidechain. Each of the tryptophan sidechains showed  
262 PCSs in most, if not all, of the mutants. As the L3 loop is near residue 172, the mutant N172C  
263 endowed Trp28 with particularly large PCSs. Tables S3 and S4 report the PCSs measured in  
264 this way for the samples labelled with C2 tags.

265 In contrast, assigning the indole N-H groups in the [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectra was much  
266 more difficult because IMP-1 is a protein prone to showing more than a single peak per proton  
267 (Figs S5 and S6). In particular, the [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectrum of wild-type IMP-1 selectively  
268 labelled with <sup>15</sup>N-tryptophan displayed six intense and at least three weak N<sup>ε1</sup>-H<sup>ε1</sup> cross-peaks  
269 (Fig. S6; Carruthers et al., 2014) and the [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectra of the tagged cysteine mutants  
270 showed evidence of heterogeneity too (Fig. S5). Nonetheless, the six most intense N<sup>ε1</sup>-H<sup>ε1</sup>  
271 cross-peaks could be assigned by comparison to the PCSs observed in the NOE-relayed  
272 [<sup>13</sup>C,<sup>1</sup>H]-HSQC spectrum and this assignment was used to measure the PCSs of the tryptophan  
273 H<sup>ε1</sup> resonances in the mutant N172C tagged with C12 tag (Fig. S8; Table S4).

274 Spectra recorded in the presence of L-captopril were very similar to those recorded  
275 without the inhibitor, except that some new, narrow C-H cross-peaks appeared in the [<sup>13</sup>C,<sup>1</sup>H]-  
276 HSQC spectra of the mutants A53C and S204C, which were suggestive of protein degradation  
277 (Fig. 3). We consequently used the better-resolved indole H<sup>N</sup> cross-peaks to identify the correct  
278 parent C-H cross-peaks. The chemical shifts of the tryptophan sidechains changed very little  
279 in response to the presence of L-captopril, except for the <sup>13</sup>C-chemical shift of Trp28, which is  
280 nearest to the ligand binding site. The PCSs of the indole protons measured in the presence of  
281 the inhibitor are listed in Tables S5 and S6.

282

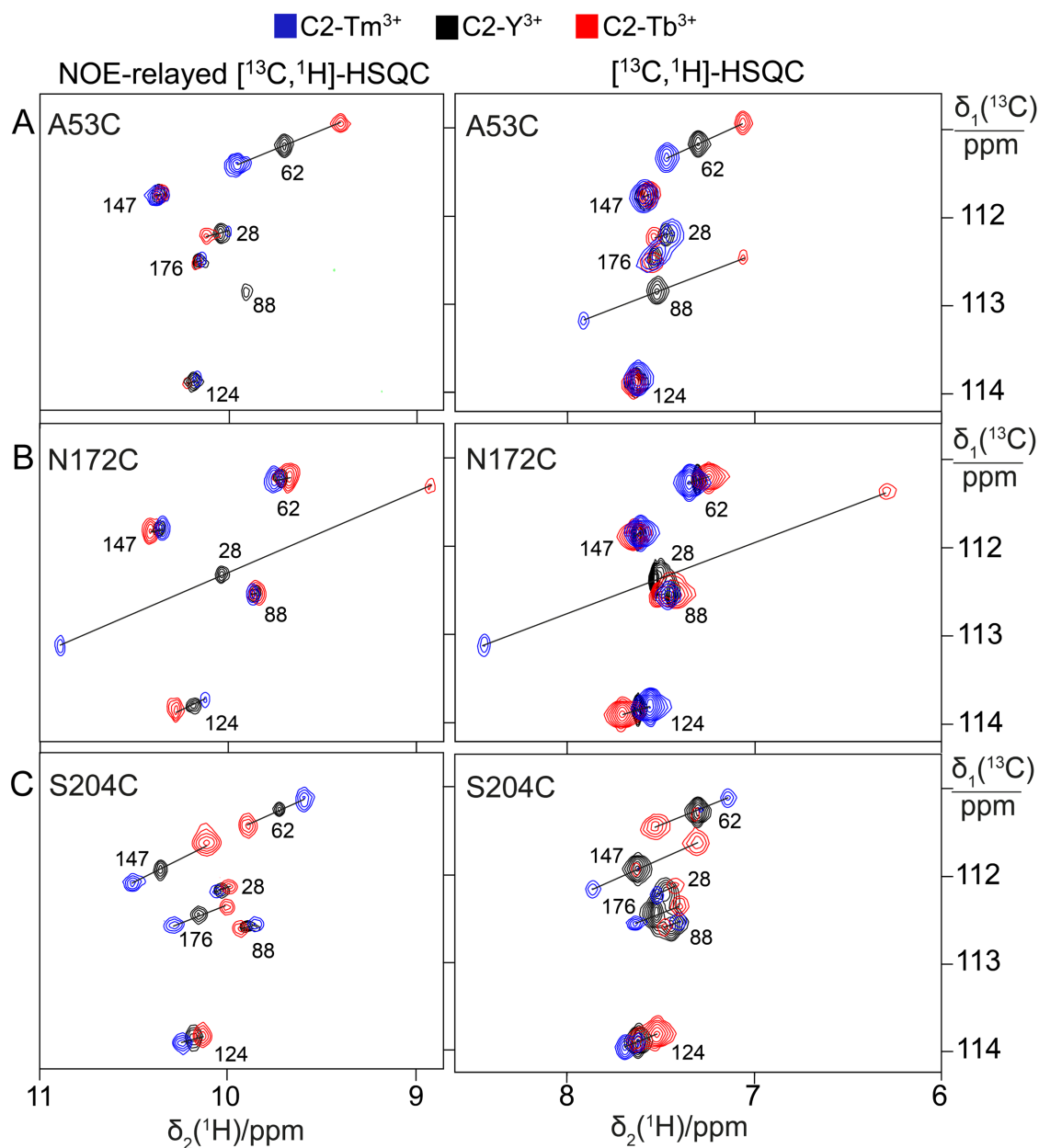
### 283 3.2 Δχ-tensor fits

284 The Δχ-tensor parameters were determined using the program Paramagpy (Orton et al., 2020),  
285 using all available <sup>1</sup>H PCSs measured of backbone amides. Comparing the Δχ tensor fits to the

286 crystal structures 5EV6 chains A and C (Hinchliffe et al., 2016) and 1DDK (Concha et al.,  
287 2000) of the free protein, the chain A of the structure 5EV6 proved to produce the smallest  $Q$   
288 factor by a small margin (Fig. S11) and was used as the reference structure of the free protein  
289 for the subsequent evaluation. Similarly, chain A of the co-crystal structure published with the  
290 inhibitor L-captopril (PDB ID: 4C1F; Brem et al., 2016) on average delivered better fits than  
291 chain B and was used as the reference structure for the NMR data recorded in the presence of  
292 L-captopril. The  $\Delta\chi$ -tensor fits of each mutant and tag used a common metal position for the  
293 data obtained with the  $\text{Tb}^{3+}$  and  $\text{Tm}^{3+}$  tags. The fits positioned the paramagnetic centres at  
294 distances between 8.2 and 9.4 Å from the  $\text{C}^\beta$  atom of the tagged cysteine residues, which is  
295 compatible with the chemical structure of the C2-tag. Figure 4 shows the correlations between  
296 back-calculated and experimental PCSs and Table S7 reports the fitted  $\Delta\chi$  tensor parameters.  
297 Very similar  $Q$  factors were obtained when using the PCSs measured in the absence of inhibitor  
298 to fit the  $\Delta\chi$  tensor to the co-crystal structure 4C1F or the PCSs measured in the presence of  
299 inhibitor to fit the  $\Delta\chi$  tensor to the crystal structure of the free protein. This indicates that the  
300 protein structure did not change very much in response to inhibitor binding. This conclusion  
301 was also indicated by the similarity between the backbone PCSs observed with and without  
302 inhibitor (Fig. S12).

303 The  $\Delta\chi$  tensors obtained with the  $\text{Tb}^{3+}$  tags were larger than those obtained with the  
304  $\text{Tm}^{3+}$  tags, which is also reflected by the consistently larger PCSs observed in the  $^{13}\text{C}$ - $^1\text{H}$   
305 correlation spectra of Fig. 2 and 3. The fits of  $\Delta\chi$  tensors to the protein backbone also yielded  
306 better  $Q$  factors for PCSs generated by  $\text{Tb}^{3+}$  than  $\text{Tm}^{3+}$  ions. Therefore, we determined the  
307 localisation spaces of the tryptophan sidechains in the first instance by using their  $^1\text{H}$  PCSs  
308 measured with  $\text{Tb}^{3+}$  tags only.

309

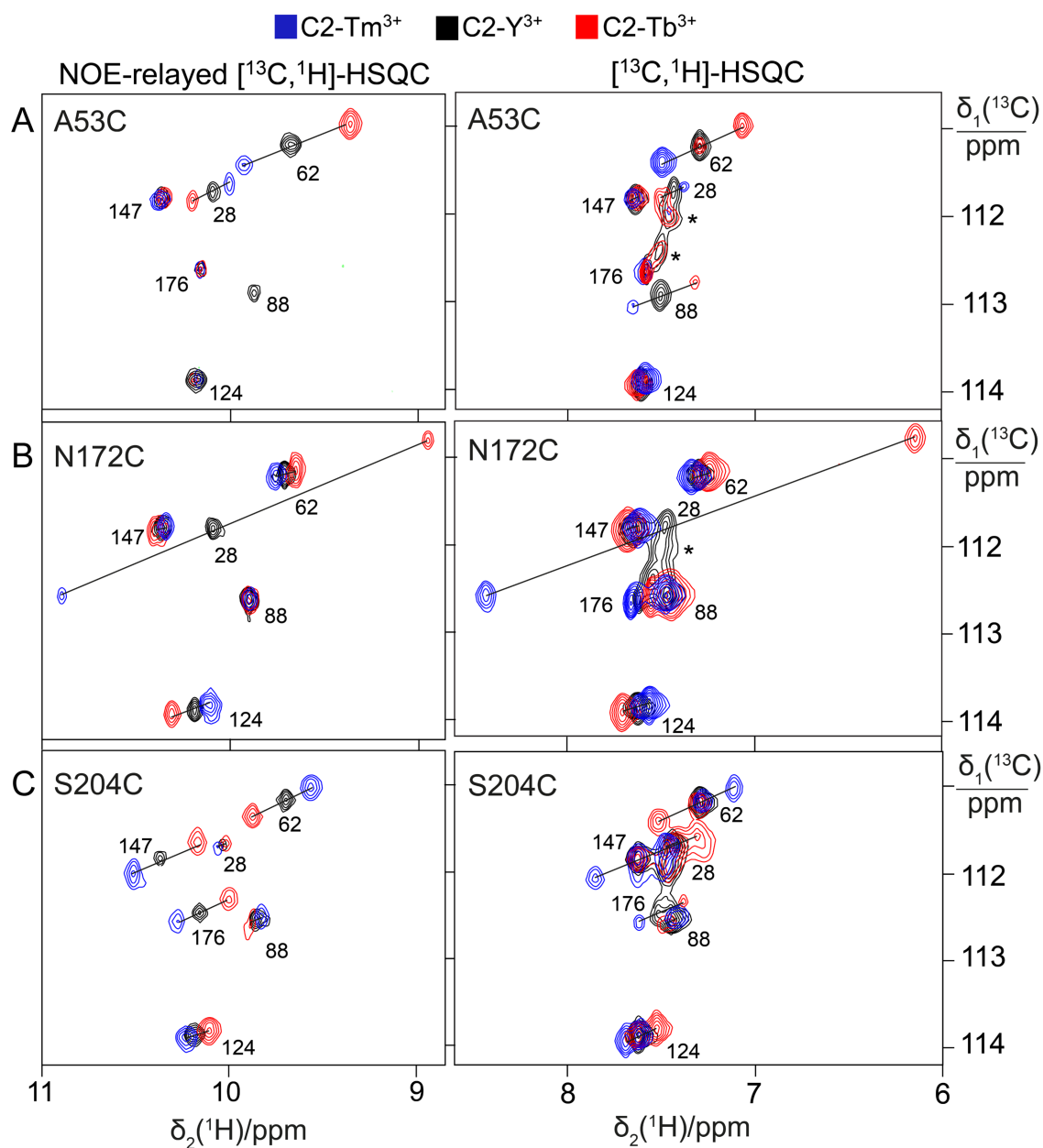


310

311 **Figure 2.** PCSs observed in  $^{13}\text{C}$ - $^1\text{H}$  correlation spectra of 0.4 mM solutions of IMP-1 mutants  
 312 tagged with C2- $\text{Ln}^{3+}$  tags and containing selectively isotope-labelled tryptophan produced from  
 313  $7\text{-}^{13}\text{C}$ -indole deuterated in the positions 2, 4, 5 and 6. The plots show superimpositions of  
 314 spectra recorded with diamagnetic (C2- $\text{Y}^{3+}$ , black) or paramagnetic (C2- $\text{Tb}^{3+}$ , red; C2- $\text{Tm}^{3+}$ ,  
 315 blue) tags. All spectra were recorded with spin-state selection in the  $^{13}\text{C}$ -dimension to record  
 316 the narrow low-field component of each  $^{13}\text{C}$ -doublet. Right panels:  $^{13}\text{C}$ , $^1\text{H}$ -HSQC spectra.  
 317 Left panels: NOE-relayed  $^{13}\text{C}$ , $^1\text{H}$ -HSQC spectra (150 ms NOE mixing time) to record the  $\text{H}^{\epsilon 1}$   
 318 resonances of the tryptophan side chains. PCSs are indicated by lines connecting the peaks of  
 319 paramagnetic and diamagnetic samples. The cross-peaks are assigned with the residue number  
 320 of the individual tryptophan residues. (A) Mutant A53C. (B) Mutant N172C. (C) Mutant

321 S204C.

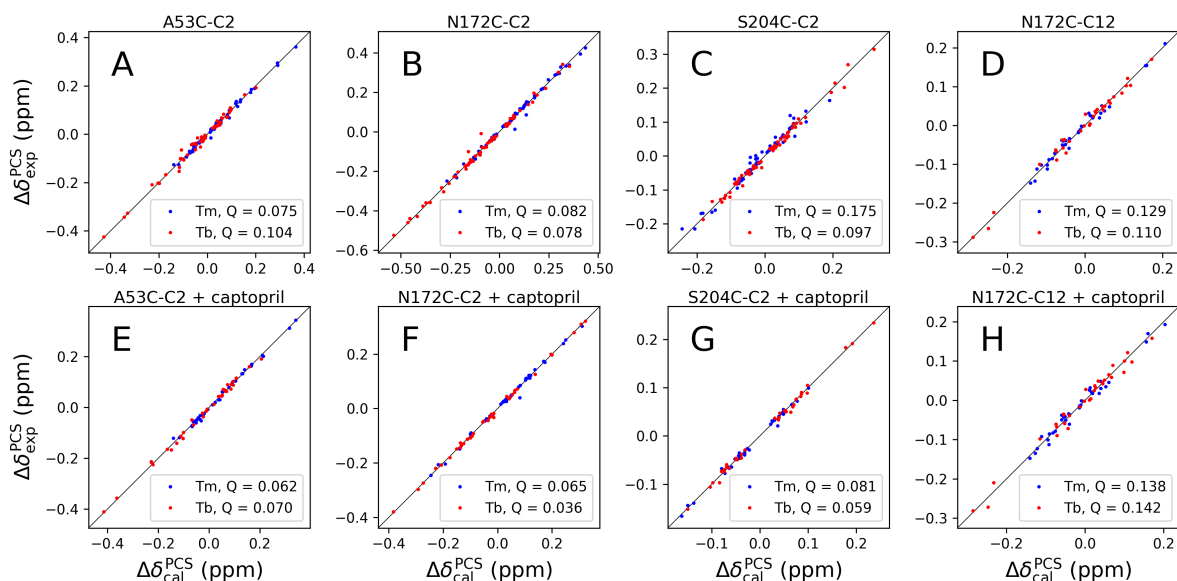
322



323

324 **Figure 3.** Effect of the presence of L-captopril on the PCSs observed in  $^{13}\text{C}$ - $^1\text{H}$  correlation  
325 spectra of 0.4 mM solutions of IMP-1 mutants. Protein preparations and experimental  
326 parameters were the same as in Fig. 2. Spectra recorded with diamagnetic ( $\text{C2-Y}^{3+}$ , black) or  
327 paramagnetic ( $\text{C2-Tb}^{3+}$ , red;  $\text{C2-Tm}^{3+}$ , blue) tags are superimposed. Right column:  $^{13}\text{C}$ , $^1\text{H}$ -  
328 HSQC spectra. Left column: NOE-relayed  $^{13}\text{C}$ , $^1\text{H}$ -HSQC spectra recorded with 150 ms NOE  
329 mixing time. Stars mark cross-peaks of species putatively attributed to protein degradation. (A)  
330 Mutant A53C. (B) Mutant N172C. (C) Mutant S204C.

331



332

333 **Figure 4.** Correlations between back-calculated and experimental  $^1\text{H}$  PCSs measured of  
 334 backbone amides of IMP-1 with C2 tags at three different sites (positions 53, 172 and 204) and  
 335 the C12 tag in position 172. Red and blue data points correspond to the PCS data obtained with  
 336  $\text{Tb}^{3+}$  and  $\text{Tm}^{3+}$  tags, respectively. (A) Mutant A53C with C2 tag. (B) Mutant N172C with C2  
 337 tag. (C) Mutant S204C with C2 tag. (D) Mutant N172C with C12 tag. (E) Same as (A) but in  
 338 the presence of captopril. (F) Same as (B) but in the presence of captopril. (G) Same as (C) but  
 339 in the presence of captopril. (H) Same as (D) but in the presence of captopril. PCS data in (A)–  
 340 (D) were used to fit  $\Delta\chi$  tensors to the structure 5EV6. PCS data in (E)–(F) were used to fit  $\Delta\chi$   
 341 tensors to the structure 4C1F.

342

### 343 3.3 Determining the localisation spaces of tryptophan sidechains

344 The  $\Delta\chi$  tensors determined of backbone amides not only enabled the resonance assignment of  
 345 the tryptophan sidechains by comparing back-calculated with experimental PCSs, but also  
 346 allowed translation of the indole PCSs into restraints that define the locations of the tryptophan  
 347  $\text{H}^{\zeta 2}$  and  $\text{H}^{\epsilon 1}$  atoms with respect to the rest of the protein. The concept of localising nuclear spins  
 348 by PCSs that are generated by lanthanoid tags at different sites is well-established (see, e.g.,  
 349 Yagi et al., 2013; Lescanne et al., 2018; Zimmermann et al., 2019). It can be visualised by  
 350 representing each PCS restraint by the corresponding PCS isosurface, which comprises all  
 351 points in space where this PCS value is generated by the  $\Delta\chi$  tensor (Fig. 5). With PCS restraints  
 352 from two different metal sites, the intersection between the respective isosurfaces defines a  
 353 line. The intersection of this line with the PCS isosurface from a third  $\Delta\chi$  tensor defines two  
 354 points. While a fourth  $\Delta\chi$  tensor could unambiguously produce a single solution, a fourth tensor

355 may not be required if one of these two points is incompatible with the covalent structure of  
356 the protein. In favourable circumstances, the constraints imposed by the covalent structure may  
357 even allow the accurate positioning of nuclear spins by PCSs generated from only two different  
358  $\Delta\chi$  tensors (Pearce et al., 2017). Therefore, the present study was successful with only three  
359 different tagging sites. Figure S13 illustrates the concept for the Trp28 H <sup>$\epsilon$ 1</sup> atom.

360 The spatial definition of the intersection point defined by the PCS isosurfaces depends  
361 on the experimental uncertainties in a non-isotropic way, as the PCS isosurfaces rarely intersect  
362 in an orthogonal manner and the PCS gradients differ for each  $\Delta\chi$  tensor. To capture a  
363 localisation space, which allows for the experimental uncertainty in the measured PCS data  
364 and fitted  $\Delta\chi$  tensors, we mapped the spatial field of root-mean-squared deviations (RMSD)  
365 between experimental and calculated PCS values and defined the boundary of the localisation  
366 space by a maximal RMSD value. In addition, uncertainties in the  $\Delta\chi$  tensors were propagated  
367 by averaging over the results from 20  $\Delta\chi$ -tensor fits performed with random omission of 20 %  
368 of the backbone PCS data. In the present work, the routine for defining the localisation space  
369 was implemented as a script in the software Paramagpy (Orton et al., 2020). Figure 6 shows  
370 the resulting localisation spaces for the H <sup>$\epsilon$ 1</sup> and H <sup>$\zeta$ 2</sup> atoms of Trp28, using the PCS data  
371 obtained for the three cysteine mutants A53C, N172C and S204C with the C2-Tb<sup>3+</sup> tag as well  
372 as the N172C mutant with the C12-Tb<sup>3+</sup> tag.

373 The localisation spaces found for the H <sup>$\epsilon$ 1</sup> and H <sup>$\zeta$ 2</sup> atoms of Trp28 were clearly different.  
374 Furthermore, the distance between them corresponded closely to the distance expected from  
375 the chemical structure of the indole ring (2.9 Å). The irregular shapes of the localisation spaces  
376 displayed in Fig. 6 purely reflect the relative geometry of the intersecting PCS isosurfaces and  
377 do not take into account any dynamic flexibility of the L3 loop or protein structure. In  
378 particular, the relevant PCS isosurfaces associated with the C2 tag at sites N172C and S204C  
379 intersect at a shallow angle, which leads to the elongated shape of the localisation space for the  
380 Trp28 H <sup>$\zeta$ 2</sup> atom (Fig. S13). For the nitrogen-bound H <sup>$\epsilon$ 1</sup> atom, the localisation space was  
381 restricted further by the additional data obtained with the C12 tag at site N172C (Fig. 6).  
382 Calculating the localisation spaces from the Tm<sup>3+</sup> data yielded very similar results (Fig. S14).  
383 The agreement of the localisation spaces of Trp28 with chain A of the previously published  
384 crystal structure 5EV6 is excellent and they are clearly incompatible with the conformations  
385 observed in chain C of the same structure or in the structure 1DDK (Fig. 1A).

386 Due to close proximity to the C2 tags in the N172C mutant, the largest PCSs were  
387 observed for Trp28 H <sup>$\epsilon$ 1</sup> but, in the absence of captopril, their exact magnitude appeared about

388 0.3 ppm smaller in the [<sup>15</sup>N,<sup>1</sup>H]-HSQC (Fig. S5b) than the NOE-relayed [<sup>13</sup>C,<sup>1</sup>H]-HSQC (Fig.  
389 2B) spectrum. The centre of the localisation space of Trp28 H<sup>ε1</sup> moved to a slightly more open  
390 L3 loop conformation when using the smaller PCS detected in the [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectrum  
391 of the N172C mutant labelled with the C2-Tb<sup>3+</sup> tag. The space still encompassed the  
392 coordinates observed in the structure 5EV6, limiting the significance of this difference in PCS.

393 None of the minor additional cross-peaks observed in any of the sample preparations  
394 could be attributed to alternative conformations of Trp28 either. In particular, the most extreme  
395 conformation observed in the crystal structure 1DDK (green in Fig. 1) predicts PCSs > 1 ppm  
396 for Trp28 H<sup>ε1</sup> in the mutant N172C with C2 tags, but we observed no PCS of this magnitude  
397 for any of the unassigned peaks.

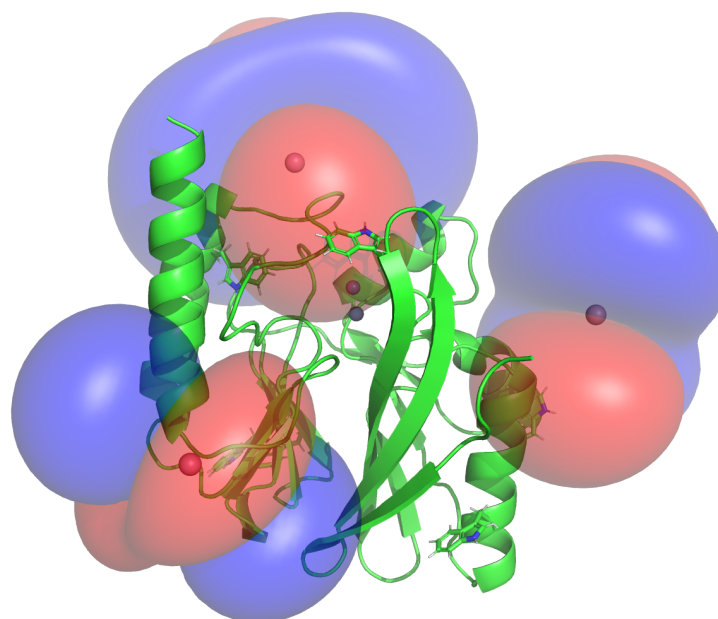
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#### 400 **3.4 Defining the localisation space with one versus two lanthanoid ions in the same tag** 401 **and at the same site**

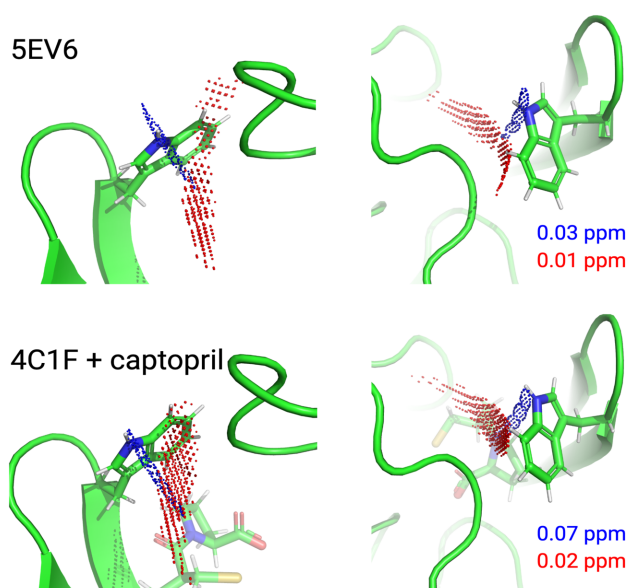
402 Unexpectedly, determining separate localisation spaces from the Tm<sup>3+</sup> and Tb<sup>3+</sup> datasets  
403 yielded more plausible results than when both datasets were used simultaneously. Careful  
404 inspection showed that the close alignment of the  $\Delta\chi$  tensors of the Tm<sup>3+</sup> and Tb<sup>3+</sup> data resulted  
405 in particularly shallow intersection angles of the respective PCS isosurfaces. In calculating the  
406 localisation space of Trp28, the PCS isosurfaces arising from the N172C mutant carried by far  
407 the greatest weight as this site is closer to residue 28 than the sites 53 and 204. Therefore, the  
408 Tm<sup>3+</sup> and Tb<sup>3+</sup> data from the N172C mutant dominated the PCS RMSD calculation and the  
409 intersection between the associated isosurfaces pulled the final localisation space to a  
410 structurally implausible location, which was unstable with respect to small perturbations in  $\Delta\chi$ -  
411 tensor orientations associated with the tensors at site 172. In contrast, considering the Tm<sup>3+</sup> and  
412 Tb<sup>3+</sup> datasets separately allowed the localisation spaces to be determined by the intersections  
413 with PCS isosurfaces from the other sites. The resulting localisation spaces consistently were  
414 compatible with crystal structures.

415



416  
 417 **Figure 5.** PCS isosurfaces of the IMP-1 mutants A53C, N172C and S204C plotted on the  
 418 crystal structure 5EV6. The respective  $\Delta\chi$  tensors were determined from the  $^1\text{H}$  PCSs measured  
 419 of backbone amides. Blue/red isosurfaces correspond to PCSs of  $\pm 1.0$  ppm, respectively,  
 420 generated with C2-Tb $^{3+}$  tags.

421



422  
 423 **Figure 6.** Localisation space of the sidechain of Trp28 defined by the PCSs from tags in the  
 424 IMP-1 mutants A53C, N172C and S204C. The left and right panels display the same results in  
 425 two different orientations. Red and blue points outline localisation spaces determined for the  
 426  $\text{H}^{\epsilon 2}$  and  $\text{H}^{\epsilon 1}$  atoms, respectively. The localisation space of the  $\text{H}^{\epsilon 2}$  atom was defined by the  
 427 PCSs and  $\Delta\chi$  tensors determined for the Tb $^{3+}$ -loaded C2 tags, while the localisation space of  
 428 the  $\text{H}^{\epsilon 1}$  atom was restricted by additional data obtained with C12-Tb $^{3+}$  tag at site N172C. The



429 boundaries of the respective localisation spaces displayed are defined by the PCS RMSD values  
430 indicated in ppm. The top panel depicts the localisation spaces determined for the free protein  
431 plotted on chain A of the crystal structure 5EV6 depicted in two different orientations. The  
432 lower panel depicts the localisation spaces determined in the presence of captopril plotted on  
433 chain A of the crystal structure 4C1F.

434

### 435 **3.5 L3 loop conformation in the presence of L-captopril**

436 Figure 6 shows that, within the uncertainty of the experiments, the localisation space of the  
437 indole sidechain of Trp28 is invariant with respect to the presence or absence of captopril.  
438 Conservation of the L3 loop conformation with and without inhibitor is supported by the close  
439 similarity in all the PCSs observed for Trp28 in the NOE-relayed [<sup>13</sup>C,<sup>1</sup>H]-HSQC spectra (Fig.  
440 2 and 3). In the [<sup>1</sup>H,<sup>15</sup>N]-HSQC spectra of the mutant N172C with C2 tag, however, the PCSs  
441 observed for Trp28 H<sup>ε1</sup> appeared somewhat smaller without than with captopril (Fig. S5b). As  
442 the PCSs of backbone amides were very similar in the absence and presence of the inhibitor  
443 (Fig. S12), this difference in PCS suggests a change in L3 loop conformation, contradicting the  
444 observations made with the selectively <sup>13</sup>C-labelled samples. As discussed above, using the  
445 smaller PCS of Trp28 H<sup>ε1</sup> did not sufficiently change its localisation space in the free protein  
446 to render it incompatible with the coordinates of the structure 5EV6. Therefore, as far as the  
447 data of the <sup>15</sup>N-labelled samples indicate a conformational change of the L3 loop between the  
448 free and bound state, it is small. We attribute the differences in PCSs observed between the  
449 selectively <sup>13</sup>C-labelled and uniformly <sup>15</sup>N-labelled samples to differences in sample  
450 preparation of unknown origin, which are also reflected by different numbers of weak  
451 unassigned cross-peaks (Figs 2, 3, S5 and S6).

452 The cross-peak intensities of the Trp28 sidechain resonances are relatively weak  
453 compared with those of the other tryptophan sidechains, suggesting that Trp28 is subject to  
454 dynamics that broaden its resonances. Its cross-peaks appeared slightly weaker in the presence  
455 than in the absence of inhibitor (Fig. 2 and 3), suggesting a change in dynamics caused by the  
456 inhibitor binding. Previous NMR studies of metallo-β-lactamases reported faster  $R_2(^{15}\text{N})$   
457 relaxation rates of the L3-loop tryptophan sidechain in the presence than in the absence of  
458 inhibitor, which was attributed to dampened dynamics (Huntley et al., 2000; Softley et al.,  
459 2020). In the presence of dynamics, the localisation spaces determined in the present work  
460 must be considered averages that do not report on the amplitude or direction of motions.

461

### 462 **3.6 Localisation spaces of tryptophan side chains other than Trp28**

463 As the tagging sites had been designed to analyse the conformation of the L3 loop, they were  
464 positioned at similar distances from the L3 loop and therefore not optimal for determining  
465 localisation spaces of the other tryptophan residues. Nonetheless, clear differences were  
466 observed in the PCSs of the H<sup>ε2</sup> and H<sup>ε1</sup> atoms (Fig. 2), allowing the separation of the respective  
467 localisation spaces, which also proved to be in excellent agreement with the conformations of  
468 the side-chain indoles of Trp62, Trp124 and Trp147 as found in the crystal structure (Fig. S15),  
469 whereas the data were insufficient to determine the sidechain conformation of Trp176.

470

### 471 **4 Discussion**

472 The L3 loop of metallo-β-lactamases is known to be flexible and, in the specific case of IMP-  
473 1, significantly assists in substrate binding and enzymatic activity (Moali et al., 2003). As the  
474 substrate is sandwiched between the di-zinc site and the L3 loop, it is tempting to think that the  
475 loop opens up for substrate binding and product release while it may be closed during the  
476 enzymatic reaction to hold the substrate and reaction intermediate in place. In contrast, some  
477 of the conformations observed in crystal structures of IMP-1 obtained in the presence and  
478 absence of the inhibitor L-captopril, revealed the loop in almost identical conformations (Brem  
479 et al., 2016). This observation is inconclusive, however, as the L3 loop forms more extensive  
480 intermolecular contacts with neighbouring protein molecules in the crystal lattice than  
481 intramolecular contacts. In addition, other crystal structures observed the loop to move by  
482 almost 3 Å in response to a different inhibitor (Concha et al., 2000). This prompted us to probe  
483 its actual location in the absence of crystal packing forces in solution, a task which is difficult  
484 to tackle by traditional NMR spectroscopic methods that rely on short-range NOEs.

485 Our results show that by furnishing IMP-1 with paramagnetic lanthanoid tags, the  
486 coordinates of the indole sidechain of Trp28, which is a key residue near the tip of the loop,  
487 can be determined with remarkable accuracy even in the free protein, where the available  
488 crystal structures position the L3 loop in a conformation without any direct contacts with the  
489 core of the protein. Indeed, the localisation space identified by the NMR data of the free protein  
490 proved to be sufficiently well-defined to discriminate between different crystal structures of  
491 IMP-1, as well as between different chains in the same asymmetric crystal unit. For example,  
492 the sidechain orientation of Trp28 observed in [Fe<sup>3+</sup>,Zn<sup>2+</sup>]-IMP-1 (4UAM; Carruthers et al.,  
493 2014) proved to be in poor agreement with the PCS data, whereas the data were in full  
494 agreement with chain A in the structure 5EV6 of [Zn<sup>2+</sup>,Zn<sup>2+</sup>]-IMP-1 without inhibitor

495 (Hinchliffe et al., 2016) and chain A in the structure 4C1F with bound L-captopril (Brem et al.,  
496 2016). This highlights the outstanding capacity of PCSs to assess small conformational  
497 differences.

498 The approach of using PCSs for local structure determination is particularly appealing  
499 in the case of difficult proteins such as IMP-1, where the sequence-specific NMR resonance  
500 assignments are incomplete due to line-broadening attributable to motions in the  $\mu\text{s}$ – $\text{ms}$  time  
501 range and additional signals are observed that either stem from protein degradation, misfolding  
502 or alternative conformations in slow exchange with the main structure. Notably, all information  
503 required to establish the  $\Delta\chi$  tensors could be obtained from resolved cross-peaks observed in  
504 sensitive  $^{15}\text{N}$ , $^1\text{H}$ ]-HSQC spectra. Similarly, the localisation information of the tryptophan  
505 sidechains could be obtained from sensitive  $^{13}\text{C}$ - $^1\text{H}$  and  $^{15}\text{N}$ - $^1\text{H}$  correlation spectra. Positioning  
506 the lanthanoid tags relatively far from the substrate binding site avoided direct interference  
507 with the binding loop structure.

508 In the face of additional signals from minor species, site-selective  $^{13}\text{C}$ -labelling of the  
509 tryptophan sidechains was particularly helpful for simplifying the  $^{13}\text{C}$ , $^1\text{H}$ ]-HSQC spectra.  
510 Gratifyingly, this could be achieved by providing suitably labelled indole without having to  
511 synthesise the full amino acid (Maleckis et al., 2021).

512 It has been pointed out previously that the accuracy with which localisation spaces can  
513 be determined is best when PCS isosurfaces intersect in an orthogonal manner (Pintacuda et  
514 al., 2006; Lescanne et al., 2018; Zimmermann et al., 2019). In the present work, we found that,  
515 counterintuitively, the provision of additional data can considerably degrade the accuracy of  
516 the localisation space. This effect arises when PCS isosurfaces intersect at a shallow angle, as  
517 the location of these intersections becomes very sensitive with regard to small errors in the  
518 relative orientations of the underpinning  $\Delta\chi$  tensors. Shallow intersection angles of PCS  
519 isosurfaces are common, when two PCS datasets are from tags and tagging sites that differ only  
520 in the identity of the paramagnetic metal ion in the tag. This situation commonly generates  $\Delta\chi$   
521 tensors of different magnitude and sign, but closely similar orientation (Bertini et al., 2001; Su  
522 et al., 2008; Keizers et al., 2008; Man et al., 2010; Graham et al., 2011; Joss et al., 2018;  
523 Zimmermann et al., 2019). Therefore, while the use of  $\text{Tm}^{3+}$  and  $\text{Tb}^{3+}$  tags is helpful for  
524 assigning the cross-peaks in the paramagnetic state, more robust results are obtained by using  
525 only one of these data sets for calculating the localisation space. Good localisation spaces were  
526 thus obtained by using only PCSs measured for  $\text{Tb}^{3+}$  tags (Fig. 6) or only PCSs measured for  
527  $\text{Tm}^{3+}$  tags (Fig. S13). In contrast, however, very different tags attached at the same site, such

528 as the C2 and C12 tags installed in the mutant N172C, produced independent  $\Delta\chi$ -tensor  
529 orientations and therefore contributed positively to localising the Trp28 H<sup>ε1</sup> atom.

530 In principle it is inappropriate to explain a set of PCSs by a single  $\Delta\chi$  tensor, if they are  
531 generated by a lanthanoid tag attached via a flexible linker, which positions the lanthanide ions  
532 at variable coordinates relative to the protein. In this situation, fitting a single  $\Delta\chi$  tensor  
533 amounts to an approximation. The effective  $\Delta\chi$  tensors obtained in this way, however, can  
534 fulfill the PCSs remarkably well (Shishmarev and Otting, 2013), as illustrated by the low  $Q$   
535 factors obtained in this work (Fig. 4), and the localisation spaces obtained for the tryptophan  
536 sidechains are correspondingly well defined.

537 The accuracy, with which localisation spaces can be determined, further depends on the  
538 accuracy with which PCSs can be measured (which critically depends on the reproducibility of  
539 the sample conditions between the paramagnetic and diamagnetic states), the accuracy of the  
540 protein structure used to fit the  $\Delta\chi$  tensors and the angle with which PCS isosurfaces of different  
541 tensors intersect. To take into account the uncertainties associated with the PCS isosurfaces, it  
542 is useful to think of each of them individually as a shell of a certain thickness (rather than a  
543 surface) that represents a compatible localisation space. Two shells of a given thickness share  
544 a smaller common space if they intersect orthogonally than if they intersect at a shallow angle.

545 The present work employed <sup>1</sup>H PCSs only, although PCSs were also observed in the  
546 indirect dimensions of the [<sup>13</sup>C,<sup>1</sup>H]-HSQC and [<sup>15</sup>N,<sup>1</sup>H]-HSQC spectra. We made this choice  
547 because the paramagnetic tags give rise to weak molecular alignments in the magnetic field,  
548 which result in residual anisotropic chemical shifts (RACS). The effect is unimportant for <sup>1</sup>H  
549 spins but significant for nuclear spins with large chemical shift anisotropy (CSA) tensors such  
550 as backbone nitrogens and aromatic carbons. Correcting for the RACS effect is possible with  
551 prior knowledge of the CSA tensors and bond orientations (John et al., 2005). We therefore  
552 chose not to measure PCSs of the heteronuclear spins in favour of improving sensitivity by  
553 accepting a lower spectral resolution in the indirect dimensions.

554 Finally, the C12 tag was designed specifically with the intent to produce a more rigid  
555 tether to the protein than the C2 tag, but this did not result in larger  $\Delta\chi$  tensors (Table S7) and  
556 the NMR spectra of IMP-1 N172C displayed more heterogeneity with the C12 than the C2 tag,  
557 suggesting that the shorter and more rigid tether combined with the fairly high molecular  
558 weight of the cyclen-lanthanoid complex may have perturbed the protein structure to some  
559 degree.

560

## 561 **5 Conclusion**

562 The current work illustrates how  $\Delta\chi$  tensors from paramagnetic lanthanoid ion tags installed at  
563 three different sites of the protein can be used to probe the conformation of a selected site in  
564 solution in unprecedented detail, provided the structure of most of the protein is known with  
565 high accuracy to allow fitting effective  $\Delta\chi$  tensors of high predictive value. Importantly,  
566 however, the method is easily compromised, if two PCS isosurfaces intersect at a shallow angle  
567 as, in this situation, inaccuracies in  $\Delta\chi$  tensor determinations have an outsized effect on  
568 positioning the localisation spaces defined by the PCSs. Therefore, improved results were  
569 obtained by not combining data from different metal ions bound to otherwise identical tags and  
570 tagging sites. In the present work, simplifying the NMR spectrum of tryptophan residues by  
571 site-selective isotope labelling proved to be of great value for sufficiently improving the  
572 spectral resolution to allow assigning the labelled resonances solely from PCSs and PREs. The  
573 strategy opens a path to detailed structural investigations of proteins of limited stability like  
574 IMP-1, for which complete assignments of the NMR spectrum are difficult to obtain.

575

576

577 **Code and data availability.** NMR spectra and pulse programs are available at  
578 <https://doi.org/10.5281/zenodo.5518294>. The script for calculating localisation spaces is  
579 available at <https://doi.org/10.5281/zenodo.3594568> and from the GitHub site of Paramagpy.

580

581 **Supplement.** The supplement related to this article is available online at: <https://doi.org/...>

582

583 **Author contributions.** GO initiated the project and edited the final version of the manuscript.  
584 HWO wrote NMR pulse programs and software to calculate localisation spaces and performed  
585 the  $\Delta\chi$  tensor and structure analysis. IDH made labelled protein samples, recorded and assigned  
586 NMR spectra, measured PCSs and wrote the first version of the manuscript. AM synthesised  
587 the isotope-labelled indole. SJ made  $^{15}\text{N}$ -labelled protein mutants with C2 tags and assigned  
588 PCSs of backbone amides. MS synthesized C2 tags with different lanthanoid ions. CB, LT and  
589 SB synthesized C12 tags with different lanthanoid ions.

590

591 **Competing interests.** The authors declare that they have no conflict of interest.

592

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