

Fe³⁺ in the Dinuclear Metallo-β-Lactamase IMP-1 Located by Pseudocontact Shifts**

Thomas J. Carruthers, Paul D. Carr, Choy-Theng Loh, Colin J. Jackson, and Gottfried Otting*

Abstract: Heterodinuclear metallo-enzymes are an important class of metallo-proteins, but determining the location of the different metal ions can be difficult. Here we present a new nuclear magnetic resonance (NMR) method that uses pseudocontact shifts (PCS) to achieve this without assumptions about the coordinating ligands. The approach is illustrated with the dinuclear [FeZn] complex of IMP-1, which is a prototypical metallo-β-lactamase (MβL) that confers resistance to β-lactam antibiotics. Results from single crystal X-ray diffraction were compromised by degradation during crystallization. With [GaZn]-IMP-1 as diamagnetic reference, the PCSs unambiguously identified the iron binding site in fresh samples of [FeZn]-IMP-1, even though the two metal centers are less than 3.8 Å apart and the iron is high-spin Fe³⁺, which produces only small PCSs. [FeZn]-MβLs may be important drug targets, as [FeZn]-IMP-1 is enzymatically active and readily produced in the presence of small amounts of Fe³⁺.

It is estimated that up to half of all proteins contain metal ion cofactors.^[1] While protein:metal ion stoichiometry can readily be determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) or atomic absorption spectroscopy, the precise location of the metal ions is much more difficult to determine, especially in heterodinuclear active sites. Two metal ions in close proximity are a common motif in metallo-enzymes, and zinc and iron are among the most abundant transition metal ions in metallo-enzymes.^[2] Established methods such as extended X-ray absorption fine structure (EXAFS) and electron paramagnetic resonance (EPR) data provide information on the metal coordination sphere. To link these data to specific metal binding sites, however, requires independent information about the identity and, often, geometry of the amino acid side chains constituting the metal binding sites. Furthermore, these methods can easily be compromised by 10% of an impurity. X-ray crystallography provides structures at atomic resolution but requires single crystals, which can be challenging to obtain and often require highly specific crystallization conditions. Even then, unambiguous identification of metal ion positions requires

anomalous X-ray scattering experiments, which can be difficult to interpret when a dinuclear site is capable of harboring different metals with different occupancies. Using NMR spectroscopy, we show that pseudocontact shifts (PCS) generated by a paramagnetic ion contain sufficient information to pinpoint the location of the paramagnetic center with an accuracy of a few Ångströms even for a difficult nucleus like high-spin Fe³⁺ that generates large paramagnetic relaxation enhancements (PRE) and only small PCSs.^[3] The method requires knowledge of the structure of the protein backbone but makes no assumptions about the metal-coordinating environment. Using the 25 kDa MβL IMP-1 as an example, we established that the enzyme readily acquires an [FeZn] heterodinuclear active site, identified the iron as high-spin Fe³⁺, and located it at one of the two metal sites with clear discrimination between the two sites, which are within 3.8 Å of each other.

IMP-1 is the prototype of over 40 members of the class B1 of MβLs that pose an increasing health risk by generating broadband resistance against β-lactam antibiotics including carbapenems.^[4] Located on mobile genetic elements, IMP-1 has been found in hospital strains of different pathogenic bacteria.^[5] Available crystal structures of IMP-1 have been modeled with two zinc atoms in the active site.^[6,7] The metal ions are essential for the activity and structural integrity of the enzyme.

We found that IMP-1 produced by *in vivo* overexpression in *Escherichia coli* in the presence of small amounts (25 μM) of FeCl₃ in the medium takes up equimolar amounts of iron and zinc as determined by ICP-OES. Even a ten-fold excess of zinc did not entirely suppress the uptake of iron. [FeZn]-IMP-1 was enzymatically active (Table S1).

To establish the binding site of the iron and the distribution of iron and zinc between the two metal sites M₁ and M₂, we initially set out to determine the single crystal X-ray structure. Notably, however, the single crystals were difficult to grow. The final successful protocol required several weeks for crystal growth, including an incubation period at 37 °C and the inclusion of high concentrations of the metal ion chelator sodium citrate, leading to decreased metal occupancies. While the overall structure of the protein (Protein Data Bank accession code 4UAM) was the same as that reported previously for the wild-type [ZnZn] enzyme,^[6] the electron density map also indicated that the crystallization conditions had resulted in partial oxidation of the cysteine residue in the active site to sulfinate, with uncertain impact on the metal binding preferences of the two sites (Figure 1, A and B). The iron ion was detected at the more buried M₁-site by using anomalous X-ray scattering, collecting data above the Fe-K and Zn-K edges (Figure 1, C to F).^[8]

[*] T. J. Carruthers, Dr. P. D. Carr, Dr. C.-T. Loh, Dr. C. J. Jackson, Prof. G. Otting
Research School of Chemistry
Australian National University
Canberra, ACT 0200 (Australia)
E-mail: gottfried.otting@anu.edu.au

[**] Financial support by the Australian Research Council is gratefully acknowledged. We thank A/Prof. Gerhard Schenk and Dr. Peter Vela for the pET-47b(+) expression vector with the *bla*_{IMP1} gene, Dr. Ruhui Qi for help with cloning, Ms. Ellen Cliff, Mr. Jia Jiunn Chew, Mr. Jeremy Neeman, and Ms. Katey Dugdale for help with the preparation of selectively ¹⁵N-labeled samples, Mrs. Viki Withers for help with ICP-OES measurements, Prof. Thomas Huber and Dr. Thitima Urathamakul for help with mass spectrometry and the staff of the Australian Synchrotron for assistance with the X-ray experiments.

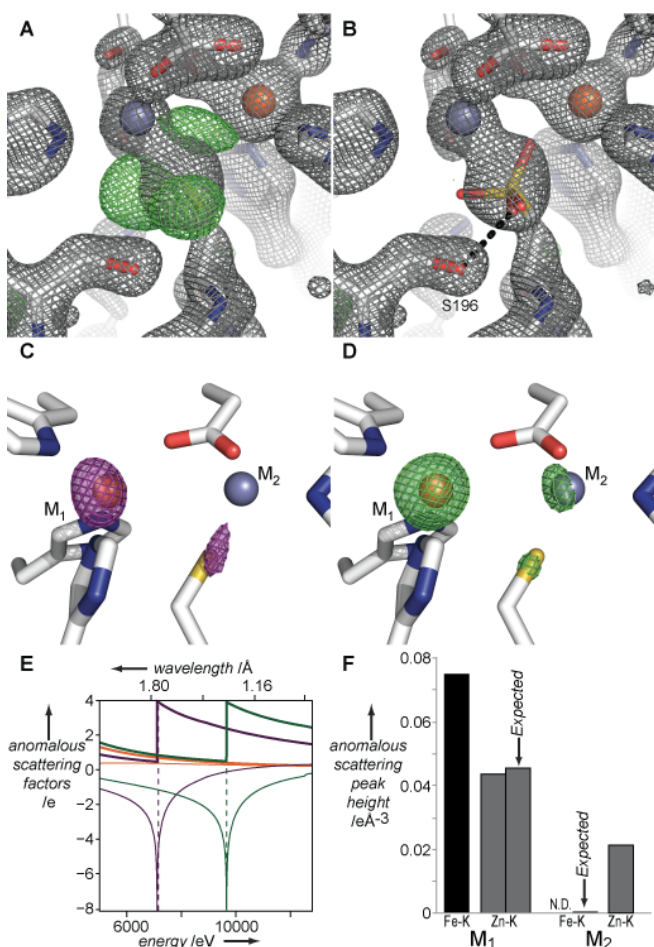


Figure 1. Active site of [FeZn]-IMP-1 determined by X-ray crystallography. The 1.8 Å structure crystallized with a citrate molecule bound to the active site (not shown). The metals in the M₁ and M₂ sites are coordinated by three histidines, and a histidine, aspartate, and cysteine, respectively and indicated by orange and grey spheres, respectively. The active site residues are represented by sticks. The electron densities of the crystal structure indicated about 80% occupancy of the M₁ site with Fe³⁺ and 40% occupancy of the M₂ site with Zn²⁺. (A) 2mFo-DFc density map (grey mesh at 2.5 σ) of the active site. The mFo-DFc difference map (green mesh at 4 σ) indicated three lobes of positive density around the sulfur of Cys158 indicating partial oxidation. (B) Same as (A) except that Cys158 was modeled with two additional conformers of cysteinesulfonic acid, each with a hydrogen bond to Ser196. (C) Anomalous scattering map measured above the Fe-K edge, highlighting the location of iron. (D) Same as (C), except that the scattering map was measured at the Zn-K edge, highlighting the locations of iron and zinc. The contour levels were chosen to depict the similar level of scattering on the sulfur atom of Cys158. (E) Wavelengths used for the anomalous scattering experiments highlighted by dashed lines in a plot of the theoretical anomalous scattering factors, f' (light lines) and f'' (heavy lines), for iron (purple), zinc (green) and sulfur (orange).^[9] (F) Anomalous scattering peak heights at the M₁ and M₂ sites. The experimental anomalous scattering peak height measured above the Zn-K edge for the M₁ site closely matches expectations if the M₁ site only contains iron.^[9] Due to low metal occupancy at the M₂ site, no anomalous scattering signal was detected at the M₂ site above the Fe-K edge, matching expectations if the M₂ site only contains zinc.

To validate the location of the iron ion in fresh solutions of IMP-1, we performed NMR experiments in the absence of citrate. Compared with [ZnZn]-IMP-1, ¹⁵N-HSQC cross-peaks of amides near the active site were absent from the NMR spectra of

[FeZn]-IMP-1 and cross-peaks of more remote amides were weakened due to PREs (Figure 3A). The PRE data were insufficiently accurate, however, to discriminate between the two metal sites (Figure S8). Discrimination between the metal sites was achieved by measuring PCSs, using samples of [FeZn]-IMP-1 and its diamagnetic analogue, [GaZn]-IMP-1.

The sequence specific resonance assignment of IMP-1 was performed using uniformly ¹⁵N/¹³C and ¹⁵N/¹³C/²H labeled samples of the [ZnZn] complex, which were prepared *in vivo* with a large excess of zinc in the medium. The backbone resonance assignments of the diamagnetic complex were readily transferred to the [FeZn] complex, because the chemical shifts differed only little between the two forms of the protein. The resonance assignments were submitted to the BioMagResBank with the accession codes 25063 and 25080. For PCS measurements, the [FeZn] and [GaZn] complexes were prepared by cell-free protein synthesis^[10] with selectively ¹⁵N-labeled amino acids and controlled supply of metal ions.

Most paramagnetic metal ions are associated with a magnetic susceptibility tensor that is anisotropic and therefore induces PCSs in the protein to which the metal ion is bound. The PCSs arise from through-space interactions between the nuclear spins and the paramagnetic center. They depend on the position of the metal ion and the orientation of the $\Delta\chi$ tensor, which describes the anisotropy of the magnetic susceptibility, relative to the protein. The PCS, $\Delta\delta^{\text{PCS}}$, of a nuclear spin is measured in ppm and described by^[11]

$$\Delta\delta^{\text{PCS}} = \frac{1}{12\pi r^3} \left[\Delta\chi_{\text{ax}} (3\cos^2\theta - 1) + \frac{3}{2} \Delta\chi_{\text{rh}} \sin^2\theta \cos 2\phi \right] \quad (1)$$

where r , θ , and ϕ are the polar coordinates of the nuclear spin with respect to the principal axes of the $\Delta\chi$ tensor, the magnitude of which is characterized by the axial and rhombic components $\Delta\chi_{\text{ax}}$ and $\Delta\chi_{\text{rh}}$, respectively. A complete description of the $\Delta\chi$ tensor is given by eight parameters (the x , y , and z coordinates of the metal ion, the α β γ Euler angles describing the orientation of the $\Delta\chi$ tensor relative to the protein coordinates, and $\Delta\chi_{\text{ax}}$ and $\Delta\chi_{\text{rh}}$). Therefore, measurement of the PCSs of at least eight different spins allows determination of the $\Delta\chi$ tensor, including the location of its origin.^[11,12]

PCSs were measured as the amide proton chemical shifts observed in [FeZn]-IMP-1 minus the corresponding chemical shifts in a diamagnetic sample. All PCSs were very small (< 0.12 ppm). [ZnZn]-IMP-1 turned out to be inadequate as a diamagnetic reference, which we attribute to small changes in chemical shifts caused by different oxidation states of the metal (Fe³⁺ versus Zn²⁺). The inadequacy of [ZnZn]-IMP-1 as a diamagnetic reference was readily apparent in ¹⁵N-HSQC spectra of selectively ¹⁵N-labeled samples. For example, the ¹⁵N-HSQC cross-peaks were frequently displaced in different directions in the ¹⁵N and ¹H dimensions (Figure 2 Top). As the nitrogen and proton of each amide group are very close compared with their distance from the paramagnetic center, similar PCSs are expected for both nuclei.^[13] This was indeed the case, when we compared [FeZn] with [GaZn] samples (Figure 2 Bottom), suggesting that the iron is in the oxidation state 3+, where gallium and iron have very similar ionic radii.

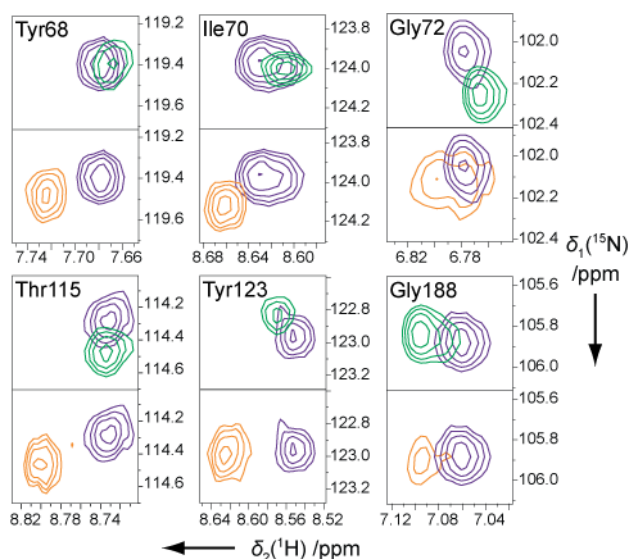


Figure 2. Selected spectral regions from ^{15}N -HSQC spectra of ^{15}N -glycine, ^{15}N -isoleucine, ^{15}N -threonine, or ^{15}N -tyrosine labeled samples of [FeZn], [ZnZn], and [GaZn] complexes of IMP-1. The spectra were recorded of 50 μM solutions of IMP-1 in 20 mM MES buffer, pH 6.5, at 37 $^{\circ}\text{C}$ on a Bruker 800 MHz NMR spectrometer. (Top) Superimposition of the ^{15}N -HSQC spectra of the [ZnZn] (green) and the [FeZn] complex (purple). (Bottom) Superimposition of the ^{15}N -HSQC spectra of the [GaZn] (orange) and the [FeZn] complex (purple).

A total of 48 PCSs were measured using [GaZn]-IMP-1 as the diamagnetic reference. Using the PCSs as input to the program PyParaTools,^[14] we fitted $\Delta\chi$ tensors to the crystal structure of the [FeZn] complex. A systematic grid search for the best location of the paramagnetic center revealed the M₁ site as the best fitting site (Figure 3).

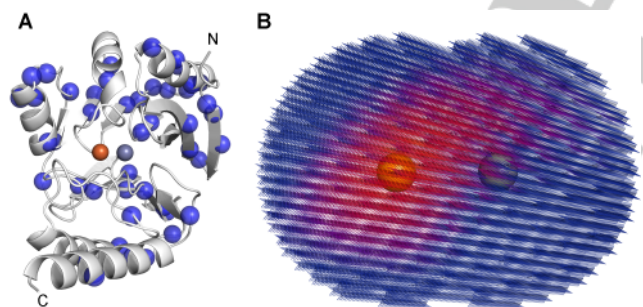


Figure 3. Identification of the paramagnetic center by PCSs. The M₁ and M₂ metal sites in the active site of IMP-1 are depicted as orange and grey spheres, respectively. (A) Ribbon drawing of the crystal structure of [FeZn]-IMP-1. Blue spheres highlight the backbone amides of all residues for which a PCS could be measured. Due to PREs all these amides are at least 11.5 Å from the M₁ site. (B) Graphical representation of a grid search around the active site for metal positions with minimal root mean squared deviation (RMSD) between experimental and back-calculated PCS values. The grid points are depicted as crosses that are colored by RMSD values from red (0.023 ppm) to blue (0.028 ppm or greater). The best results cluster around the M₁ site.

To assess the oxidation and spin state of the iron, we measured the total paramagnetic susceptibility of [FeZn]-IMP-1 by the experiment by Evans *et al.*^[15] The result indicated a high-

spin state with five unpaired electrons, confirming the oxidation state of the iron as Fe^{3+} (Figure S12).

To the best of our knowledge, this is the first time that PCSs have been used for the purpose of accurately locating a Fe^{3+} ion in a protein. High-spin Fe^{3+} is known to be associated with a large but mostly isotropic magnetic susceptibility tensor, resulting in large PREs and small PCSs.^[3] Nonetheless, it is much easier to measure accurate PCSs than PREs, which can in part be attributed to the relative insensitivity of PCSs with regard to protein motions and non-specific intermolecular effects. To measure the small PCSs associated with Fe^{3+} , however, a suitable diamagnetic reference proved to be of critical importance. Ga^{3+} is an established structural analogue of Fe^{3+} in dinuclear metallo-enzymes.^[16] In the case of IMP-1, the [GdZn] complex is, in subtle ways, non-isostructural with the [ZnZn] complex, as chemical shift differences were observed far from the metal site.

The present work illustrates the challenge associated with determining the identity, location, and oxidation states of transition metal ions in dinuclear enzymes, as the metal ions may be distributed between the sites with heterogeneous populations (e.g. [FeZn] versus [ZnFe]). The NMR approach presented here thus adds a uniquely powerful tool to the analysis of metallo-enzymes. In particular, NMR spectroscopy would readily detect the presence of any significant heterogeneity in metal composition or location. By using ^{13}C or ^{15}N direct detection, the accuracy with which the paramagnetic center can be located could be enhanced further, provided that the protein is soluble in high concentration.^[17]

It is intriguing to speculate on the biological significance of the iron-zinc promiscuity in IMP-1. It is well known that iron stimulates bacterial growth and that bacteria are particularly sensitive to β -lactam antibiotics during cell division. Therefore, maintaining active enzyme by substituting iron for zinc could provide an advantage under conditions when zinc is scarce but bacterial growth is promoted by iron.

Although mixed-metal active sites with Fe^{3+} are common in dinuclear metallohydrolases,^[18] and a glyoxalase II from the wider metallo- β -lactamase superfamily of proteins has been shown to be active with a dinuclear [FeZn] center containing Fe^{3+} ,^[19] all dinuclear M β LS from the subclasses B1-B3 have so far been assumed to act predominantly as pure zinc enzymes.^[20] The only [FeZn]-M β L investigated to date in some detail has been L1, a subclass B3 M β L, which was shown to maintain enzymatic activity in the presence of substoichiometric amounts of iron.^[21] The ready formation and significant enzymatic activity of the [FeZn] complex of IMP-1 are thus likely features also of many other members of the B1 subclass of M β LS, which is the clinically most relevant subclass.^[4] The ready uptake of Fe^{3+} poses a new challenge for drug development.

Keywords: dinuclear metallo-enzymes • IMP-1 • metallo- β -lactamase • NMR spectroscopy • pseudocontact shifts

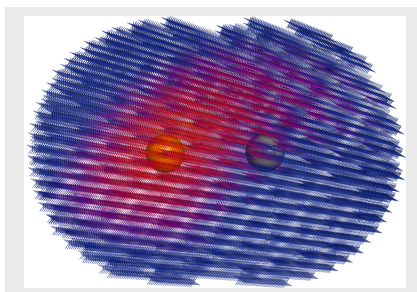
[1] K. J. Waldron, J. C. Rutherford, D. Ford, N. J. Robinson, *Nature* **2009**, 460, 823–830.

- [2] N. Sträter, W. N. Lipscomb, T. Klabunde, B. Krebs, *Angew. Chem.* **1996**, *108*, 2158–2191; *Angew. Chem. Int. Ed.* **1996**, *35*, 2024–2055.
- [3] I. Bertini, C. Luchinat, *Coord. Chem. Rev.* **1996**, *150*, 1–256.
- [4] W.-H. Zhao, Z.-Q. Hu, *Crit. Rev. Microbiol.* **2011**, *37*, 214–226.
- [5] T. R. Walsh, M. A. Toleman, L. Poirel, P. Nordmann, *Clin. Microbiol. Rev.* **2005**, *18*, 306–325.
- [6] a) N. O. Concha, C. A. Janson, P. Rowling, S. Pearson, C. A. Cheever, B. P. Clarke, C. Lewis, M. Galleni, J.-M. Frère, D. J. Payne, J. H. Bateson, S. S. Abdel-Meguid, *Biochemistry* **2000**, *39*, 4288–4298; b) J. H. Toney, G. G. Hammond, P. M. D. Fitzgerald, N. P. Sharma, J. M. Balkovec, G. P. Rouen, S. H. Olson, M. L. Hammond, M. L. Greenlee, Y.-D. Gao, *J. Biol. Chem.* **2001**, *276*, 31913–31918; c) H. Kurosaki, Y. Yamaguchi, T. Higashi, K. Soga, S. Matsueda, H. Yumoto, S. Misumi, Y. Yamagata, Y. Arakawa, M. Goto, *Angew. Chem.* **2005**, *117*, 3929–3932; *Angew. Chem. Int. Ed.* **2005**, *44*, 3861–3864; d) H. Kurosaki, Y. Yamaguchi, H. Yasuzawa, W. Jin, Y. Yamagata, Y. Arakawa, *ChemMedChem* **2006**, *1*, 969–972.
- [7] a) Y. Yamaguchi, T. Kuroki, H. Yasuzawa, T. Higashi, W. Jin, A. Kawanami, Y. Yamagata, Y. Arakawa, H. Kurosaki, *ChemBioChem* **2005**, *12*, 1979–1983; b) L. B. Horton, S. Shanker, R. Mikulski, N. G. Brown, K. J. Phillips, E. Lykissa, B. V. Venkataram Prasad, T. Palzkill, *Antimicrob. Agents Chemother.* **2012**, *56*, 5667–5677.
- [8] C. J. Jackson, P. D. Carr, H.-K. Kim, J.-W. Liu, P. Herrald, N. Mitić, G. Schenk, C. A. Smith, D. L. Ollis, *Biochem. J.* **2006**, *397*, 501–508.
- [9] E. A. Merritt, <http://skuld.bmsc.washington.edu/scatter>
- [10] K. Ozawa, M. J. Headlam, P. M. Schaeffer, B. R. Henderson, N. L. Dixon, G. Otting, *Eur. J. Biochem.* **2004**, *271*, 4084–4093.
- [11] I. Bertini, C. Luchinat, G. Parigi, *Prog. NMR Spectrosc.* **2002**, *40*, 249–273.
- [12] C. Schmitz, M. J. Stanton-Cook, X.-C. Su, G. Otting, T. Huber, *J. Biomol. NMR* **2008**, *41*, 179–189.
- [13] M. John, G. Otting, *ChemPhysChem* **2007**, *8*, 2309–2313.
- [14] M. Stanton-Cook, X.-C. Su, G. Otting, T. Huber, <http://comp-bio.anu.edu.au/mscook/PPT>.
- [15] a) D. F. Evans, *J. Chem. Soc.* **1959**, 2003–2005; b) S. K. Sur, *J. Magn. Reson.* **1989**, *82*, 169–173.
- [16] a) M. Merckx, B. A. Averill, *Biochemistry* **1998**, *37*, 8490–8497; b) S. J. Smith, A. Casellato, K. S. Hadler, N. Mitić, M. J. Riley, A. J. Bortoluzzi, B. Szpoganicz, G. Schenk, A. Neves, L. R. Gahan, *J. Biol. Inorg. Chem.* **2007**, *12*, 1207–1220.
- [17] C. Caillet-Saguy, M. Delepiere, A. Lecroisey, I. Bertini, M. Piccioli, P. Turano, *J. Am. Chem. Soc.* **2006**, *128*, 150–158.
- [18] N. Mitić, S. J. Smith, A. Neves, L. W. Guddat, L. R. Gahan, G. Schenk, *Chem. Rev.* **2006**, *106*, 3338–3363.
- [19] O. Schilling, N. Wenzel, M. Naylor, A. Vogel, M. Crowder, C. Makaroff, W. Meyer-Klaucke, *Biochemistry* **2003**, *42*, 11777–11786.
- [20] C. Bebrone, *Biochem. Pharmacol.* **2007**, *74*, 1686–1701.
- [21] a) Z. Hu, T. S. Gunasekera, L. Spadofora, B. Bennett, M. W. Crowder, *Biochemistry* **2008**, *47*, 7947–7953. b) Z. Hu, G. Periyannan, B. Bennett, M. W. Crowder, *J. Am. Chem. Soc.* **2008**, *130*, 14207–14216.

Entry for the Table of Contents

COMMUNICATION

Pseudocontact shifts measured by nuclear magnetic resonance spectroscopy are shown to present a unique tool for accurate determination of the location of paramagnetic metal ions in the active site of dinuclear metallo-enzymes. The method identified and characterized a [FeZn]-complex of the metallo- β -lactamase IMP-1.



Thomas J. Carruthers, Paul D. Carr,
Choy-Theng Loh, Colin J. Jackson, and
Gottfried Otting*

Page No. – Page No.

**Fe³⁺ in the Dinuclear Metallo- β -
Lactamase IMP-1 Located by
Pseudocontact Shifts**