Supplementary Material (ESI) for Chemical Communications

^{68}Zn isotope exchange experiments reveal an unusual kinetic lability of the metal ions in the di-zinc form of IMP-1 metallo-β-lactamase

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Electronic Supplementary Information

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Experimental details

General

Unless otherwise stated, all reagents were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO). All buffers were prepared using MilliQ water (Millipore, Bedford) and treated with Chelex resin (NH_4^+ form). To minimize contamination by residual trace elements, all containers (only plastic ware was used in these studies) and pipet tips were treated with HNO_3 (1% v/v) for 24 h and rinsed acid free with MilliQ water prior to use. Zn-68 (metal) was purchased from Pennwood Chemicals (Great Neck, NY). An aqueous stock solution of Zn-68 was prepared by dissolving the metal in 6N HCl and subsequent dilution with MilliQ water to a final concentrations of 200 mM with respect to both Zn(-68)Cl_2 and HCl. The concentration of Zn^{2+} in the stock solution was ascertained by spectrophotometric analysis using PAR (see below) and ITV-ICP-MS.

Purification of IMP-1

IMP-1 was expressed in *Escherichia coli* BL21(DE3) carrying pCIP4 (kindly provided by Dr. Moreno Galleni, Laboratoire d'Enzymologie & Centre d'Ingénierie des Protéines, Institut de Chimie, Université de Liège, Belgium) and purified as documented by Laraki et al.[1] The homogeneity of the protein was confirmed by SDS-polyacrylamide gel electrophoresis. Gel filtration of IMP-1 (10-20 μM) revealed the enzyme to elute solely at an effluent volume corresponding to that of the monomeric species, indicating that protein aggregation is unlikely at the concentration used in the exchange experiments. The presence of two Zn^{2+} in purified IMP-1 was established by ESI-MS under non-denaturing conditions,[2] as well as by ITV-ICP-MS. The concentration of the enzyme was determined from its absorbance at 280 nm (ε = 44,380 M^{-1} cm^{-1}). In these studies, IMP-1 (10-20 μM) in HEPES (50 mM, pH 7.3) containing $ZnSO_4$ (50 μM) served as the stock solution.

Assay procedures

Metallo-β-lactamase activity was determined spectrophotometrically, using IMP-1 at a concentration of 4 nM, and nitrocefin (Oxoid, Basingstoke, U.K.) as the chromophoric substrate at a concentration of 0.1 mM.[3] The inhibition of MBL activity by Zn^{2+} was

monitored by including $ZnCl_2$ (at various concentrations) in the assay medium. The chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) was employed in the spectrophotometric determination of Zn^{2+}.[2] For the determination of extraneous Zn^{2+} in protein preparations subsequent to gel filtration (see below), advantage was taken of the faster rate of complexation of PAR with free Zn^{2+} relative to that with protein-bound metal ions.[2]

Sample Preparation

The buffer medium (50 mM HEPES, pH 7.3) of the IMP-1 stock solution was switched to NH_4OAc (25 mM, pH 7) either by dialysis or by gel filtration on Biogel P4 resin (super fine, BioRad), since only volatile buffer systems are compatible with both ITV-ICP-MS and ESI-MS measurements. It is important to note that dialysis of IMP-1 against NH_4OAc buffer for 16 h did not result in a change in the protein's metal content (determined by ESI-MS). This observation indicates that NH_4OAc is not inducing the facile exchange of Zn^{2+} in IMP-1.

In a typical experiment, in a final volume of 0.3 mL, IMP-1 (7 μM) was treated with $Zn(-68)Cl_2$ (140 μM) to achieve a tenfold excess of the metal ion over that present in the protein. The reaction was allowed to proceed at 298 K. At desired intervals, aliquots (50 μL) of the reaction mixture were subjected to rapid gel filtration (on P4 resin; with 25 mM NH_4OAc (pH 7) buffer serving both as equilibration and elution media) to recover the protein free of extraneous Zn^{2+}. Fractions (50 μL) collected were assessed for the presence of protein (catalytic activity using nitrocefin as substrate) and for free Zn^{2+} ions (with PAR). Samples which were positive for enzymatic activity and negative with respect to the PAR assay (indicating the successful separation of extraneous metal ions from the protein) were analyzed by ITV-ICP-MS.

Exchange with Cd(II)

In a final volume of 0.5 mL, IMP-1 (10 μM in HEPES buffer) was diluted (1:10) in NH_4OAc (10 mM, pH 7) containing BSA (0.25 mg). Following introduction of $CdCl_2$ (final concentration: 100 μM) and either 5 or 40 min of incubation at 298 K, extraneous metal ions were removed by rapid gel filtration on P4 resin, with 25 mM NH_4OAc (pH 7)

buffer serving both as equilibration and elution media. The final sample solution containing ca. 5 μM of IMP-1 was analyzed by ESI-MS under non-denaturing conditions.

Inductively coupled plasma (ICP)-mass spectrometry

The isotopic ratio of ^{68}Zn and ^{64}Zn ($R^{68/64}$) in IMP-1 after treatment of the enzyme with Zn-68 was determined by inductively coupled plasma mass spectrometry (ICP-MS), using a Sciex Elan 250 quadrupole mass spectrometer (PE-SCIEX, Concord, Ontario, Canada) coupled to an in-house built in-torch vaporization (ITV) sample introduction system (Figure S1). Measurements were performed in dual-mass mode for the detection of ^{64}Zn and ^{68}Zn isotopes (see Table S1 for detailed ITV-ICP-MS operating conditions). For external calibration of the ICP-MS, standard solutions (3, 10, 30, 100 μg/L) were prepared by serial dilution of a 1000 mg/L Zn-standard solution (SCP Science, Quebec, Canada) with MilliQ water. Solutions without added Zn^{2+} served as blanks. Calibration curves were constructed by linear regression. Samples (3 μL) were placed onto a sample holder (rhenium coiled filament) prior to vaporization (Figure S2). All samples were measured in quadruplicate. The ratio of ^{68}Zn and ^{64}Zn ($R^{68/64}$) was deduced from the integrated signals and served to determine the *tracer-to-tracee* ratio (ttr) and the average number (N) of Zn^{2+} exchanged in IMP-1.

Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS studies were performed in positive ion mode on a Micromass Q-Tof Ultima™ Global mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray electrospray ionization source. The instrument was calibrated over a mass-to-charge ratio (m/z) range of 600-3200 as previously documented[2], using a mixture of bovine trypsinogen and horse heart myoglobin (10 pmol/μL each in water/acetonitrile [50:50 (v/v)] containing 0.1 % formic acid). Spectra of IMP-1 exposed to $CdCl_2$ under non-denaturing (native) aqueous conditions were obtained after introduction of the protein sample into the ESI source from a syringe pump (Micromass) at a flow rate of 6 μL/min. Spectra were recorded using the following ESI conditions: source temperature (110 °C), desolvation temperature (200 °C), nitrogen desolvation gas flow (450 L/hr), cone voltage (100 V). Data was accumulated at a rate of 2.0 s per spectrum, and 100 such

scans were combined and baseline subtracted. Deconvolution of the raw data was achieved using the MaxEnt program, a component of the Micromass MassLynx software package. The relative abundances of the diZn and ZnCd hybrid species were estimated from the peak heights of both the centered raw data at two charge state distributions (+10 and +11) and the centered deconvoluted peaks (both methods agreed within ± 2 %).

Tracer-to-tracee ratio (ttr) formalism

Stable isotopes of metal ions have been widely employed in the study of trace element metabolism in humans and animal models.[4] In the majority of cases, one (or two) isotopically enriched trace element solutions are administered orally or intravenously (or both), and the amount of each isotope in the system under investigations (e.g. tissue, urine) is determined by analytical techniques such as ICP-MS.

Since naturally abundant and isotopically enriched solutions of trace element are always mixtures of isotopes (except for those elements for which only one stable nuclide exists; e.g. ^{55}Mn, ^{59}Co) the conversion of experimentally determined isotope ratios into readily interpretable data is not straightforward. Thus, the *tracer-to-tracee* ratio (ttr) formalism[5] has been introduced to permit the expression of stable isotope data from tracer experiments in a more transparent and convenient fashion. The ttr is defined as the quotient of the amount of tracer (infused material; isotopically enriched trace element) and the amount of tracee (material originally present; normally trace element of natural abundance) in a given system. The ttr for a tracer experiment involving a trace element with z stable isotopes can be deduced from the measured isotope ratio, $R^{x/y}$, of the enriched isotope, x, and a reference isotope, y, in the following manner:

$$ttr = \frac{R^{x/y} - r^{x}_{tracee}}{r^{x}_{tracer} - R^{x/y}} \times \frac{\sum_{i=1}^{z} r^{i}_{tracer}}{\sum_{i=1}^{z} r^{i}_{tracee}}$$

where r^i is the isotope ratio of nuclide i and reference isotope y.

We have adapted the ttr formalism for the use in stable isotope studies involving a purified protein, IMP-1. Using enriched Zn-68 and ^{64}Zn as a reference, the ttr value can be expressed as:

$$ttr = \frac{R^{68/64} - r^{68}_{tracee}}{r^{68}_{tracer} - R^{68/64}} \times \frac{\sum_{i=1}^{5} r^{i}_{tracer}}{\sum_{i=1}^{5} r^{i}_{tracee}}$$

The isotope ratios of tracer and tracee used in the determination of ttr values are summarized in Table S2. Figure S3 demonstrates the relationship between $R^{68/64}$ and ttr.

In the case of stable isotope studies with isolated proteins and under conditions where neither tracee nor tracer are removed (closed system), the upper limit of the ttr value (ttr_{max}) corresponds to the ratio of tracer and tracee in the sample. ttr_{max} can only be achieved at equilibrium, i.e. when the exchange process is complete. In the case of IMP-1, which was supplemented with a 10-fold molar excess of Zn-68 with respect to Zn^{2+} in the enzyme, a ttr value of 10 (ttr_{max}) would be expected if both metal ions in the enzyme had exchanged.

The average number of metal ions exchanged (N) in a n-nuclear system can be derived from the following equation:

$$N = n \times \left(\frac{ttr}{ttr + 1} \right)$$

In the case of binuclear diZn IMP-1 (n = 2), a ttr value of 1 would be indicative of the exchange of one of the two Zn^{2+} present in IMP-1 with Zn-68 (N = 1). From the equation above it also follows that N < n (for n > 0). Consequently, in the case of IMP-1 at equilibrium (ttr_{max} = 10), 1.82 (20/11) Zn^{2+} would have been replaced by the tracer (Zn-68), whereas 0.18 (2/11) Zn^{2+}, originally present in the protein, would have remained.

References

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(2) Siemann, S.; Brewer, D.; Clarke, A. J.; Dmitrienko, G. I.; Lajoie, G.; Viswanatha, T. *Biochim. Biophys. Acta* **2002**, *1571*, 190–200.
(3) Siemann, S.; Clarke, A. J.; Viswanatha, T.; Dmitrienko, G. I. *Biochemistry* **2003**, *42*, 1673–1683.
(4) Woodhouse, L. R.; Abrams, S. A. In *Advances in Isotope Methods for the Analysis of Trace Elements in Man*; Lowe, N., Jackson, M., Eds.; CRC Press: Boca Raton, 2001; pp 1–22.

(5) (a) Toffolo, G.; Shames, D. M.; Stevanato, A.; Cobelli, C. In *Advances in Isotope Methods for the Analysis of Trace Elements in Man*; Lowe, N., Jackson, M., Eds.; CRC Press: Boca Raton, 2001; pp 43–57. (b) Cobelli, C.; Toffolo, G.; Bier, D. M.; Nosadini, R. *Am. J. Physiol.* **1987**, *253*, E551–564. (c) Cobelli, C.; Toffolo, G.; Foster, D. M. *Am. J. Physiol.* **1992**, *262*, E968–975. (d) Buckley, W.T. *Proc. Nutr. Soc.* **1988**, *47*, 407–416.

Table S1. ITV-ICP-MS operating conditions used for this work

ICP-			
RF Generator		Plasma Therm Inc., Model HFP 2500 F	
Frequency/MHz		27.25, crystal controlled	
RF Forward Power/W		1000	
RF Reflected Power/W		<5	
Outer-Tube Gas/L.min^{-1}		16.0	
Intermediate-Tube Gas/L.min^{-1}		1.0	
Mass Spectrometer-			
Model		Sciex Elan 250	
		Setting	*Voltage/V*
Bessel Box: Barrel (B)		50	5
Bessel Box: Plate Lenses		30	-18
Einzel Lenses: E1, E3		95	-19
Einzel Lenses: E2		Factory Set	-130
Stop Lens (S2)		40	-8
Software Settings-			
Machine Config.:	Scan Program	Scan.real	
	Port Speed	9600	
	Port Parity	None (N)	
	Field Separator	Tab (T)	
	Device Type	General Purpose Transient Sampler (G)	
Transient Scans:	Preliminary Transient Scans	Yes (Y)	
	Scanning Mode	Elem. (E)	
	Measurement Mode	Transient (T)	
	Dwell Time/ms	10	
	Masses Monitored	^{64}Zn and ^{68}Zn	
ITV-			
Carrier Gas/L.min^{-1}		1.2	
Drying Power, Duration/W, min		~1, 1.5	
Vaporization Power/W		25 W	
Re Coil Insertion Position/mm		18 (from carrier gas inlet)	
Sampling Depth/mm		11 (from the end of load coil)*	

* Determined based on maximum signal intensity by ITV-ICP-MS.

Table S2. Zinc isotope abundance and isotope ratios of tracee and enriched Zn-68 tracer

	Abundance		Isotope Ratio [a]	
Isotopes	Tracee [b]	Zn-68 Tracer [c]	Tracee	Zn-68 Tracer
64	0.4889	0.0058	1.0000	1.0000
66	0.2781	0.0044	0.5688	0.7586
67	0.0411	0.0038	0.0841	0.6552
68	0.1857	0.9830	0.3798	169.4828
70	0.0062	0.0030	0.0127	0.5172
SUM [d]	**1.0000**	**1.0000**	**2.0454**	**172.4138**

[a] Isotope ratios are defined as the quotient of the amount of each isotope and the reference isotope ^{64}Zn present in tracer and tracee.

[b] Tracee is defined as the endogenous material naturally present in the system. In this study the tracee is Zn^{2+} bound to IMP-1. The values for the natural abundance of zinc were taken from: *Handbook of Chemistry and Physics (51st Edition)*; Weast, R. C., Ed.; CRC Press, Cleveland, 1970-1971.

[c] Tracer is defined as the infused material into a system. Here, the tracer is isotopically enriched Zn-68. The values were obtained from the supplier (Pennwood Chemicals, Great Neck, NY).

[d] The sum of isotope ratios was employed in the determination of ttr values (see equation in the inset of Figure S3).

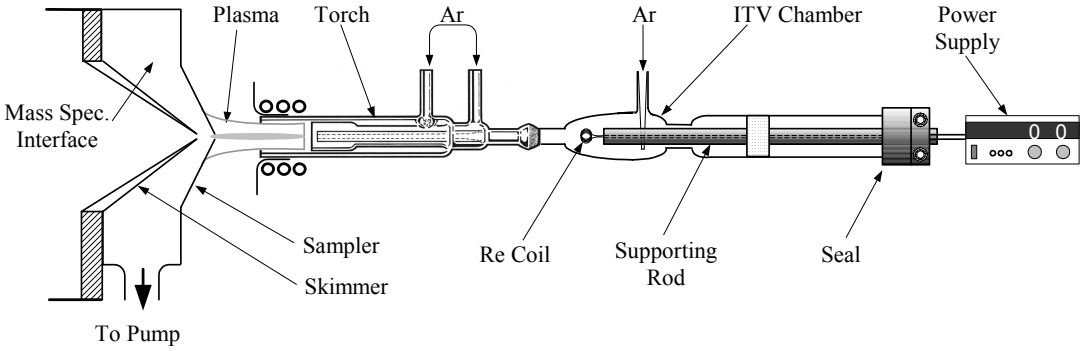


Figure S1: Schematic diagram of an ITV-ICP-MS system (illustration not to scale).

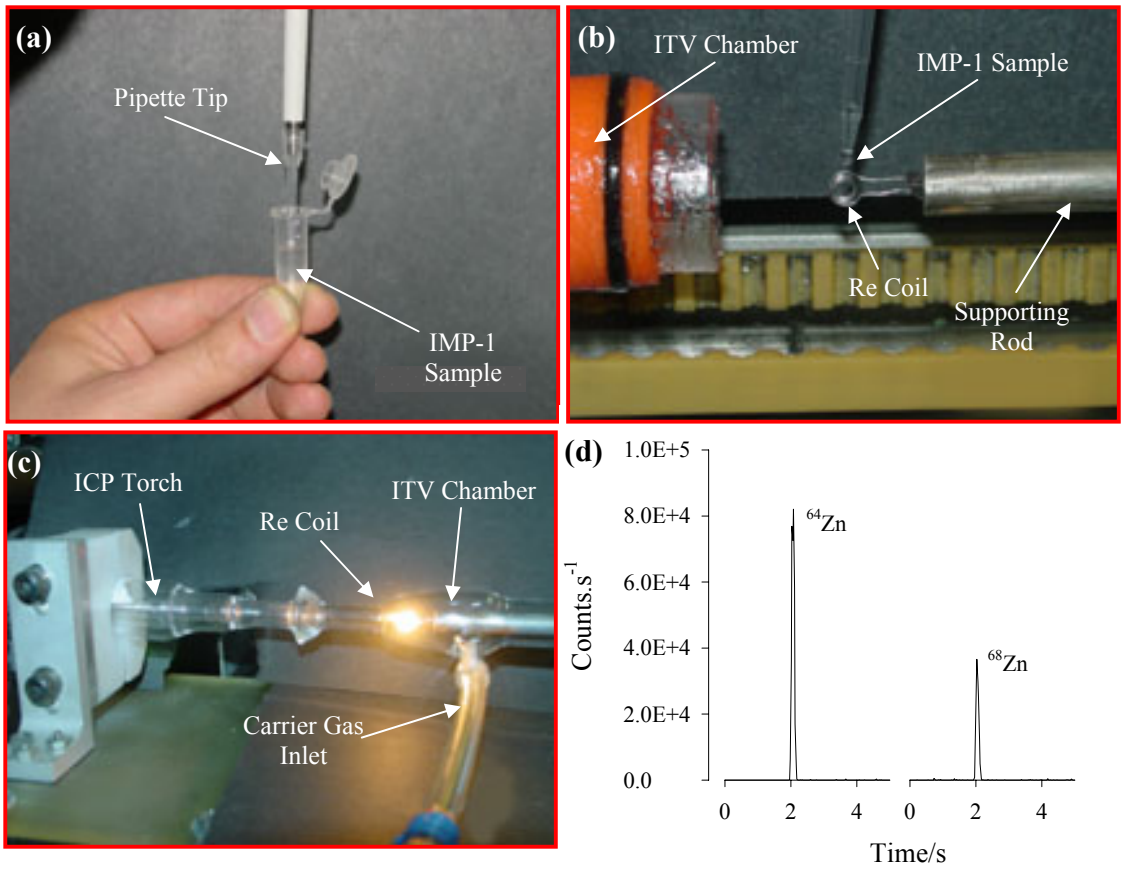


Figure S2: Photographs show the a) a 3-µL aliquot containing IMP-1 withdrawn from a microcentrifuge tube using a pipette and b) the sample aliquot deposited on the ITV Re coil. The ITV supporting rod, along with the Re coil, was retracted out of the ITV chamber for sample deposition. c) After the solution was dried by applying a low electrical power to the Re coil (~1 W, 90 s), the sample residue was vaporized at high power (25 W). The photograph shows the bright glow of the Re coil during the vaporization step. d) Typical transient signals obtained at mass 64 and 68 from 3 µL of a 10-ppb standard Zn solution. The signals are from the same sample aliquot.

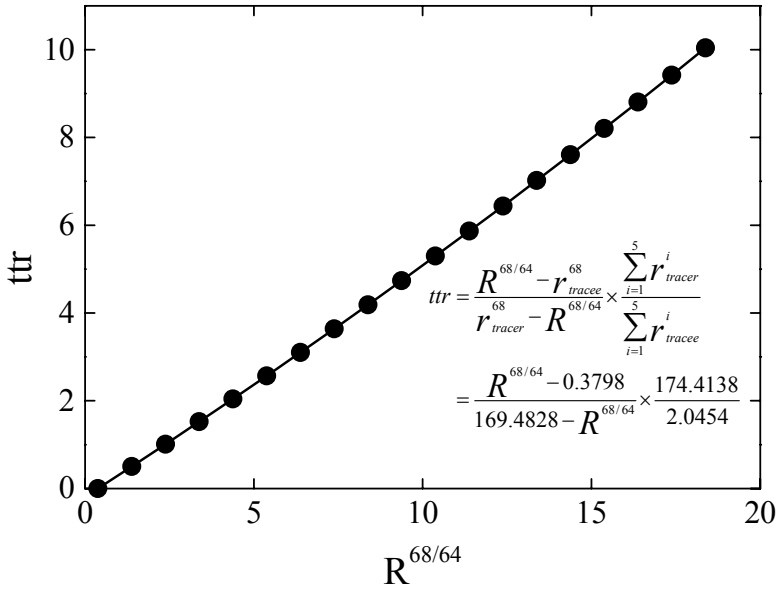


Figure S3: Relationship between isotope ratio of ^{68}Zn and ^{64}Zn ($R^{68/64}$) and *tracer-to-tracee* ratio (ttr). The equation in the inset of the figure provided the basis for the determination of ttr values. Isotope ratios of tracee and tracer are presented in Table S2.

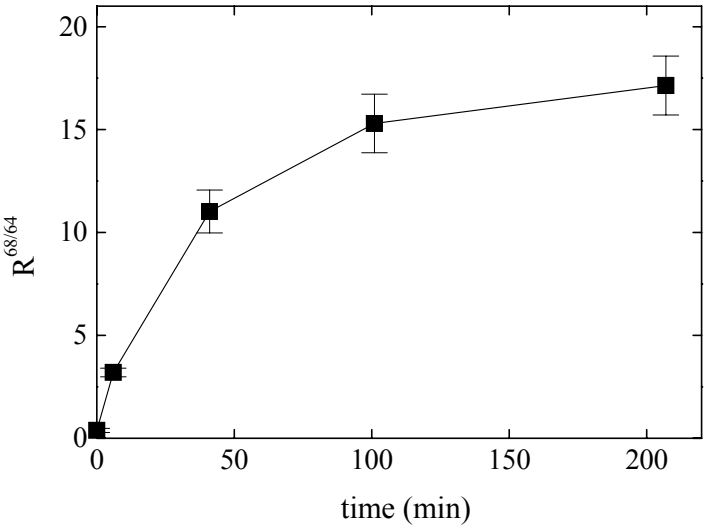
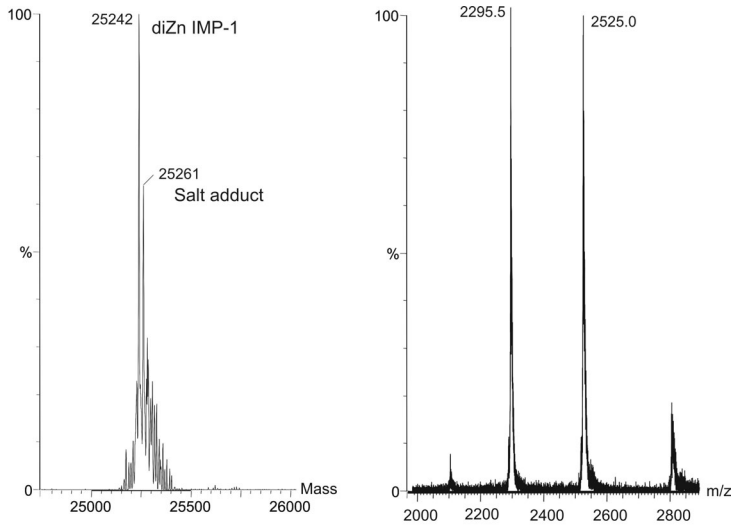


Figure S4: Time-dependence of isotope ratios of ^{68}Zn and ^{64}Zn ($R^{68/64}$) after exposure of IMP-1 to Zn-68. The isotope ratios were determined by ITV-ICP-MS subsequent to the removal of extraneous Zn^{2+} by rapid gel filtration. The data point at t = 0 corresponds to the experimentally determined $R^{68/64}$ isotope ratio found in native diZn-IMP-1, which has not been treated with the Zn-68 tracer.

A



B

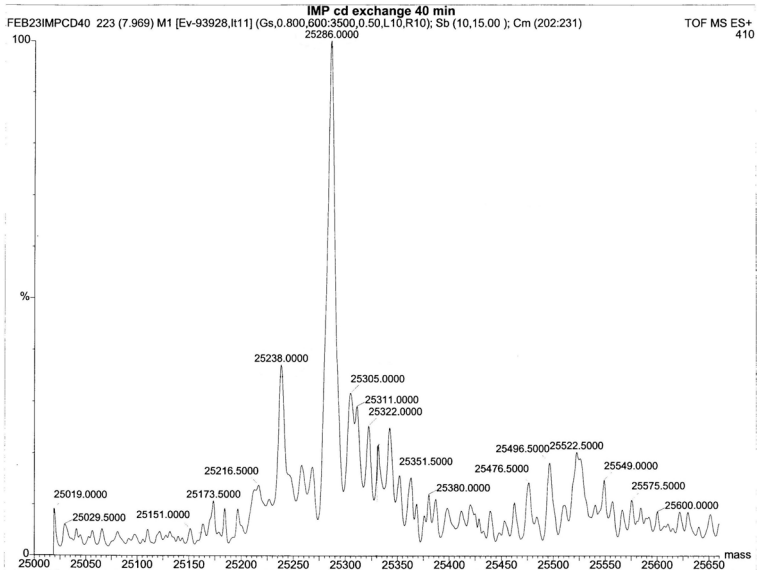
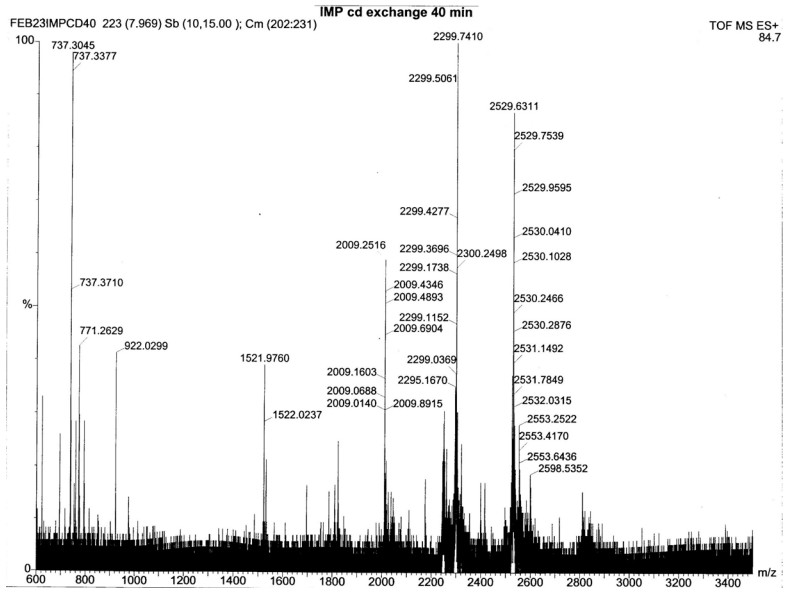


Figure S5: ESI-MS spectra of IMP-1. Spectrum A: Deconvoluted spectrum (left panel) and raw data spectrum (right panel) of native diZn IMP-1. The signal at 25,242 Da corresponds to the native protein containing two Zn(II) ions (see ref. 6 in text). The peak at 25,261 Da presumably arises from an adduct of the diZn protein with one ammonium ion. Spectrum B: Deconvoluted mass spectrum of native IMP-1 after Cd(II) exchange for 40 min. While the peak at 25,238 Da can be attributed to diZn IMP-1, the signal at 25,286 Da arises from the ZnCd hybrid (also compare Figure 1). A peak at 25,333 Da (25,286 + 47 Da), indicative of a diCd protein is not observed. Spectrum C (see next page): Raw data corresponding to spectrum B. Spectrum D: Expansion (of the 25,000-25,500 Da mass range) of the spectrum presented in Figure 1 of the Communication.

C



D

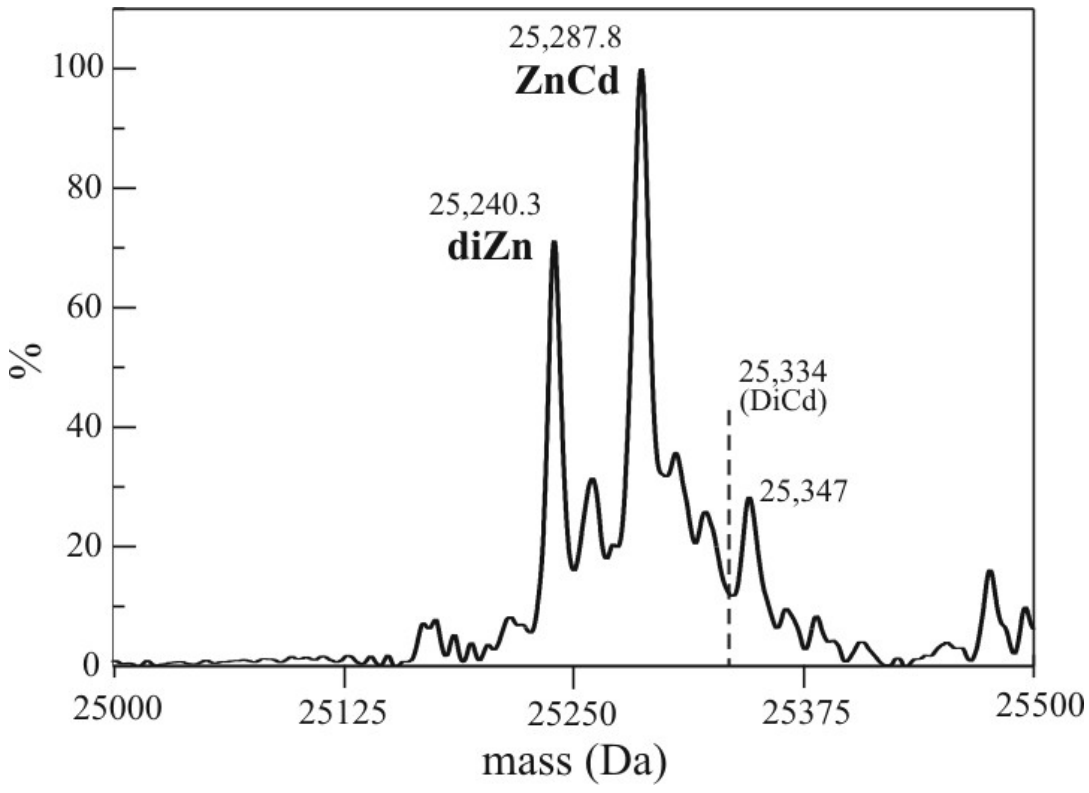


Figure S5: continued