^{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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The apparently paradoxical behaviour of facile exchange (kinetic lability) of tightly bound (thermodynamic stability) zinc ions in the enzyme IMP-1 metallo-β-lactamase with Zn-68 and cadmium ions, as indicated by in-torch vaporization inductively-coupled plasma mass spectrometry (ITV-ICP-MS) and electrospray-ionization mass spectrometry (ESI-MS), is consistent with the involvement of a third metal ion in promoting Lewis acid/base type exchange processes.

Zinc (Zn^{2+}) is an essential element because of its requirement in a multitude of cellular processes.[1,2] The zinc ion's Lewis acidity, redox inertness and exceptionally flexible coordination to functional groups of proteins appears to form the basis for its versatility in biological systems.[3] A review of the three-dimensional structures of more than 200 Zn-dependent proteins recorded to date suggests that the role of this metal ion falls into four distinct categories: (i) catalytic, (ii) structural, (iii) co-catalytic, and (iv) inter-facial.[2]

In recent years, zinc-dependent metallo-β-lactamases (MBLs) have received considerable attention in view of their role in microbial resistance to β-lactam antibiotics.[4] All MBLs have two distinct Zn^{2+} binding sites, which closely resemble those found in members of zinc proteins with co-catalytic sites. However, MBLs appear unique in that they are catalytically competent with just one metal ion present in the active site.[5,6]

The current report concerns the unusual dynamics of the two Zn^{2+} ions in IMP-1 from *Pseudomonas aeruginosa*, a metallo-β-lactamase of particular interest due to its potential for facile dissemination *via* horizontal gene transfer,[7] and to gain mutation-dependent enhanced catalytic efficiency.[8] While one of the two Zn^{2+} ions in IMP-1 is coordinated to three histidine residues (forming the His-site), the other metal ion is bound to an aspartate, cysteine, and histidine residue (forming the DCH-site).[9] Both Zn^{2+} in the enzyme are tightly bound as indicated by their retention even after prolonged dialysis[7] or upon exposure to chelators.[6] These findings appear to be in accordance with the observation that metal ions in the active sites of most Zn-dependent proteins (*e.g.* carbonic anhydrase, alkaline phosphatase, aspartate transcarbamoylase, and carboxypeptidase A) are usually very tightly bound and exhibit very little propensity for exchange with extraneous metal ions (such processes often requiring days or weeks to proceed).[10] However, despite IMP-1's very high affinity for its metal ions, we demonstrate that the enzyme is capable of undergoing rapid transformation to the ZnCd hybrid species upon exposure to Cd^{2+}. In order to determine whether this exchange phenomenon is specifically induced by Cd^{2+} (by virtue of its affinity for thiolate ligands) or is a reflection of an intrinsic kinetic lability of Zn^{2+} in IMP-1 akin to that in metallothioneins,[11] we have also monitored the propensity of the protein's metal ions for exchange with Zn-68[12] serving as the tracer. These studies have revealed that both tightly bound Zn^{2+} in the active site of IMP-1 are amenable to facile displacement by exogenous Zn^{2+}.

In the experiments with Cd^{2+}, IMP-1 was exposed to $CdCl_2$ (50-fold molar excess with respect to IMP-1's Zn^{2+}) and an aliquot withdrawn after 5 min was rendered free of extraneous metal ions by rapid gel filtration. Electrospray ionization (ESI)-MS analysis of the recovered protein under non-denaturing conditions (Fig. 1) revealed the presence of two distinct species, the parent di-Zn protein (35%) and the ZnCd hybrid (65%). A di-Cd species was not observed, even after prolonging the period of incubation to

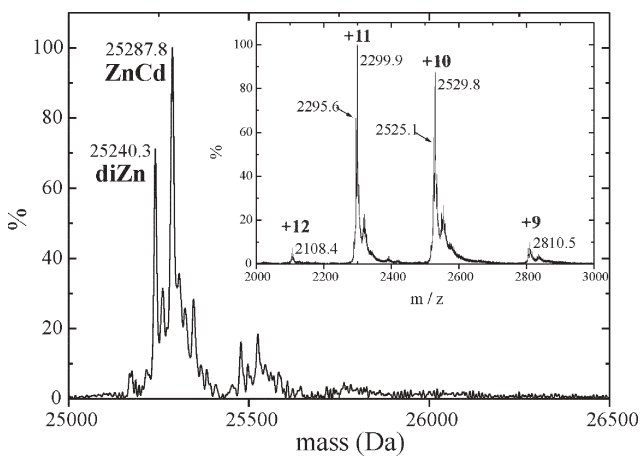

Fig. 1 Deconvoluted ESI mass spectrum of native IMP-1 after Cd^{2+} exchange. The corresponding raw data is shown in the inset (bold numbers denote charge states). The signal at 25 240.3 Da has been previously assigned to the di-Zn form of IMP-1.[6] Exchange of one Zn^{2+} with Cd^{2+} is expected to yield a mass increment of +47 Da (observed value: +47.5 Da).

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† Electronic supplementary information (ESI) available: Experimental details, tables of isotope abundances, ITV-ICP-MS diagram and operating conditions, conversion of ^{68}Zn/^{64}Zn isotope ratios into ttr values, time-dependence of measured isotope ratios, ESI-MS spectra. See DOI: 10.1039/b514227j

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40 min (with the ESI mass spectrum showing a 4:1 ratio of ZnCd hybrid and di-Zn species, data not shown).

To monitor the dynamics of Zn^{2+} exchange, the di-Zn protein (with the metal ion in its natural abundance) was exposed to a medium of enriched Zn-68 (10-fold molar excess with respect to the protein's metal content). At desired intervals, aliquots of the reaction mixture were withdrawn and rendered free of the extraneous metal ions by rapid gel filtration. In-torch vaporization (ITV) inductively-coupled plasma mass spectrometry (ICP-MS), a new technique for analysis of small samples without compromising accuracy and reliability,[13] was subsequently employed to determine the ratio of ^{68}Zn to ^{64}Zn, $R^{68/64}$ in the samples. The extent of Zn^{2+} exchange was determined by adapting the *tracer-to-tracee* (ttr) methodology (usually employed in studies on trace element metabolism in humans and animal models),[14] using the equations given in Fig. 2.[15]

Results presented in Fig. 2 reveal that both Zn^{2+} of IMP-1 are amenable to replacement, with approximately 60% of the metal ions in the protein being exchanged five minutes after the initiation of the process. After 100 min, the observed ttr value was close to that expected for a complete exchange of metal ions ($ttr_{max} = 10$), indicating the attainment of equilibrium between the reactants. The observed kinetics are consistent with one of the Zn^{2+} of IMP-1 being more prone to exchange than the other.

As outlined previously, metal ions in the active sites of most Zn-dependent proteins are usually tightly bound and resist exchange with extraneous metal ions. Notable exceptions are metallothioneins (MTs), which are capable of rapidly transferring their tightly bound Zn^{2+} to other acceptor proteins and participating in exchange with exogenous metal ions.[11] These somewhat paradoxical features (*i.e.* thermodynamically very stable Zn-protein complexes contrasting the kinetic lability of the metal ions), which facilitate the proteins' function in cellular Zn^{2+} homeostasis, distribution and enzyme regulation, are thought to be related to the presence of two unique Zn^{2+}/thiolate clusters and their redox chemistry.[11]

In the case of Zn_4-SmtA, a cyanobacterial metallothionein, exchange of 1.4 (out of three susceptible) metal ions with ^{67}Zn under conditions similar to those used in the current study was

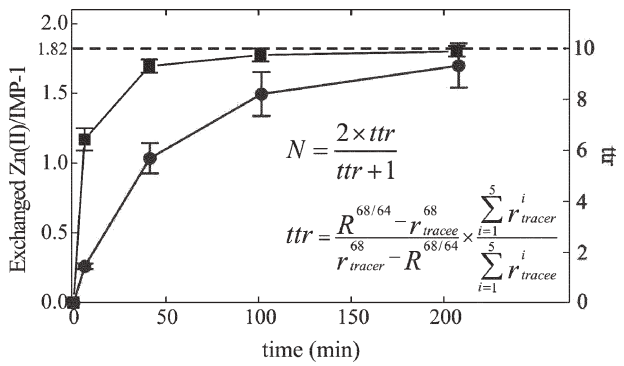

Fig. 2 Time-course of Zn^{2+} exchange in IMP-1. Number of Zn^{2+} exchanged per molecule of enzyme, *N*, (squares); tracer-to-tracee ratio, ttr (circles). *N* and ttr values were obtained from the recorded ratio of ^{68}Zn and ^{64}Zn according to the equations in the figure and the data presented in the ESI.† The dashed line represents maximal ttr and *N* values, expected from the 10-fold excess of Zn-68 over Zn^{2+} in IMP-1 employed in this experiment.

observed after 1 h of exposure to the isotope tracer.[16] Thus, the very rapid (within 5 min) isotopic exchange of Zn^{2+} and incorporation of Cd^{2+} reported here with IMP-1, are remarkable and deserve some consideration with respect to a possible mechanism.

Carboxypeptidase A and thermolysin serve as examples for Zn^{2+} in the active sites of proteins capable of interacting with their extraneous metal ion counterparts (as $[Zn(OH)]^+$) in a substrate like fashion with concomitant inhibition of catalytic function.[17] The ability of extraneous Zn^{2+} to inhibit the catalytic activity of IMP-1 ($IC_{50} \approx 1$ mM)[18] appears to provide support for the concept of its binding in the vicinity of the active site of the protein. Thus, the interaction of exogenous Zn^{2+} with IMP-1 could be envisioned to be analogous to that with the electrophilic carbon of its β-lactam substrate resulting in a native IMP-1:Zn^{2+} complex (tri-Zn species).

In such a complex, the Zn^{2+} in the His site may be positioned to function as a Lewis acid catalyst, which should render the bound exogenous Zn^{2+} more electrophilic, and thus more able to bind to one or more ligands provided by the DCH site.[18] Such ligand exchange (or sharing) between the exogenous and DCH site Zn^{2+} ions would likely facilitate the dissociation of the latter metal ion. Similarly, Zn^{2+} in the DCH site is poised to potentially catalyze ligand exchange between the His site and the third Zn^{2+}. In such a model, the ease of exchange might be determined by the Lewis acidity of the metal ions involved.

Given the nature of the ligands in the two sites, it is reasonable to suggest that Zn^{2+} in the His site is the more effective Lewis acid. Consequently, one would expect the exchange of Zn^{2+} in the DCH site to be more rapid. Similarly, one would predict that Cd^{2+} incorporation should occur at the DCH site. The lower Lewis acidity of thiolate-bound Cd^{2+} compared to that of Zn^{2+} in the DCH site may explain the inability of IMP-1 to incorporate a second Cd^{2+} in its active site.

As noted before, the opposing features of thermodynamic stability and kinetic lability of protein bound Zn^{2+} are intrinsic to metallothioneins and are thought to be related to the unique thiolate environment around the protein's Zn^{2+} ions.[11] The observations recorded in the current report suggest that the kinetic lability of tightly bound Zn^{2+} can also be an inherent feature of coordination environments other than that restricted exclusively to thiolate clusters found in metallothioneins. Interestingly, the basis for the kinetic instability of tightly bound Zn^{2+} in proteins as a prelude to transfer to other vicinal acceptors has been recently addressed in considerable detail.[19] Recent studies on Zn^{2+}–peptide complexes have revealed a rapid equilibrium of 'bound' and 'unbound' states of Zn^{2+} ligands, a feature that is likely to facilitate rapid ligand exchange processes, and may thus contribute to the kinetic lability of such complexes.[19] Such dynamic interactions may indeed provide the basis for the transfer of the Zn^{2+} ions between proteins. In light of these considerations, it seems plausible that similar equilibria (of bound and unbound ligands) in combination with the innate feature of the incoming exogenous counterpart to function as a Lewis acid catalyst, may serve as the basis for the observed kinetic lability of Zn^{2+} in the active site of IMP-1.[20]

Our data certainly do not preclude, however, the possibility of other mechanisms for the observed facile metal ion-induced ion exchange process. For example, it is also plausible that zinc

binding at a site remote from the dizinc catalytic site of the enzyme might induce subtle conformational changes in the protein which loosen the grip of the active-site metal binding ligands on the catalytic metal ions sufficiently to provide a low energy pathway for metal ion exchange. In any event, the mechanistic aspects of the exchange process are of fundamental interest and are currently being probed experimentally in these laboratories.

Finally, the current report illustrates the applicability of ITV-ICP-MS and the ttr methodology to monitor stable isotope exchange processes in proteins of limited availability. Furthermore, the ligand exchange processes catalysed by extraneous transition metal ions, all of which are Lewis acids, may form the basis for metal-substitution reactions (*e.g.* by dialysis of a Zn-protein with other metal ions). Thus, exposure of native IMP-1 and other MBLs to extraneous transition metal ions may provide a direct approach to metal-substitution in these enzymes for spectroscopic studies.[21]

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