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Chelator-facilitated chemical modification of IMP-1 metallo-β-lactamase and its consequences on metal binding ☆

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ABSTRACT

A method involving the reversible chemical modification of an active site, zinc-binding cysteine residue (Cys221) for the specific removal of one of the two zinc ions in the metallo-β-lactamase IMP-1 was explored. Covalent modification of Cys221 by 5,5′-dithio-bis(2-nitrobenzoic acid) was greatly enhanced by the presence of dipicolinic acid, and subsequent removal of the modifying group was easily achieved by reduction of the disulfide bond. However, mass spectrometric analyses and an assessment of IMP-1's catalytic competence are consistent with the maintenance of the enzyme's binuclear status. The consequences arising from chemical modification of Cys221 are thus distinct from those reported for Cys → Ala/Ser mutants of IMP-1 and other metallo-β-lactamases, which are mononuclear.

Introduction

The requirement for zinc (Zn^{2+}) for so many biological processes mainly derives from its abundance in the biosphere, and from a unique combination of its chemical properties, including redox-inertness, high Lewis acidity, exceptionally flexible coordination geometry, and its ability to accommodate hard, borderline and soft bases [1–4]. Based on the analysis of hundreds of crystal structures of zinc proteins, the function of the metal ion can be classified as (i) catalytic, (ii) structural, (iii) co-catalytic, or (iv) interfacial [5,6].

Zn^{2+}-dependent metallo-β-lactamases (MBLs) have attracted considerable attention in recent years by virtue of their role in rendering microorganisms resistant to a wide spectrum of β-lactam antibiotics [7–11]. Although MBLs are characterized by the presence of two vicinal Zn^{2+}-binding sites [12], these proteins differ from those harboring co-catalytic centres by the absence of an amino acid bridging ligand between the two metal ions. Furthermore, the role of the second metal ion in MBLs is not clearly defined at the present time since the mononuclear forms of some of these proteins have been shown to be catalytically competent [13–15].

IMP-1 from *Pseudomonas aeruginosa* is an MBL of particular interest due to its potential for facile dissemination via horizontal gene transfer [16]. The two Zn^{2+} ions of IMP-1 are bound in two distinct sites: the Zn1 site (coordination to three histidine residues, 3H site) and the Zn2 site (coordination to an aspartate, a histidine, and a cysteine residue, DCH site) [17]. Both metal ions are tightly bound as indicated by their retention in the protein even after prolonged dialysis and exposure to chelators [13,16]. Although treatment with the chelator DPA leads to rapid inactivation of the enzyme within minutes ($k_{inact}/K_I = 187\ M^{-1}\ s^{-1}$) due to the formation of an enzyme:Zn^{2+}:chelator ternary complex, the actual removal of one of the metal ions is a very slow process (~10 h) [13]. Furthermore, the reconstitution of Zn^{2+}-deficient forms of IMP-1 to the diZn enzyme has not been possible, presumably due to conformational aberration(s) in the protein following its prolonged exposure to chelators [13,18]. Hence, new methods for the rapid generation of fully reconstitutable mononuclear IMP-1 (and other MBLs) are needed to elucidate the role of the second Zn^{2+} ion in the enzyme's catalytic mechanism.

The current report explores a strategy for the generation of mononuclear IMP-1 (outlined in Fig. 1) by removal of Zn2 through its displacement from Cys221 (a ligand coordinating this metal ion [12,17]) via chemical modification of this amino acid residue with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), followed by the removal of the modifying group with tris(2-carboxyethyl)phosphine (TCEP). The rationale behind the above strategy stems from the enhanced nucleophilicity of zinc-coordinated thiols relative to that of their uncoordinated counterparts [19], a feature that would facili-

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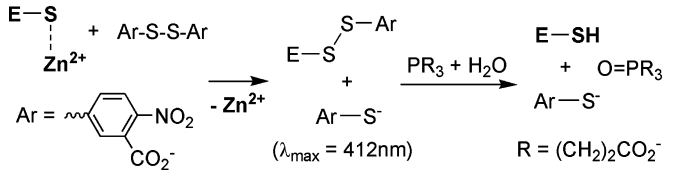

Fig. 1. Proposed two-step reaction protocol for the removal of cysteine-bound Zn^{2+} from IMP-1.

tate their participation in disulfide exchange reactions, and thus render the Zn^{2+} ion more labile.

Materials and methods

Chemicals and buffer preparation. 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. The concentration of residual Zn^{2+} ions after Chelex treatment was determined to be $\leqslant$20 nM.

Purification of IMP-1. IMP-1 was expressed in *Escherichia coli* BL21(DE3) carrying pCIP4, and purified as documented previously [16]. The homogeneity of the protein was confirmed by SDS–polyacrylamide gel electrophoresis. The molecular weight of purified IMP-1 (M_r = 25,113 ± 0.5 Da), as determined by electrospray-ionization mass spectrometry (ESI-MS), was in agreement with that deduced from its amino acid sequence [20]. The presence of two Zn^{2+} ions in the protein was established by ESI-MS under non-denaturing conditions, and with the chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) [13]. The concentration of the enzyme was determined from its absorbance at 280 nm (ε = 44,380 M^{-1} cm^{-1}) [21].

Assessment of enzymatic activity. MBL activity was determined spectrophotometrically as described previously [21], using nitrocefin (Oxoid, Basingstoke, UK) as the chromophoric substrate. The effect of DTNB, methyl methanethiosulfonate (MMTS), and 2-nitro-5-thiocyanobenzoate (NTCB) on IMP-1's catalytic function was assessed by pre-incubating the enzyme (4 nM) in Hepes buffer (50 mM, pH 7.3) containing bovine serum albumin (BSA; 100 μg/mL) with the desired modifying agent in a final volume of 950 μL. The reaction was initiated by the addition of an aliquot (50 μL) of a nitrocefin stock solution (2 mM), and the progress of substrate hydrolysis was monitored spectrophotometrically at 482 nm.

Chemical modification of IMP-1 by thiol-modifying agents. The interaction of thiol-modifying agents with IMP-1 was also monitored using ESI-MS. In a final volume of 200 μL, IMP-1 (2 μM) was incubated in Hepes buffer (50 mM, pH 7.3) in the presence of BSA (0.1 mg/mL) and the desired modifying agent (1 mM) for 15 min at 25 °C. The degree of modification of Cys221 in IMP-1 was assessed by ESI-MS (see below).

Modification of IMP-1 by DTNB in the presence of dipicolinic acid (DPA). The rate enhancement of the chemical modification of IMP-1 by DTNB in the presence of DPA was assessed spectrophotometrically by monitoring the release of the chromophoric 2-nitro-5-thiobenzoate (NTB) anion at 412 nm. In a typical assay (90 μL), IMP-1 (3 μM) in Hepes buffer (50 mM, pH 7.3) was allowed to equilibrate at 25 °C for 1 min. The reaction was initiated by the introduction of DTNB (10 μL of a 5 mM stock solution in buffer), and the change in absorbance at 412 nm was monitored for 5 min. After 5 min, an aliquot (1.5 μL) of a freshly prepared DPA stock solution (50 mM in buffer) was added to the sample. Following thorough mixing for ~30 s, the change in absorbance was continued to be monitored at 412 nm. The final concentrations of IMP-1, DTNB, and DPA in the assay were thus 2.66 μM, 493 μM, and 739 μM, respectively. Following completion of the assay (after ~9 min), an aliquot (10 μL) was withdrawn from the mixture for the determination of enzymatic activity. The remainder of the sample was used for ESI-MS analysis, or for experiments designed to remove the NTB moiety from the protein.

Electrospray-ionization mass spectrometry. ESI-MS studies were performed in positive ion mode on a Micromass Q-Tof Ultima™ Global mass spectrometer (Micromass, Manchester, UK) using previously documented calibration procedures and instrumental parameters [13]. Prior to ESI-MS analysis, the buffer medium of the enzyme preparations was replaced by ammonium acetate (10 mM, pH 7) by dilution and subsequent filtration (3×) with the aid of Microcon centrifugal concentrators (Millipore, Bedford, MA). This procedure typically resulted in final enzyme concentrations of 3–10 μM with concomitant dilution (approximately 1000-fold) of any contaminating reagents (e.g. DTNB, NTB, DPA, etc.). For some samples, removal of the NTB moiety from chemically modified IMP-1 preparations was achieved by their treatment with TCEP at a final concentration of 250 μM at room temperature for 2 min prior to analysis by ESI-MS. In the case of measurements performed under denaturing conditions, protein samples were denatured using a mixture of water/acetonitrile (50:50 [v/v]) containing 0.1% (v/v) formic acid.

Results

Chelator-induced chemical modification of IMP-1's Cys221

Initial efforts to achieve selective modification of Cys221 of IMP-1 by exposure to such thiol-specific modifying agents as DTNB [22,23], NTCB [24], and MMTS [25] revealed the amino acid's thiol function to be incapable of facile interaction with the above reagents (Table 1). In light of this finding, the possibility of thiol-

Table 1
Inhibition of IMP-1 by thiol-modifying agents and degree of modification.

	Residual activity[a] (%)	Peaks in mass spectrum[b] (Da)	Degree of modification[c] (%)
DTNB	80	A: 25,114; B: 25,311 (Δ = 197)[d]	30
MMTS	90	A: 25,113; B: 25,160 (Δ = 47)[d]	14
NTCB	>99	A: 25,113	–

[a] Residual activity was assessed after incubation of IMP-1 (4 nM) with the desired thiol-modifying agent at a final concentration of 1 mM for 15 min at 30 °C using nitrocefin as the substrate. Untreated protein served as the control (100%) in these studies.

[b] IMP-1 (2 μM) was incubated in Hepes buffer (50 mM, pH 7.3) in the presence of BSA (0.1 mg/mL) and the desired modifying agent (1 mM) for 15 min at 25 °C. The degree of modification of Cys221 in IMP-1 was assessed by ESI-MS under denaturing conditions. While two peaks were observed in the mass spectra of IMP-1 treated with DTNB and MMTS, only one signal corresponding to that of the unmodified protein was observed for the NTCB-exposed enzyme.

[c] The heights of the centred peaks A and B (when present) provided the basis for the estimation of the degree of modification of IMP-1.

[d] Δ values denote the difference in mass between the species corresponding to peaks B and A. The expected Δ values for modification of a cysteine residue by DTNB and MMTS are 198 Da (NTB moiety) and 47 Da (CH_3S group), respectively.

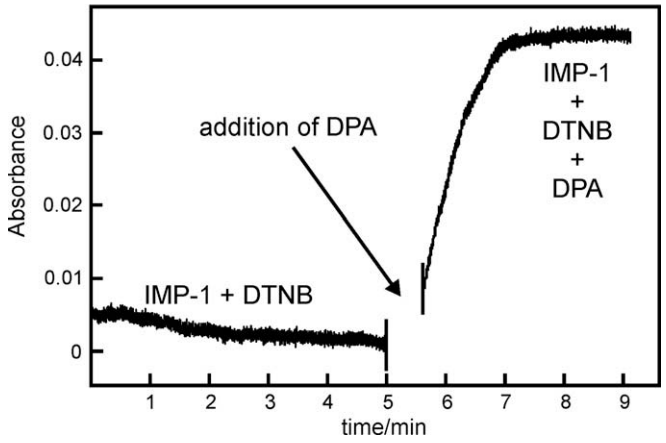

Fig. 2. Progress of the reaction of IMP-1 with DTNB in the absence and presence of DPA. IMP-1 (3 μM) was treated with DTNB (500 μM) in Hepes buffer (50 mM, pH 7.3), and the absorbance at 412 nm was monitored for 5 min. DPA (740 μM) was subsequently added to the mixture, and the release of the NTB anion was continued to be recorded at 412 nm.

modifying agents functioning in synergy with DPA (a chelator shown to cause the slow removal of one of the Zn^{2+} ions in IMP-1 [13]) was investigated. As shown in Fig. 2, introduction of DPA to IMP-1 pre-exposed to DTNB resulted in a rapid increase in absorbance at 412 nm, a feature characteristic of the release of the NTB anion (see Fig. 1). Completion of the reaction was achieved within 2 min after the addition of the chelator, and modification of IMP-1 under these conditions led to the loss of its catalytic function. These results suggest that the interaction of DPA with (presumably) Zn2 in IMP-1 leads to a destabilization of the coordinative bond between Cys221's sulfur atom and the metal ion, thus facilitating the reaction of the former with DTNB. Since the formation of an IMP-1:Zn^{2+}:DPA ternary complex has been shown to proceed within 1–2 min [13], a period of time identical to that observed in the current study for the release of the NTB anion, the interaction of DPA with Zn2 appears to govern the overall rate of modification of Cys221 by DTNB.

Confirmation of the modification of IMP-1's Cys221 was also achieved by electrospray-ionization mass spectrometry (ESI-MS) of the protein under denaturing conditions. As shown in Fig. 3, a comparison of mass spectra of untreated IMP-1 (panel A) with that of the modified preparation (panel B) revealed an increase in the molecular mass in the latter sample by 199 Da (from 25,113 to 25,312 Da), a finding consistent with the incorporation of an NTB moiety (198 Da) into the protein (see Table 2). Analysis of modified IMP-1 by ESI-MS under non-denaturing (native) conditions (Fig. 4A and Table 2) revealed the mass of the protein to be that expected of a species associated with one NTB moiety (198 Da) and one Zn^{2+} ion (64 Da). Thus, the chelator-induced chemical modification of IMP-1's Cys221 would appear to serve as a convenient strategy for the facile removal of Zn2, and hence for the generation of the mononuclear form of the enzyme. However, the observed loss of the Zn^{2+} ion is only apparent, as indicated by the observations recorded below.

Removal of modifying group by TCEP

Removal of the NTB moiety present in modified IMP-1 preparations was readily achieved by treatment with TCEP (250 μM for 2 min), as indicated by the ESI-MS spectra recorded under denaturing conditions, which were identical to that shown in Fig. 3A. Interestingly, analysis of these samples by ESI-MS recorded under non-denaturing conditions revealed IMP-1 to be associated with two

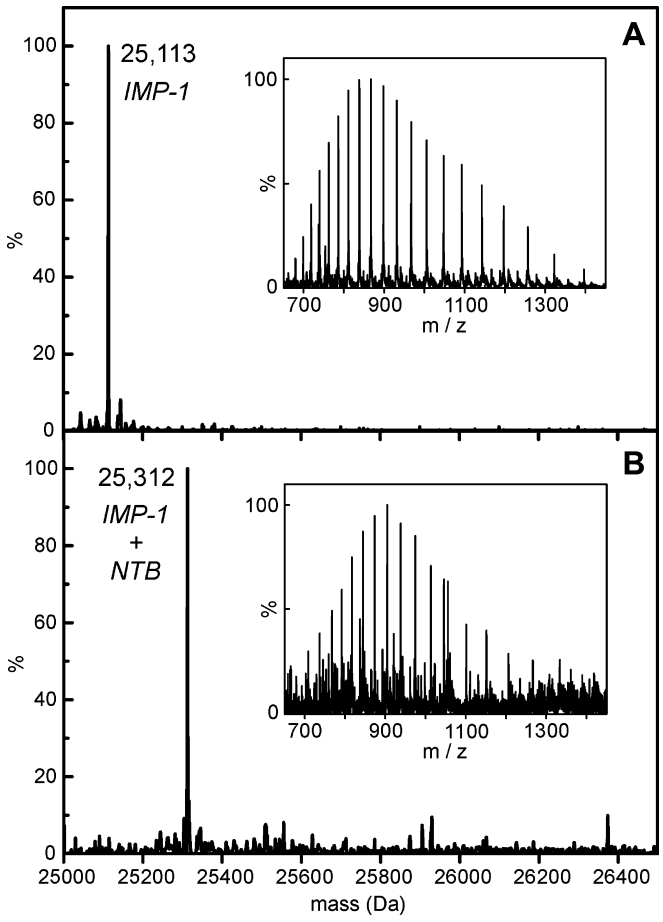

Fig. 3. ESI-MS spectra of IMP-1 recorded under denaturing conditions. (A) Unmodified IMP-1. (B) IMP-1 modified with DTNB. Raw spectra are shown in the insets of the figure.

(instead of one, as expected from the previously mentioned observations) Zn^{2+} ions (Fig. 4B, Table 2). It is pertinent to note that mass spectrometric analysis of unmodified monoZn-IMP-1, generated by previously documented procedures [13], revealed the protein to remain mononuclear (data not shown), thus ruling out the possibility of artefactual re-introduction of the second Zn^{2+} ion into TCEP-treated IMP-1 as a consequence of metal contamination in the mass spectrometer. Furthermore, the reduction of the disulfide bond by TCEP led to the restoration of catalytic function to ~85% of that of the diZn enzyme, a value significantly higher than that expected for its mononuclear variant (~50%; see [13]). Hence, both the high degree of restoration of catalytic activity and the observation of two Zn^{2+} ions in the mass spectrum of IMP-1 after its treatment with TCEP clearly suggest that none of the metal ions were removed from the enzyme during its exposure to DPA and DTNB. The inability to detect both Zn^{2+} ions in chemically modified

Table 2
Summary of calculated and observed masses of IMP-1 species.

IMP-1 species	Calculated mass (Da)				Observed mass (Da)
	IMP-1	Zn^{2+}	NTB	Sum	
Apo[a]	25,113	—	—	25,113	25,113
diZn[b]	25,113	2 × 64	—	25,241	25,242
NTB-modified[a]	25,113	—	198	25,311	25,312
NTB-modified[b]	25,113	64	198	25,375	25,374

[a] Measured under denaturing conditions.
[b] Measured under non-denaturing (native) conditions.

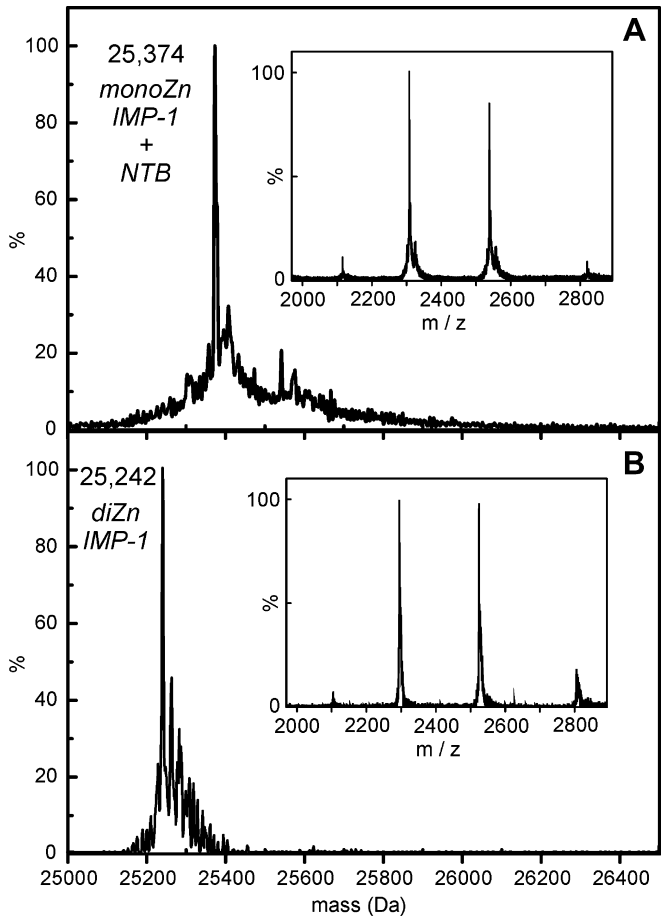

Fig. 4. ESI-MS spectra of IMP-1 recorded under non-denaturing conditions. (A) IMP-1 modified with DTNB. (B) DTNB-modified IMP-1 preparation after treatment with TCEP. Raw spectra are shown in the insets of the figure.

IMP-1 (prior to TCEP exposure) by ESI-MS under non-denaturing conditions (Fig. 4A) may be a consequence of the loss of Zn2 during ionization of the sample, an event not inconceivable in view of the expected weaker affinity of the modified enzyme for the metal ion (see Discussion).

Discussion

The role of the second Zn^{2+}-binding site (and its occupant) in MBLs remains to be unequivocally established. Contrary to an earlier proposal of mononuclear MBLs being the sole contributor to the hydrolysis of β-lactams under physiological conditions [15], recent studies on BcII have suggested that the mononuclear form is catalytically incompetent [26–28]. Such dichotomy in these reports emphasizes the need for ready access to reconstitutable mononuclear forms of MBLs to facilitate a comparative study with their parent binuclear counterparts about their true catalytic competence on a wide spectrum of β-lactam substrates.

In the case of IMP-1, the clinically relevant MBL from *P. aeruginosa*, both Zn^{2+} ions in IMP-1 are tightly bound [13,16]. Furthermore, the generation of Zn^{2+}-deficient variants of the enzyme by chelator-mediated removal of one or both metal ions is not only a very slow and cumbersome process but also irreversible [13,18]. The results of the current study clearly suggest that modification of the enzyme's Cys221 residue in the presence of a chelator does not lead to facile removal of the second Zn^{2+} ion of IMP-1. It is interesting to note that replacement of this amino acid by either serine or alanine by site-directed mutagenesis has been shown to result in the production of the mononuclear form of the protein in the case of IMP-1 [18], CcrA [29] (the MBL from *Bacteroides fragilis*), and BcII [30] (the MBL from *Bacillus cereus*). Hence, the observation that IMP-1 can still retain both its Zn^{2+} ions even after the modification of its Cys221 residue with DTNB appears to suggest that consequences arising from the chemical modification of this amino acid residue are not identical with those elicited by its replacement by site-directed mutagenesis.

As regards the location and coordination environment of Zn2, removal of the thiolate function of Cys221 (either by the formation of a disulfide bond reported herein, or by site-directed mutagenesis) can be expected to significantly lower the metal ion's affinity for the DCH site. Indeed, the above noted loss of one of the Zn^{2+} ions upon replacement of Cys221 with alanine or serine is a clear reflection of such phenomenon. Furthermore, numerous studies on disulfide-containing 3d transition metal complexes have revealed the interaction between the sulfur donor centres and the metal ions to be very weak [31,32]. Thus, a significant interaction between the disulfide (established between Cys221 and the NTB moiety) and Zn2 in IMP-1 appears highly unlikely. It is not inconceivable, however, that modification of the enzyme by DTNB is accompanied by a change in the arrangement of Zn^{2+} ligands or a translocation of Zn2 to the putative, vicinal third binding site in the protein, which is proposed to be involved in the facile exchange of protein-bound metal ions and their extraneous counterparts [33]. Indeed, such an additional site has also recently been reported to occur in BcII [34]. Regardless of whether Zn2 remains at the DCH site in an altered coordination environment after modification of Cys221, or translocates to the vicinal third binding site, such changes are transient in nature since removal of the modifying group by TCEP results in a protein preparation virtually identical to that of untreated binuclear IMP-1 with respect to its mass spectrum and its catalytic competence.

In conclusion, the retention of the binuclear status of IMP-1 after chelator-facilitated chemical modification of one of its active site ligands reported herein, not only attests to the high thermodynamic stability of the protein-metal complex, but also underscores the difficulty in generating a true mononuclear form of the enzyme from the parent holoprotein. Furthermore, another significant aspect of the current report concerns the observation that consequences arising from the chemical modification of an active site amino acid residue may not be identical with those elicited by its replacement by site-directed mutagenesis, a phenomenon which has been noted in several other instances [35,36].

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