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The Metal Binding Site of Zoocin A

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Abstract

Direct metal analysis of the bacteriolytic exoenzyme zoocin A failed to unequivocally identify a putative metal cofactor. Hence, indirect experiments utilizing NMR were undertaken to settle this question. Cd²⁺ as a surrogate metal ion was reconstituted into EDTA-treated, metal-free recombinant zoocin, and ¹¹³Cd-NMR was employed to explore binding in the protein for this ion. The Cd-substituted enzyme was found to have 75% of native streptococcolytic activity. A major ¹¹³Cd resonance at 113.6 ppm was observed which with time split into resonances at 113.6 and 107.2 ppm. A minor ¹¹³Cd resonance at 87.3 ppm was observed which increased in intensity with time. These Cd chemical shifts are indicative of two nitrogen atoms and two oxygen atoms ligating directly to the metal site. Based upon conserved amino acid residues in a homologous protein of known structure, LytM, the ligands in zoocin are tentatively assigned to H45, D49, H133, and some combination of water or buffer ions as the fourth oxygen donor in zoocin A. Comparison of the combined intensities for ¹¹³Cd-substituted zoocin with a known quantity of another Cd-substituted protein gave Cd-binding as approximately stoichiometric (1.2 +/- 0.2) with protein. Additional metal-removal and reconstitution experiments on the recombinant catalytic domain of zoocin implicate Zn²⁺ as the metal cofactor. Therefore, the evidence supports zoocin as a single Zn ion binding metalloenzyme.

Keywords

zoocin; lysostaphin; LytM; zinc metallopeptidase; Cd-NMR

Introduction

Select strains of *Streptococcus equi* subspecies *zooepidemicus* secrete an extracellular protein zoocin A which has potent bacteriolytic activity against related strains of streptococci, including the common pathogens that cause group A streptococcal sore throat and dental caries [1,2]. With the rapid rise in drug-resistant bacteria, novel approaches are needed for the control of human and animal pathogens. The potential use of lytic enzymes such as zoocin A, to treat streptococcal infections, lysostaphin [3,4] to treat methicillin- and vancomycin-resistant *Staphylococcus aureus* infections, or the phage lysin PlyG [5] to treat *Bacillus anthracis* infections are just a few examples of such enzymes that offer an attractive alternative to conventional antibiotic therapy.

Zoocin A has been cloned and its primary sequence determined [6]. The protein is composed of two domains, an N-terminal catalytic domain (CAT) followed by a threonine-proline rich linker sequence, and then the target-recognition domain (TRD), which is responsible for

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specific recognition of cell wall architecture on sensitive strains [7]. Zoocin is a member of a superfamily of endopeptidases that selectively hydrolyze peptide cross bridges in peptidoglycans of the cell wall. For members who are bacteriolytic, the enzyme is secreted extracellularly and attacks sensitive strains thereby weakening their cell wall and causing its subsequent rupture. For members who are autolysins, the enzyme acts on the hosts own cell wall short of rupture to allow for cell expansion during growth. The precise site of hydrolysis for zoocin has not yet been demonstrated, but probably involves alanyl residues in the cross bridges of sensitive strain peptidoglycans since the enzyme is a penicillin-binding protein [8]. Other members of this family have been shown to be glycyl-glycine peptidases. CAT has strong homology, including circa 40% identity, to the N-terminal domain of lysostaphin, an extracellular bacteriolytic Zn-endopeptidase specific for glycyl residues from *Staphylococcus simulans* biovar *staphylolyticus* that targets the cell wall of related staphylococcal species including *S. aureus* [9]. LytM is an autolysin from *Staphylococcus aureus* that is also a Zn-endopeptidase specific for glycyl residues [10]. The catalytic region of LytM shows homology to both zoocin (35% identities, 54% positive matches by BLAST 2 alignment) and lysostaphin (48% identities, 63% positive matches). Zoocin has been over-expressed and purified from *Escherichia coli* (rZOO) as have the catalytic (rCAT) and target-recognition (rTRD) domains, each with only half of the putative TP-linkers and all with an N-terminal MRGS(H)₆ hexahistidine tag for Ni-affinity column purification [7].

The homology of CAT to the active domains of lysostaphin and LytM makes it probable that zoocin is also a zinc metallopeptidase, but, direct experimental evidence for the presence of zinc in zoocin has been difficult to obtain. Standard metal analysis of purified and exhaustively dialyzed zoocin proved problematic. Inductively coupled plasma emission spectrometry (ICP) did detect elevated levels of zinc in rZOO and rCAT, but not in rTRD, but at a less than stoichiometric value of circa 0.3 equivalents per equivalent of protein. Other metals were detected at higher or comparable values, including K (0.6 eq.), Na (2.3 eq.), P (5 eq.), Ni (0.25 eq.), B (0.1 eq.), and Ca (0.1 eq.), which brought into question the validity of the results. rZOO, rCAT, and rTRD were also mass analyzed in a FT-MS instrument with electrospray sample injection in the hope that the apparent mass would indicate the presence of zinc. All three proteins bound large amounts of K⁺ and Na⁺ which obscured whether any Zn⁺² was present. The detection of potassium and sodium adducts in mass spectral samples that are supposed to be free of these metals is a common occurrence and does not necessarily imply such binding is physiologically relevant. These difficulties forced adoption of indirect methods based upon NMR to probe the presumed metal binding site of zoocin. The results provide additional information on the identity of metal ligands in the active site and lay a necessary foundation for identifying the active site amino acid residues.

Materials and Methods

Natural abundance recombinant protein samples and ¹⁵N-enriched rCAT were prepared as described previously and quantified by extinction coefficients at 280 nm as given previously [11]. The blue copper protein azurin was isolated from *Pseudomonas aeruginosa* (ATCC19429) as described previously and quantified by its extinction coefficient of 5.7 mM⁻¹ at 625 nm or 14.0 mM⁻¹ at 280 nm for the Cu⁺²-holoprotein [12]. Metal analysis by ICP was performed at the Chemical Analysis Laboratory of the University of Georgia. FT-MS analysis was performed at the University of Alabama on a Bruker BioApex 47E instrument with electrospray inlet with the kind assistance of Dr. Carolyn Cassady. ¹¹³Cd-NMR experiments were performed on a Bruker Avance 500 spectrometer in 5 mm or 10 mm probes. Two dimensional NMR experiments were performed on a Bruker Avance 600 spectrometer in a 5 mm triple resonance probe.

Apo-rZOO was prepared by treatment with 5 mM disodium EDTA, or 10 mM 1,10-phenanthroline in 10 mM potassium phosphate buffer, pH 6.8, for 6-8 hr, followed by dialysis against four changes of 10 mM sodium acetate buffer, pH 5.6, and at least 5 hr in between buffer changes. Apo- ^{15}N -rCAT was prepared directly in a 5 mm NMR tube. To a solution 0.4 mM in protein in 10 mM potassium phosphate buffer, pH 7.0, was added an aliquot of 200 mM disodium EDTA in the same buffer at pH 7.0, to a final concentration of 4 mM EDTA. Two dimensional NMR spectra were recorded as described below and under Results. Apo-azurin was prepared by dialysis of Cu^{+1} azurin against 50 mM potassium cyanide in 25 mM tris-acetate, pH 8.0, followed by dialysis versus just tris-acetate, pH 8.0.

^{113}Cd reconstitution was accomplished by addition to apo-proteins of 94% enriched $^{113}\text{CdCl}_2$ (Cambridge Isotope Labs) normally to a final concentration of 5 mM. Protein concentrations ranged from 0.7 to 2.1 mM, so the $^{113}\text{Cd}^{+2}$ was in 2.4 to 7-fold excess. In select experiments 0.9 equivalents of $^{113}\text{Cd}^{+2}$ was added to test the effect of sub-stoichiometric reconstitution. Apo- ^{15}N -CAT was unstable and began to precipitate shortly after addition of EDTA. After obtaining a final NMR spectrum after 24 hr, ZnCl_2 was added to a final concentration of 12 mM (4-fold excess over EDTA) to regenerate rapidly the metal-bound form. Excess Zn^{+2} and EDTA were removed by dialysis and the precipitate removed by centrifugation. Treatment with Zn^{+2} failed to re-solubilize precipitated protein and only 4% of the original holoprotein could be recovered. As described below however, this was sufficient to obtain a two-dimensional NMR spectrum by using longer data averaging.

Initial ^{113}Cd -NMR spectra were taken with wide spectral widths in order to locate resonances of interest at correct chemical shifts without fold-ins. Once found, the spectral width was decreased in order to improve digital resolution and noise reduction. While ^{113}Cd has relatively good sensitivity for a metal, data acquisition still takes hours rather than seconds. Spectra such as Fig. 1A were obtained on a 2.1 mM protein sample using 8196 data points over a spectral width of 198 ppm with a digital resolution of 2.7 Hz employing a 45° transmitter pulse, an acquisition time of 0.19 s, a recycle delay of 1 s, and accumulation of 57,224 transients at 302K. Exponential line broadening of 100 Hz was used in the processing, which is close to a digital matched filter. The natural linewidths (full width at half height) were 132 Hz which is similar to the 110 Hz linewidths observed for ^{113}Cd -substituted gene 32 protein from bacteriophage T4 which is comparable in size to rZOO (232 residues compared to 274 for rZOO) [13]. ^{113}Cd chemical shifts are reported versus external 0.1 M natural abundance $\text{Cd}(\text{ClO}_4)_2$.

Two dimensional ^{15}N - ^1H homonuclear single quantum correlation spectra (^{15}N -hsqc, see reference [14] and citations to the earlier literature) were obtained at 299K at a protein concentration of 0.4 mM before and immediately after the addition of EDTA in 10 mM potassium phosphate, pH 7, plus 10% deuterium oxide. A standard Bruker pulse program, hsqcetgpsi with Z-gradients, was used. In the ^1H dimension, 1024 data points were spread over a 16 ppm window, while in the ^{15}N dimension 128 data points were spread over a 35.3 ppm window. Acquisition time was 0.053 s, broadband GARP ^{15}N decoupling was employed during acquisition with a recycle delay of 1 s, and the entire time to complete one data set was 1.25 hr. Data were processed with the NMRPipe program [15] and apodized with two sine squared functions and zero-filled to 2048 (^1H) and 1024 (^{15}N) points. The same spectral parameters were employed to obtain spectra after reconstitution with Zn^{+2} , dialysis to remove excess ZnCl_2 and EDTA, and centrifugation to remove precipitated protein, except the number of transients averaged was increased so that the experiment required 24 hr to complete.

Streptococcolytic activity of rZOO and Cd-rZOO was determined by use of a dye-release assay with Remazol brilliant blue-labeled, heat-killed cells of *Streptococcus pyogenes* FF22 as substrate as previously described [7].

Numbering amino acid residues for native zoocin, its recombinant forms, and homologs related to it, gets very confusing because of the insertion of hexa-histidine tags, variable threonine-proline linker sequences, and post-translational proteolytic cleavage of the homologs. Discussion of specific amino acid residues in zoocin and any of its recombinant forms will use the sequence numbering of NCIB entry gi/2804351, while residues for lysostaphin will use NCIB entry gi/2072411. Discussion of the homolog LytM will use the numbering scheme adopted in the publication of its crystal structures [16,17], which are 25 higher than the sequence in NCIB entry gi/42543452 which is missing a 25 residue leader sequence.

Results and Discussion

Apo-rZOO could be prepared by treatment with either EDTA or 1,10-phenanthroline as described in the second paragraph of the Materials and Methods section. EDTA treatment became the preferred method for most of the experiments to be described. As will be further discussed, relatively low levels and short exposure time for EDTA proved efficient at removing metal, suggesting that the binding affinity is not extraordinarily high. This might explain why the metal analyses done on exhaustively dialyzed zoocin showed less than a stoichiometric amount of zinc present.

Cd^{+2} is an excellent surrogate/mimic for Zn^{+2} because of its ionic radius and ligand preferences. The ^{113}Cd isotope is NMR active with relatively good sensitivity and has been used to probe metalloprotein active sites [18-20]. ^{113}Cd -NMR of apo-rZOO reconstituted with excess $^{113}\text{Cd}^{+2}$ confirmed that this ion indeed bound to the protein. A major resonance was detected at 113.6 ppm relative to $\text{Cd}(\text{ClO}_4)_2$ as 0 ppm, with a minor peak at 87.3 ppm as shown in Fig. 1A. An important feature of biological ^{113}Cd -NMR is that it has been well established that the chemical shift of protein-bound $^{113}\text{Cd}^{+2}$ is a sensitive indicator of the identity of the ligand atoms directly bound to the metal [18] and to the number of ligands [21]. The chemical shifts of both major and minor peaks for ^{113}Cd -rZOO fall well within the window that represents two nitrogens and two oxygens binding to the metal.

Crystal structures have been determined for inactive [16] and active [17] forms of the zoocin/lysostaphin homolog LytM. The inactive form has side chain nitrogens of H210 and H293, and side chain oxygens of N117 and D214 as ligands, while the active form has H210, H293, D214, and water or a buffer anion as ligands. The active form of LytM is produced by proteolytic cleavage of the N-terminal portion of the whole length inactive LytM that contains N117. There is no such activation sequence for zoocin and its N-terminal domain is CAT while TRD is C-terminal. There is therefore no homology to the N-terminal domain of LytM and hence no homolog for N117. However, both zoocin and lysostaphin have homologs to the other active site residues in LytM. For LytM H210, these are H289 (lysostaphin) and H45 (zoocin); for LytM D214, these are D293 (lysostaphin) and D49 (zoocin); and for LytM H293, these are H362 (lysostaphin) and H133 (zoocin).

The minor peak of ^{113}Cd -rZOO is in the same window for 2N+2O ligands and so cannot represent an atom type switch, but it could represent an alteration in the identity of the donor. Active LytM was crystallized in different forms and a confusing substitution of the fourth O-ligand was observed [17]. Depending upon crystallization buffer, phosphate, cacodylate, or tartrate was found, while the expectation was that water would be the physiologically relevant ligand in analogy to other zinc metalloproteases. In its preparation, ^{113}Cd -rZOO was exposed to phosphate, acetate, and chloride containing buffers, and the major and minor peaks could represent some variable combination of these plus water as fourth ligand. The situation is complicated by the observation that after storage for 1 week, the main ^{113}Cd peak at 113.6 ppm split into two peaks at 113.6 and 107.2 ppm as shown in Fig. 1B, and they decreased in intensity while the 87.3 ppm peak increased. This could represent further ligand heterogeneity

with presumably acetate or chloride (in the NMR buffer) displacing phosphate (from the preparation buffer) or water. Another possibility is that the active site is experiencing some conformation change slow on the NMR time scale that while not an actual ligand substitution reaction, does alter the site geometry and perturb the Cd resonances. Whatever its cause, the evolution of the ^{113}Cd -NMR spectra did not affect the biological activity of ^{113}Cd -rZOO. Apo-rZOO had negligible activity while freshly prepared ^{113}Cd -rZOO showed 80% of the dye-release activity of native rZOO and a sample re-assayed after 7 days of storage showed 85% activity with the increase not being statistically significant.

There was concern that the hexa-histidine tag of rZOO might constitute a metal binding site. This is unlikely because at the pH of the experiment, 5.6, the unstructured histidine rings will all be protonated and hence poor ligands for Cd^{+2} . Nevertheless, it was possible to test for binding to hexa-histidine by observing the ^{113}Cd -NMR spectrum of rTRD with the same hexa-histidine tag in the presence of an excess of $^{113}\text{Cd}^{+2}$. No peaks were observed in any window associated with Cd bound to protein and the added ^{113}Cd appeared as a broad (half-width at half height of 1720 Hz) resonance at 15.5 ppm due to a complex of Cd^{+2} with buffer acetate in rapid exchange with chloride ligand(s) as confirmed in a protein-less control experiment.

Azurin is a blue copper electron transport protein found especially in pseudomonads. Cd^{+2} is also a good surrogate/mimic for Cu^{+2} , and the preparation and ^{113}Cd -NMR of Cd-azurin has been reported [22]. Apo-azurin was prepared by a standard protocol to remove its single Cu cofactor. In this case, visible spectrophotometry was used to ensure that copper removal was greater than 95% because of the intense visible band at 625 nm for Cu^{+2} -azurin. Reconstituted ^{113}Cd -azurin gave rise to a ^{113}Cd resonance at 371.2 ppm as reported previously [22]. This resonance for a known quantity of ^{113}Cd -azurin was used as an intensity standard for ^{113}Cd -rZOO. T_1 for ^{113}Cd -rZOO was not determined due to spectrometer time limitations and the relatively weak signals for ^{113}Cd -rZOO. But the use of a sub-ninety degree excitation pulse and a recycle delay of $5 \times T_1$ as reported for azurin [22], suggests that relaxation would not affect the intensity measurements. Using reasonable estimates for the accuracy of peak integration in a somewhat noisy spectrum such as Fig. 1A, the Cd content of ^{113}Cd -rZOO was measured as 1.2 ± 0.2 equivalents of Cd^{+2} per equivalent of rZOO.

The initial work was done with a slight excess of Cd^{+2} because the ease and rapidity with which the native protein lost Zn^{+2} suggested that metal binding might not be very strong, yet for optimal detection the site should be fully occupied. The appearance of multiple ^{113}Cd -resonances raised the issue of whether some of these might reflect additional binding sites in the protein. Several considerations argue against this. Metal-binding reactions are generally fast, while the new peaks appeared over days. On the other hand the buffer exchange reactions observed in the crystallography of LytM were slow compared to the time to grow crystals. The quantification of the Cd content in rZOO versus the ^{113}Cd -azurin standard is only approximate, but is accurate enough to tell the difference between one versus two equivalents per protein. The total area of protein ^{113}Cd resonances stayed the same in the presence of excess Cd^{+2} while the original dominant peak decreased in intensity and the minor peaks increased. Samples were reconstituted with 0.9 equivalents of $^{113}\text{Cd}^{+2}$. A comparable three peak pattern was still seen, but there was an effect. The line widths of the 107 and 87 ppm resonances were much broader, increasing to 500 Hz, while the 113.6 ppm resonance retained a narrower linewidth of 140 Hz (132 Hz in Fig. 1A). Addition of more $^{113}\text{Cd}^{+2}$ to a total of 2 equivalents sharpened the lines to what was seen previously. It is even more difficult to integrate accurately the broader lines, but, the total resonance area appeared approximately constant. At the present time there is no obvious explanation of what must be a dynamic process on the NMR time scale, however, these resonances are still within the 2N+2O ligand window and that remains the main conclusion.

While these results are supportive of zoocin being a single metal peptidase, zinc has not been directly identified as that metal. Additional evidence for Zn^{+2} binding came from the preparation of apo-rCAT and its reconstitution with Zn^{+2} . The ^{113}Cd -NMR experiments were done with rZOO where the large protein size (29.2 kDa) contributes to a dramatic lowering of the ^{113}Cd T_1 relaxation time from minutes to seconds. The next experiments were done on uniformly ^{15}N -labelled rCAT (16.7 kDa) in order to simplify the spectral features to be examined.

A two dimensional ^{15}N - ^1H homonuclear single quantum correlation spectrum (^{15}N -hsqc) shows the chemical shifts of main chain peptide and side chain amide nitrogens and the chemical shift of the attached protons [14]. The spectral region encompassed is frequently referred to as the “fingerprint” region of a protein because it is very sensitive to the folded conformation of the protein and is an excellent probe of minor conformational changes that may occur in that fold. ^{15}N -rCAT was treated with an excess of EDTA and its ^{15}N -hsqc spectrum recorded as a function of time. Changes could be seen within the first hour of exposure and were complete after 24 hr. Changes affected select, but not all, crosspeaks as illustrated in Fig. 2. The subset of affected peaks was interpreted as peptide amides at or near the metal binding site. Complete assignment of these crosspeaks is not currently available, but additional work on such is in progress. Once obtained, the data should identify the active site region by specific residues and confirm or disprove the site assigned previously in this article based upon homologies with LytM.

Apo- ^{15}N -rCAT was dialyzed to remove metal and EDTA. Unfortunately, apo- ^{15}N -CAT was labile and precipitated during this treatment so that only 4% of the starting protein could be recovered. Apo-rZOO had been much more stable. After reconstitution with excess zinc and sample clean-up to remove excess reagents and precipitated protein, another ^{15}N -hsqc spectrum was recorded. While the noise level was higher due to protein loss, it could be seen to have reverted to the original holo- ^{15}N -rCAT spectrum as illustrated in Fig. 2C.

The body of evidence is thus supportive of zoocin being a Zn-metallopeptidase with a single Zn^{+2} ion at its active site in the CAT domain.

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Abbreviations

rZOO, recombinant zoocin A; rCAT, recombinant catalytic domain of zoocin A; rTRD, recombinant target recognition domain of zoocin A; ICP, inductively coupled plasma emission spectrometry; FT-MS, Fourier transform mass spectrometry; ^{15}N -hsqc, ^{15}N - ^1H homonuclear single quantum correlation spectroscopy; NCIB, National Center for Biotechnology Information, see their web site at www.ncbi.nlm.gov; T_1 , spinlattice relaxation time.

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Fig. 1.
A. ^{113}Cd -NMR spectrum of ^{113}Cd -rZOO in 10 mM sodium acetate buffer, pH 5.6, at 302K in the presence of excess ^{113}Cd . The relative intensities of the major peak at 113.6 ppm to the minor peak at 87.3 ppm is 0.84 to 0.16. B. Sample as in A, but after storage for 1 week. The relative intensities for the peaks at 113.6, 107.2, and 87.3 ppm are 0.39, 0.30, and 0.31. The excess ^{113}Cd appeared as a resonance at 15.5 ppm.

Fig. 2.

A select region of the ^{15}N -hsqa spectrum of ^{15}N -rCAT illustrating how metal removal perturbed select fingerprint NH crosspeaks, while reconstitution with Zn^{+2} restored a native-like spectrum: A, native ^{15}N -rCAT; B, after incubation with EDTA; C, after reconstitution with Zn^{+2} and sample clean-up to remove excess reagents and precipitated protein. The spectrum represented in C was weaker and had considerably more noise than A or B, because of massive protein loss due to the instability of apo-rCAT. So the contour level for the two-dimensional plot had to be raised to avoid the noise level and this makes the crosspeaks in C appear artificially thinner.