FOR THE RECORD

The identification of metal-binding ligand residues in metalloproteins using nuclear magnetic resonance spectroscopy

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(RECEIVED April 17, 1998; ACCEPTED June 1, 1998)

Abstract: The identification of metal-binding ligands in metalloproteins is an important step in gaining detailed information regarding the environment of the active site. Traditionally, techniques such as ¹¹³Cd-substitution for the active metal followed by isotope-filtered NMR techniques have been used to this end. However, for medium to high molecular weight proteins (>20 kDa), these experiments may not be beneficial due to extensive ¹H spectral overlap. Here, we describe an alternative approach, where metal-binding ligands such as histidine and cysteine are specifically ¹⁵N backbone labeled, excess EDTA is added and changes to {¹H-¹⁵N} HSQC spectra are followed. Under these conditions, the amide groups of all ¹⁵N labeled histidine and cysteine residues, which were either ligands or residues close to the active site, were identified unambiguously for metallo- β -lactamase from *Bacteroides fragilis*.

Keywords: metal binding ligands; metallo- β -lactamase; metalloprotein; NMR

NMR spectroscopy has been used successfully to characterize both the environment of the metal and its ligands in metalloenzymes and metalloproteins. For example, the substitution of the NMR active metal ¹¹³Cd, for biologically significant metals such as zinc, iron, and calcium, has allowed both the coordination geometry and ligand types to be determined with great accuracy (Coleman, 1993). The ¹¹³Cd-based NMR experiments have also been used to identify side-chain proton resonances of the common zinc-binding ligands, cysteine and histidine in small protein, and model peptide systems (Frey et al., 1985; South et al., 1989; Blake et al., 1994; Gardner & Coleman, 1994). However, the unambiguous identi-

cation of specific ligands using these methods becomes increasingly difficult at higher molecular weights (>20 kDa), since severe overlap in both the ¹H aliphatic and aromatic regions can obscure individual resonances. Here we describe a method for identifying metal-binding ligands in large metalloproteins involving the specific ¹⁵N backbone labeling of metal-ligand types (His and Cys), and the addition of EDTA to sequester the metal from the protein. Specific assignment of ligand-binding residues is accomplished by first identifying resonances in ¹⁵N HSQC spectra, which are affected by the addition of EDTA and then correlating these to ¹³C nuclei of the preceding residue through CBCA(CO)NH (Grzesiek & Bax, 1992) or C(CO)NH-TOCSY (Grzesiek et al., 1993) experiments on the holoprotein.

This approach was applied to the 25.5 kDa metallo- β -lactamase (CcrA) from *Bacteroides fragilis* (Yang et al., 1992), which is a binuclear zinc-containing enzyme exhibiting the broadest range of resistance to traditional lactam-based antibiotics (Payne, 1993). The crystal structure of CcrA shows that, in addition to water/hydroxide molecules and a side-chain carboxylate (Asp103), four of the eight histidines (99, 101, 162, and 223) and one of three cysteines (104) are involved in metal binding (Concha et al., 1996). Due to its large size, poor signal dispersion is evident for both the aromatic and aliphatic ¹H regions. Combined with its propensity to aggregate at concentrations greater than 125 μ M (S. Scrofani, P.E. Wright, & H.J. Dyson, unpubl. results), the identification of metal ligands through approaches such as ¹¹³Cd substitution is impractical.

The ¹⁵N HSQC spectrum of CcrA specifically labeled with ¹⁵N-cysteine shows intense signals for each of the three cysteine residues (Fig. 1A). However, the lack of unambiguous sequential connectivities and the fact that the preceding residue is identical for two of the three cysteines (Gly precedes both Cys155 and Cys181) made it difficult to differentiate between them. Conversely, Cys104 was readily identified through the characteristic C α /C β shifts of its preceding residue, Asp103. The addition of four equivalents of EDTA to this specifically labeled CcrA, resulted in the immediate loss of signal for two of the three cysteines. The third cysteine was virtually unaffected (Fig. 1B) and was assigned

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Fig. 1. ^{15}N HSQC experiments on (A) ^{15}N Cys and (C) ^{15}N His specifically labeled CcrA in 95% $^1\text{H}_2\text{O}$ /5% $^2\text{H}_2\text{O}$. The spectrum acquired immediately after addition of four equivalents of EDTA is shown for (B) ^{15}N Cys- and (D) ^{15}N His-labeled CcrA. The asterisk indicates the presence of a His residue in the unfolded conformation.

to Cys155 since this residue is distant from the metal site. The loss of signals for Cys104 and Cys181 is directly related to the loss of the Zn^{2+} upon addition of EDTA, presumably due to an increase in local flexibility as a result of the loss of the metal ions. Although Cys104 does not appear from the crystal structure to be coordinated to the metals (Concha et al., 1996), it is nevertheless in close proximity to the metal site. The remaining cysteine resonance is assigned to the ligand Cys181 by elimination.

Metallo- β -lactamase possesses eight histidine residues, four of which are involved in metal binding (Concha et al., 1996). Resonances are observed for each of the eight histidines in the ^{15}N HSQC spectrum of specific ^{15}N -His labeled CcrA (Fig. 1C). However, the identification of ligand-binding candidates using the same technique described for cysteines was more difficult, since only three of the eight histidines (89, 138, 223) had been assigned unambiguously using triple resonance techniques. The combined use of

HSQC experiments with ^{15}N His labeled CcrA and CBCA(CO)NH experiments with the uniformly ^{15}N , ^{13}C -labeled protein allowed His162 to be assigned straightforwardly, since it was the only unassigned histidine with a glycine as the preceding residue. However, difficulties were encountered in assigning the remaining histidines since the $\text{C}^\alpha/\text{C}^\beta$ chemical shift patterns of the preceding residues were not unique. In particular, the preceding residue for both His63 and His99 (metal ligand) is asparagine, whereas His101 (metal-ligand) and His234 are located adjacent to Trp100 and Glu 233, respectively. Although in principle histidines 101 and 234 should be differentiated easily using the C(CO)NH-TOCSY experiment, poor magnetization transfer precluded the extension of assignments beyond the C^β nucleus for Glu233.

The addition of excess EDTA to the solution was found to be key in making the specific assignments. Upon addition of EDTA, the cross peak for the previously assigned His223 was lost (Fig. 1D), consistent with observations made with the ligand Cys181 in ^{15}N -Cys CcrA (Fig. 1B). The cross peak belonging to one of the two histidines preceded by Asn also disappears under these conditions; it was identified as the ligand residue His99, thereby allowing His63 to be assigned by elimination. Of the two remaining unassigned resonances, one disappears completely upon the addition of EDTA, allowing it to be assigned specifically to the remaining metal ligand His101, and identifying the remaining nonligand resonance as His234. This method has enabled us to complete the sequence-specific resonance assignments for CcrA (Scrofani et al., 1998). Specific resonance assignments can be obtained in a short time using this method, and it does not require the use of auxotrophic strains, since the levels of labeling are sufficient for NMR studies when the labeled amino acids are added at the time of induction.

Peak intensities in the zinc-containing protein were significantly weaker for three of the histidines (63, 89, and 162) than for those of the other histidines (Fig. 1C). From the crystal structure (Concha et al., 1996), His63 and His89 exhibit comparatively higher temperature factors and are located in turn regions; their broadened signals may be due to local motions intermediate on the NMR timescale. However, the metal ligand His162 exhibits comparable or smaller temperature factors to the remaining histidines and is located in a relatively well-structured region of the crystal structure. Whether the weak signal of His162 is due to mobility or is related to its metal-binding role remains to be seen, and ^{15}N backbone dynamics experiments are currently under way to address this question.

While the metallo- β -lactamases from *Bacillus cereus* (BCII) and *B. fragilis* have the same zinc binding ligands, there are significant differences in the binding affinity of the metals. In BCII, kinetic studies indicate that the binding affinity of each zinc ion differ by three orders of magnitude (Baldwin et al., 1978; Sutton et al., 1987), whereas both metals appear to possess similar binding affinities in CcrA (Concha et al., 1996; Crowder et al., 1996). A possible cause for this significant difference in metal affinity may involve the substitution of Cys104 in CcrA for an arginine in BCII. In contrast to CcrA (Concha et al., 1996), the crystal structure of BCII shows the presence of a single zinc ion, equivalent to Zn1 in CcrA (Carfi et al., 1995). Although it is uncertain whether the absence of the second zinc is an artifact of the experimental conditions, the arginine is located in close proximity to the ligands of the pentacoordinate zinc and will likely cause major differences in local electrostatic interactions from those of the cysteine in CcrA (Concha et al., 1996). As described earlier, addition of a

fourfold excess of EDTA to selective ^{15}N backbone Cys-labeled CcrA shows that Cys104 disappears along with the metal-binding residue Cys181, whereas the distant cysteine, Cys155 is unaffected (Fig. 1A,B). The fact that the nonligand Cys104 resonance disappears suggests that it is intimately associated with the active site and may be involved in electrostatic interactions, which allow both metal ions to bind in CcrA with similar strength.

A combined approach utilizing triple resonance experiments with fully $^{13}\text{C}/^{15}\text{N}$ -labeled protein and selective ^{15}N -His and -Cys (or other ligand) labeled protein in the presence and absence of EDTA is a convenient method for identifying likely candidates for ligands in metal proteins. In the case of CcrA, the addition of EDTA caused the loss of specific cross peaks in the ^{15}N - ^1H HSQC spectrum, most probably due to a change in the conformation and/or dynamics of the polypeptide in the vicinity of the zinc binding site as a consequence of loss of the metal. Other systems will most likely show different levels of influence of the addition of metal chelators. For example, if the conformation of the metal site in the absence of metal is quite similar to that in the metal-bound protein, shifts of the cross peaks due to a change in the local electrostatic environment after loss of the metal ion might be observed, rather than loss of the peaks altogether. In summary, the use of metal chelators as a means of identifying metal ligands in specifically-labeled proteins has been of use in the sequence-specific assignment of the resonances of a sizable zinc protein, and should prove a useful technique in other systems.

Materials and methods: The plasmid pT7CcrANDEO2 was transformed into *Escherichia coli* BL21(DE3) cells. A 40 mL culture grown in LB media was grown overnight (30 °C) and used to inoculate 1 L LB media. Specific ^{15}N labeling of cysteine and histidine backbone amides were individually prepared by adding the appropriate labeled amino acid (80 mg/L) at the time of induction (1 mM IPTG). This was followed immediately by the addition of 100 μM ZnSO_4 . Cultures were grown for an additional 2 h before harvesting. The purification protocol will be described elsewhere.

The gradient $\{^1\text{H}-^{15}\text{N}\}$ HSQC experiments were performed using standard methods (Zhang et al., 1994). Spectra were acquired on a Bruker AMXII-500 spectrometer at 295 K. ^1H chemical shifts were referenced to an external DSS standard (0 ppm), and indirectly for the ^{15}N dimension (Wishart et al., 1995). Quadrature detection was achieved using the States-TPPI method (Marion et al., 1989) in the indirect dimension. For each spectrum, 128 real t_1 (^{15}N) and 512 complex t_2 (^1H) points were acquired with spectral widths of 1,315.8 and 4,032.3 Hz, respectively.

Acknowledgments: Support to S.D.B.S from the Fulbright Postdoctoral Fellowship is gratefully acknowledged. This grant was supported by Grant GM 56879 from the National Institutes of Health. We thank Dr. Michael J. Osborne for useful discussions.

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