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Characterisation of a novel cysteine/histidine-rich metal binding domain from *Xenopus* nuclear factor XNF7

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A 42 amino acid synthetic peptide corresponding to a newly defined cysteine/histidine-rich protein motif called B-box, from the *Xenopus* protein XNF7 has been characterised. The metal-binding stoichiometry and dissociation constant for zinc were determined by competition with the chromophoric chelator Br_2BAPTA, demonstrating that one zinc atom binds per molecule of peptide despite the presence of seven putative metal ligands, and represents the first application of this method to measuring zinc stoichiometry of proteins and/or peptides. Cobalt binding studies indicate that the motif binds zinc more tightly than cobalt, that cysteines are used as ligands and that the cation is co-ordinated tetrahedrally. Circular dichroism and NMR studies both indicate that the B-box peptide is structured only in the presence of zinc, copper and to a lesser extent cobalt.

Protein motif; Zinc finger; Metal binding

1. INTRODUCTION

Many cellular processes such as transcription, RNA processing and replication are regulated by protein–DNA and protein–protein interactions. For this purpose numerous protein structural motifs such as the homeodomain, basic domain, leucine zipper, helix-loop-helix and the zinc finger have evolved (for reviews see [1–3]). Each of these domains is usually characterised by the conservation of some primary sequence which generally leads to a conservation of tertiary structure [4]. Amongst these motifs are a number of well-characterised zinc-binding domains which include the classical TFIIIA-like zinc finger and the steroid receptor and GAL4 DNA-binding domains (for reviews see [5,6]). A common feature of these types of structural motifs is the use of cysteines and/or histidines for zinc binding and tertiary structure stabilisation, although there appears to be little overall structural similarity. A number of other putative cysteine/histidine-rich metal binding motifs have been identified on the basis of sequence homology searches [7–9] and it is increasingly clear that there are a large number of diverse metal-binding domains which use cysteine and histidine residues for metal ligation and structural framework formation. Interestingly not all of these domains have DNA-binding properties, for example the cysteine-rich domain of protein kinase C which is implicated in binding phorbol esters [10]. The HIV Tat protein is another example, where the cysteine-rich domain mediates homo-dimerisation in a zinc-dependent manner [11].

Recently, we have identified a novel cysteine-rich motif called the RING finger which defines a new family of proteins all of which have putative functions involving nucleic-acid interaction [12–14]. The motif is simply defined as Cys-X(2)-Cys-X(9–27)-Cys-X(1–3)-His-X(2)-Cys-X(2)-Cys-X(4-48)-Cys-X(2)-Cys where X can be any amino acid and the bracketed numbers refer to the number of residues in the intervening sequences. We have shown that a peptide corresponding to the RING1 finger domain binds zinc with high affinity and DNA non-specifically in a zinc-dependent manner [13]. Amongst the RING finger family, there are a number of proteins which possess a second cysteine/histidine rich motif C-terminal to the RING finger, termed the B-box domain [15]. The motif is defined as Cys-X(2)-His-X(7)-Cys-X(7) - Cys-X(2)-Cys - X(5) - His -X(2) - His where X can be any amino acid and the bracketed numbers refer to the number of residues in the intervening sequences, although there are clear preferences for particular amino acids at certain positions between different family members [16]. Interestingly, B-box-containing proteins also include a predicted α- helical coiled-coil domain C-terminal to the B-box, thus forming a tripartite motif of the RING finger, B-box and α-helical coiled-coil [16,17] The spacing between the three ele-

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ments of the motif is highly conserved amongst the family suggesting that the positions of each domain relative to the others is of functional importance [16,17].

The B-box family comprises a number of transcription factors, ribonucleoproteins (RNPs) and proto-oncogenes which includes the ret finger protein (RFP) [18], PML [17–21] and T18 [17,22]. These proteins are oncogenic in humans and mice when found as translocations that include the RING and B-box fingers and α-helical coiled-coil domain recombined with other genes. PML and T18 possess two B-box like domains which appear to form a subgrouping of the B-box family as highlighted by the presence of an extra potential cysteine metal ligand [16]. The other members of the family include RPT-1 [23], SS-A/Ro [24], XNF7 [25] and PwA33 [26]. In order to understand the function of the B-box domain, both within this diverse family of proteins, and in relation to the RING finger and α-helical coiled-coil domains of the conserved tripartite motif, we have synthesised and purified a peptide corresponding to the B-box motif from the *Xenopus* protein XNF7. Here, we report the first data for the metal binding properties and secondary structure of a B-box domain. Due to the sequence similarities between B-box domains, we believe that the data for the XNF7 B-box will be applicable to other B-box domains.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis and purification*

The 42 amino acid peptide corresponding to the B-Box motif (residues 219–260 in the XNF7 sequence; [25]) was synthesised on a Model 431A Applied Biosystems Solid Phase Synthesiser on 4-hydroxymethylphenoxycarbonyl resin using 9-fluorenylmethyloxy-carbonyl for temporary α-amino group protection [27]. Each amino acid was coupled twice as a hydroxybenzatriazole active ester automatically formed prior to use. Cleavage from the resin and deprotection of the peptide was achieved with 90% (v/v) trifluoroacetic acid containing 7.5% (w/v) phenol, 0.2% (v/v) ethanedithiol, 0.5% (v/v) thioanisole and water at 20°C for 2 h. The yield of the 42-mer was approximately 20% and the peptide was subsequently purified by reverse-phase preparative HPLC on a Brownlee Aquapore ODS C18 column using 0.8% (v/v) trifluoracetic acid and acetonitrile gradient. The purity and molecular weight of the synthetic peptide was assessed by mass spectrometry. A few micrograms of the pure B-box peptide (peak fraction from HPLC separation) were analysed by Matrix-Assisted Laser Desorption mass spectrometry [28]. A single species was observed with an experimental molecular weight (M[H$^+$]) of 4,869 Da which is in excellent agreement with the calculated mass of 4,868.5 Da for the fully reduced form of the peptide. The concentration of B-box peptide was measured optically using a calculated extinction coefficient of 0.26 for 1 mg/ml solution measured at 280 nm. The sample was purified in the presence of ethanedithiol and in general subsequent experiments had trace amounts of ethanedithiol present.

2.2. *Optical binding studies using Br_2BAPTA*

Dissociation constants for the interaction of B-Box with Zn^{2+} were estimated by competition with the chromophoric chelator using the method originally developed for studying Ca^{2+}-protein interactions [29,30]. 5–5′-Br_2BAPTA (Br_2BAPTA, 5–5′-dibromo-1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) (40 μM) was titrated with Zn^{2+} in the presence and absence of 40 μM B-Box in a 10 mM Tris-HCl buffer (pH 7.5). Changes in the absorbance of the chromophore at 263 nm were monitored as Zn^{2+} was added.

2.3. *Optical binding studies of cobalt and zinc to the XNF7 B-box peptide*

Typically, a solution (1 ml) containing 160 μM of B-box peptide (10 mM Tris-HCl, pH 7.5) was titrated with solutions of $CoCl_2$ in the same buffer. The peptide was also titrated against solutions of $ZnCl_2$ in the presence of 100 μM $CoCl_2$. The binding reaction was monitored on an HP 8452 diode array spectrophotometer using a 1 cm path length at room temperature, and each spectrum was the average of three recorded spectra. The spectra have been corrected by subtracting the contribution from the peptide and buffer alone and also for dilution effects.

2.4. *Circular dichroism of the XNF7 B-box peptide*

Samples for CD spectroscopy were prepared by dilution of stock peptide solution into a buffer containing 20 mM PIPES (pH 7.6) plus either 0.05 mM EDTA or the required metal ion (as the chloride), generally at a concentration of 0.2 mM. Far UV CD spectra were recorded from 260 to 195 nm using a Jasco J-600 spectropolarimeter operated with a time constant of 0.5 s. The spectra were recorded at 22°C for peptide solutions of ~0.05 or 0.02 mg/ml in 2 or 5 mm fused silica cuvettes, respectively, and were the average of not less than four scans. All data reported here are presented as molar circular dichroism, $\Delta\varepsilon$, ($M^{-1}\cdot cm^{-1}$) based upon a mean residue weight of 112.7. Residue molar ellipticity [Q] ($deg\cdot cm^2\cdot decimol^{-1}$) may be obtained from the relationship $[Q]_{mrw} = 3{,}300\cdot\Delta\varepsilon$.

2.5. *NMR studies of the B-box peptide*

Approximately 10 mg of peptide were dissolved in 2 ml of 10 mM sodium phosphate, 100 mM KCl pH ~7.5 in either ^2H_2O or ^1H_2O depending on the requirements of the experiment. Small volumes of a concentrated $ZnCl_2$ solution were then added and the resulting degree of metal ligation was monitored via 1D ^{1}H NMR techniques (see section 3). Once the peptide was fully ligated (as determined from the NMR spectrum) the samples were concentrated to a final volume of 0.5 ml using Centricon 300 concentrators. The proton chemical shifts were referenced to 0.1 mM internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). All NMR experiments were carried out at 30°C. Sample concentration varied from 1.0 to 1.5 mM as determined by UV absorbance. Samples were not soluble at higher concentrations. 1D NMR experiments were collected at 14.1 T on a Varian Unity spectromenter and apodised with 2 Hz line broadening and Fourier transformed.

3. RESULTS AND DISCUSSION

3.1. *The B-box domain*

The boundaries of the B-box domain were chosen on the basis of a multiple sequence alignment of the B-box family and secondary structure predictions of the resultant alignment [31]. Consideration was also given to the known positions of the adjacent predicted α-helical coiled-coil domains and phosphorylation sites for XNF7 which exist between the RING finger domain and B-box [25,32]. This resulted in a domain comprising 42 amino acids bounded by predicted β-turn regions adjacent to both the C-terminal α-helical coiled-coil domain and N-terminal phosphorylation sites, respectively.

3.2. *BAPTA metal binding titrations*

Initial studies using CD methods (see below) indi-

cated that the B-box peptide preferentially bound zinc. In order therefore to determine both the number of zinc atoms bound per molecule of peptide and also the dissociation constant for zinc, we used a method developed for monitoring calcium binding to calmodulin [29,30]. This method works equally well for zinc and has the advantage that it can be carried out at low peptide concentrations. Thus, the chromophore Br_2BAPTA was titrated with zinc in the presence and absence of B-Box peptide as described in section 2 and the results are shown in Fig. 1. Titrations performed in the absence of peptide were used to estimate a dissociation constant for the Zn^{2+}-dye complex. The data were analysed using standard Marquardt non-linear least-squares methods with the assumption of 1:1 stoichiometry. The dissociation constant was calculated to be 92 ± 20 nM (average of three determinations).

Data obtained in the presence of peptide were analysed using the method developed by Linse et al. [29,30]. The data could be adequately described with the assumption that B-Box binds a single Zn^{2+} ion with a dissociation constant of 61 ± 12 nM (average of four determinations). However, we cannot exclude the possibility that B-Box binds a second Zn^{2+} ion with significantly lower affinity. Computer simulations (not shown) demonstrate that the binding of a second Zn^{2+} ion with a dissociation constant > 10 μM or higher would be observed with this method suggesting that any additional Zn^{2+} binding would be non-specific. A comparison of the B-box sequences suggests that up to seven residues could ligate the divalent cation. However, the

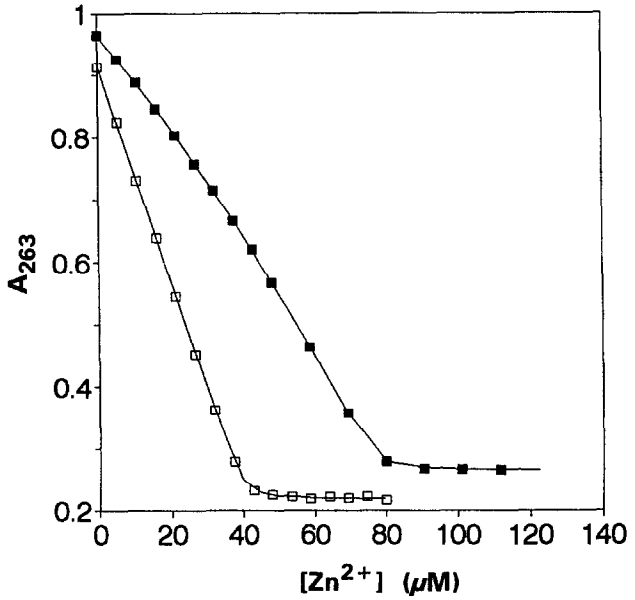

Fig. 1. Br_2BAPTA-zinc competition assays. Absorbance at 263 nm (A_{263}) is plotted against zinc concentration for the titration of 40 μM 5,5′-Br_2BAPTA in the presence (filled boxes) and absence (open boxes) of 40 μM B-Box. The initial and final A_{263} values are higher in the presence of B-Box which is due to peptide absorption and is assumed not to change upon Zn^{2+} binding.

data presented above precludes this possibility. The remaining conserved residues may be important for protein–protein interactions or in stabilisation of the tertiary fold. Another possible role for the other conserved ligands could be the formation of intermolecular dimers using divalent cations as observed for the HIV Tat protein [11], which could explain the precipitation observed in the presence of excess divalent cation (see below).

3.3. *Optical metal-binding studies*

The optical spectral properties of cobalt bound to synthetic zinc finger peptides and purified proteins have been studied extensively and provide information both on the nature of the amino acid ligands and the geometry of ligation [33–37]. A series of titrations of the purified XNF7 B-box peptide with solutions of cobalt showed absorption maxima at λ = 640–660 nm and shoulders at λ = 320 and 365 nm (Fig. 2). The 640–660 nm maxima are due to d–d transitions of Co (II) in a tetrahedral co-ordination, whilst the charge transfer bands at 300–365 nm are due to S-Co (II) co-ordination [33]. The results shown in Fig. 2 are therefore consistent with cobalt binding to the peptide using a number of cysteines as ligands in a tetrahedral co-ordination system. During the course of the cobalt titration, we observed no changes in the overall spectrum except at high concentrations of cobalt when the peptide precipitated and diminished the absorption characteristics of the cobalt–B-box complex. It should be noted, that CD measurements for the B-box peptide and cobalt (see below) show that cobalt, compared to zinc or copper, does not induce maximal secondary structure formation. Therefore, interpretations of the optically observed cobalt–peptide complex may be misleading. However, it is clear that titrations of the B-box peptide (160 μM) with zinc (10 μM) in the presence of 100 μM cobalt diminishes the characteristic cobalt transitions (Fig. 2b), suggesting that zinc displaces the cobalt and binds more tightly to the peptide than cobalt. However, it is possible that the metal binding sites for zinc and cobalt could be overlapping and not identical.

3.4. *Circular dichroism and 1D NMR studies*

Structure formation upon metal ligation was monitored using circular dichroism and NMR techniques. Fig. 3 shows the far-UV CD spectrum of the B-box peptide in aqueous solution and in the same buffer containing zinc. This region of the CD spectrum monitors the secondary structure content of the peptide. The spectrum of B-box in aqueous solutions shows an intense band at ~198 nm indicating that the peptide contains a high percentage of random or non-regular secondary structures. The addition of Zn^{2+} produces a significant increase in the amount of regular secondary structure, as shown by reduction in the intensity of the 198 nm band and the appearance of significant intensity at wavelengths >210 nm. The intensity observed at 222

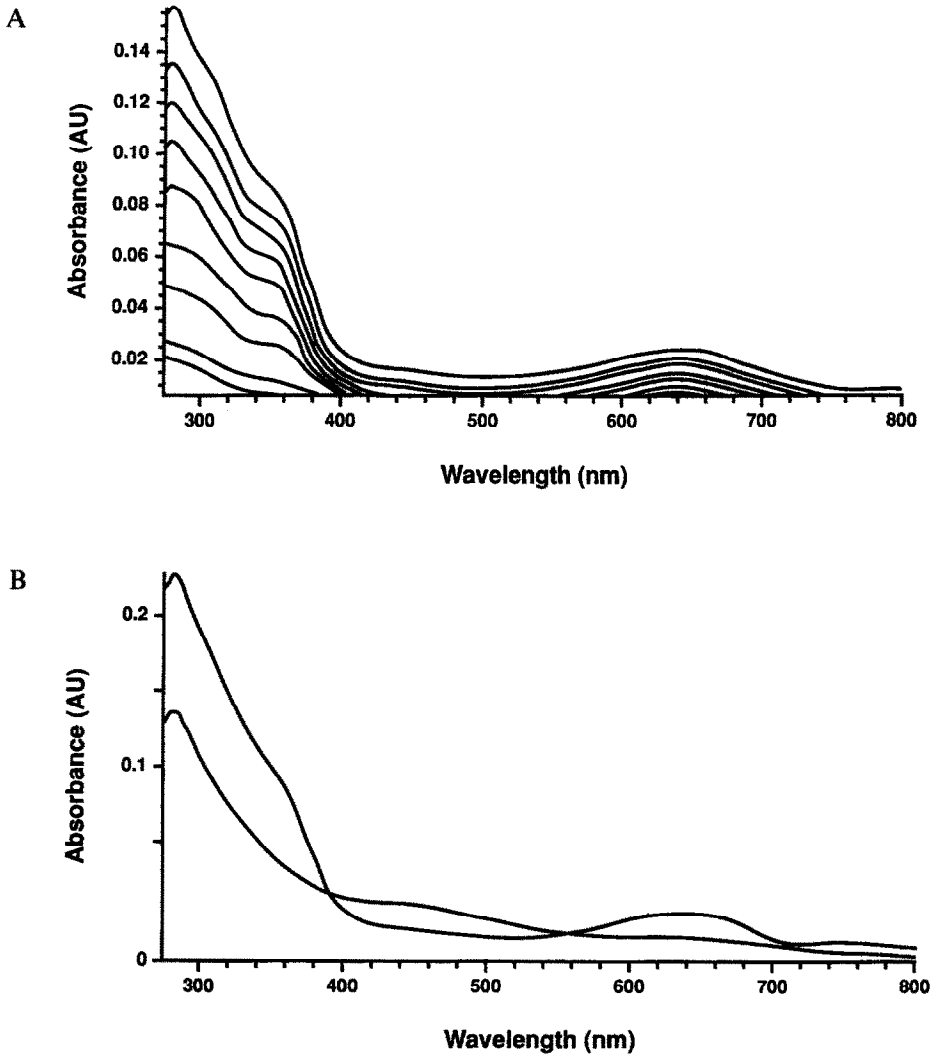

Fig. 2. Co^{2+} titration of the B-box peptide. (A) The spectra shown represent a single titration experiment of B-box peptide (158 μM). Co^{2+} was added from 2 to 27 μM in 5 μM increments and from 27 to 57 μM in 10 μM increments. (B) The spectra shown represents the addition of 10 μM Zn^{2+} (lower line) to a cobalt-peptide complex comprising 158 μM peptide and 100 μM Co^{2+} (upper line).

nm in the presence of Zn^{2+} suggests that the metal induces a maximum of 20–25% α-helix. The addition of excess EDTA to the Zn^{2+} containing solution reversed the Zn^{2+} induced change, as expected (see Fig. 3). Other divalent cations have been tested to determine whether they induce changes in secondary structure, the results of which are shown in Fig. 3. Cd^{2+} and Ca^{2+} induced only small changes in comparison to that observed with Zn^{2+}. Cu^{2+} binding induces a similar amount of helix as Zn^{2+} as does Co^{2+} but to a lesser extent (Fig. 3). Recently, copper has been shown to bind with high affinity to the normally zinc-binding estrogen receptor DNA-binding domain, using two cysteines per copper atom for ligation. The physiological significance of this observation is however unclear and may be artefactual [34].

In order to monitor any conformational changes that occur upon ligation of metal ion, 1D NMR spectra were collected in the presence and absence of zinc. Fig. 4A shows a spectrum of the peptide before addition of zinc. As seen with the CD data, there is no evidence for significant structure in the apo-form of the peptide. However, once the peptide is fully ligated a number of spectral features appear (Fig. 4B) which are indicative of both secondary and tertiary structure formation. Two of the aliphatic resonances are shifted upfield by at least 0.5 ppm to ~ 0.4 ppm. Such large upfield shifts are characteristic of the aliphatic groups coming into close proximity to aromatic ring currents as a result of tertiary structure formation. Also, a number of CαH resonances are shifted downfield of the HDO signal which is consistent with the formation of an anti-parallel β-sheet [39]. The chemical shifts of the aromatic ring protons move significantly downfield upon zinc ligation, especially the C2 protons of the histidine rings. This shift is consistent with the expected change in chemical environment which is either a result of direct

metal ligation or indirectly by subsequent tertiary structure formation. Finally, amide protons are only protected against exchange with bulk solvent in the presence of zinc suggesting that only when ligated does the peptide form a stable hydrogen-bonding network. This is indicative of secondary structure formation.

3.5. *Summary*

A synthetic peptide corresponding to the newly defined XNF7 B-box motif has no secondary structure in the absence of divalent metal ions, as shown by CD and 1D NMR. The peptide however undergoes a structural transition upon the addition of zinc or copper. Metal-binding studies of the B-box domain using the chromophore Br_2BAPTA indicate that the molar binding stoichiometry for Zn^{2+} to peptide is 1:1 with a measured K_d of 61 ± 12 nM. Cobalt-binding studies show that cobalt binds to the B-box peptide tetrahedrally using cysteine as ligands but less tightly than zinc and that zinc can displace the cobalt. Together, these metal-binding studies suggest that not all of the conserved cysteines and/or histidines are involved in direct metal ligation. This poses the question as to what is the function of the non-liganded cysteines and/or histidines in the B-box domain. One possible function could be inter-molecular dimerisation, as observed for the HIV Tat protein [11], although the preliminary NMR measurements reported here suggest a B-box monomer in solution. This question must await further investigation.

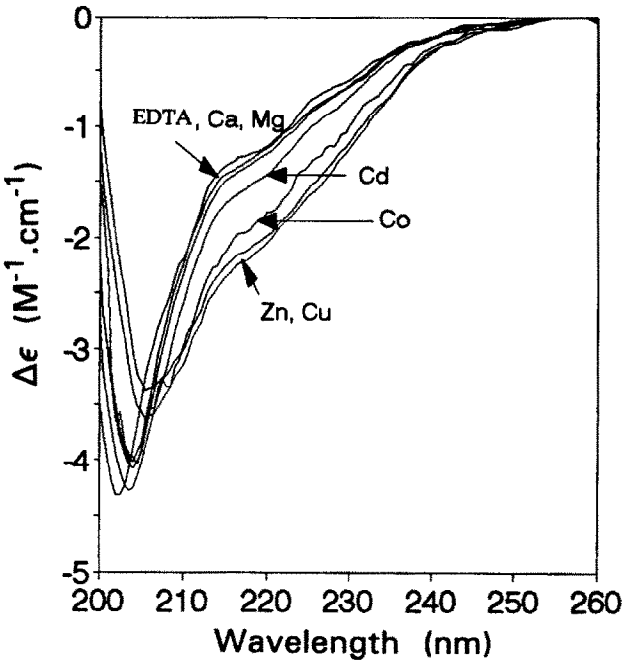

Fig. 3. CD spectra of XNF7 B-box peptide. Peptide concentration was approximately 4 μM in 5 mm cuvettes. The concentration of the divalent cation was 0.2 mM and was added as the chloride. EDTA was added in excess to the Zn^{2+}-containing solution after complexation with the peptide.

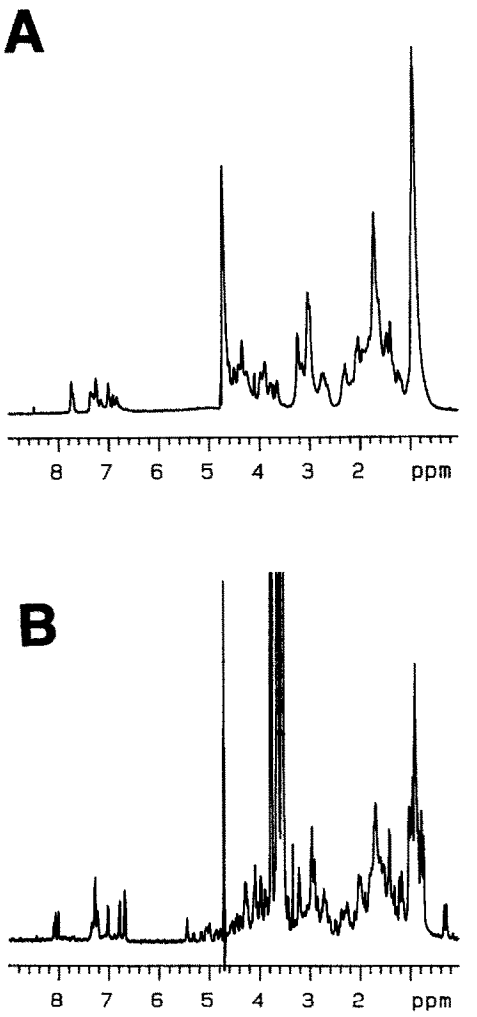

Fig. 4. One dimensional ^{1}H NMR spectra in ^2H_2O. Panel A shows the peptide (~ 1 mM) before addition of Zn^{2+} and panel B after addition of 1 mM Zn^{2+}. Both spectra were collected at pH 7.5 and 303 K. The large peak in the aliphatic region of panel B is ethanedithiol.

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