

Zinc is required for folding and binding of a single zinc finger to DNA

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A synthetic peptide corresponding to zinc finger 31 of the *Xenopus* protein Xfin adopts a folded conformation in the presence of zinc. The same peptide in the absence of zinc is not folded in a stable tertiary conformation, as determined by NMR. Binding experiments have shown that the peptide binds non-specifically to DNA only in the presence of zinc. Moreover, competitive DNA binding experiments indicate interaction with 3.9 ± 0.4 base pairs.

Zinc finger; DNA-binding; 2D NMR

1. INTRODUCTION

Zinc fingers represent an important structural motif found in a large family of eukaryotic transcriptional regulatory proteins [1–3]. Transcription factor IIIA (TFIIIA) isolated from *Xenopus laevis* [4,5] was the first protein to be identified as a zinc finger protein. It is now apparent that there are different classes of zinc fingers; this paper is focused on fingers of the TFIIIA type where the zinc is coordinated by two Cys and two His ligands. Circular dichroism and NMR spectroscopy have been used to demonstrate the folding of single zinc fingers from TFIIIA [6], ADR1 [7–9], Xfin [10,11], and SW15 [12]. The folding and the formation of a stable tertiary structure for these peptides depends on the presence of Zn^{2+} [6–12].

The TFIIIA protein contains nine zinc finger motifs with 7–11 zinc atoms per molecule [5]. Zinc is proposed to be coordinated to each of the nine finger motifs, forming an independently folded domain. However, recent reports cast doubts upon the role of zinc in stabilization of the folded zinc finger structure. Studies by Wu and co-workers suggest that only two mol of zinc/mol of TFIIIA protein are required to maintain the correct conformation for sequence-specific binding to either DNA or 5 S RNA [13,14]. In addition, Roesch and coworkers recently reported structural studies of a synthetic 30-residue zinc finger peptide corresponding to

the seventh finger domain from the mouse *Kruppel*-like gene *mKr2* [15], with the surprising finding that the peptide 'clearly possesses a well defined tertiary structure' in the absence of zinc. The solution structure proposed for zinc-free *mKr2* differs significantly from the three-dimensional structure of the Xfin-31 [10] and ADR1b [9] zinc fingers.

At present the literature contains conflicting reports concerning the exact role of the zinc in dictating both three-dimensional structure and interactions with DNA. In view of this, we undertook a direct comparison of the same zinc finger peptide, corresponding to finger 31 of the protein Xfin [16,17] in the absence and presence of Zn^{2+} to clarify the ambiguity surrounding the role of the metal. The present work and the work reported earlier [10,11] show unequivocally that the Xfin-31 zinc finger peptide is folded in a stable tertiary conformation only in the presence of Zn^{2+} . In the absence of Zn^{2+} the peptide does not show stable tertiary structure detectable by NMR. Furthermore, there is no detectable binding of the Xfin-31 peptide to DNA in the absence of Zn^{2+} but non-specific binding occurs when Zn^{2+} is present.

2. MATERIALS AND METHODS

2.1. Peptide preparation

The Xfin-31 peptide (Ac-YKCGLCERSFVEKSALSRHQRVHK-NH₂) was synthesized as described previously [11]. Prior to injection of the sample for HPLC, the peptide was treated with excess dithiothreitol (DTT) to reduce any disulfide bridged forms. The purified sample was lyophilized and stored under argon at -20°C . The sample used for binding assays and NMR studies was >95% pure as determined by analytical HPLC. The peptide concentration was determined by measurement of tyrosine absorbance in 6 M guanidine hydrochloride at 275.5 nm ($\epsilon_{275.5} = 1450 \text{ M}^{-1}\cdot\text{cm}^{-1}$) [18,19]. The contribution to the absorbance by the two cysteine residues was also

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Abbreviations: NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; COSY, scalar correlated spectroscopy

taken into account ($\epsilon_{215} = 1.45 \text{ M}^{-1} \text{ cm}^{-1}$) [20]. The zinc complex was prepared by a previously described procedure [11].

2.2. DNA for peptide binding assays

Two different double stranded deoxynucleotides were synthesized with an Applied Biosystems DNA Synthesizer Model 380B. The single stranded oligonucleotides were deblocked overnight in aqueous ammonia (0.5 M) and gel purified. Aliquots of the single stranded oligonucleotides were labeled at the 5' end with [γ - ^{32}P]ATP and polynucleotide kinase prior to renaturation with an excess of unlabeled oligonucleotide. Equal amounts of each complementary strand (estimated from UV absorbance) were mixed in 0.1 M sodium carbonate buffer, pH 7.5, heated to 95°C and allowed to hybridize by slow cooling to 22°C. The DNA sequences used were:

S S 26-mer (C-block): 5' GATCTACTTGGATGGGAGACCGCTG 3'
B-block 24-mer: 5' GATCGATGGTTCGAATCCATCCTC 3'

where the sequence of only one strand is given. In addition, a 50 base pair *EcoRI-HindIII* polylinker fragment from pUC19 was used as a non-specific DNA. For some experiments, the double stranded material was gel-purified prior to performing peptide binding experiments. Oligonucleotides were concentrated by ethanol precipitation and resuspended in binding buffer (see below). An aliquot was taken for measurement of DNA concentration by UV absorbance using an extinction coefficient of $1 \text{ OD}_{260\text{nm}} = 50 \mu\text{g/ml}$.

2.3. Gel mobility shift assays for peptide-DNA complexes

DNA binding studies of Xfin-31 peptide were carried out in a buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, 10% (v/v) glycerol with 0.5–1 ng of labeled double stranded synthetic oligonucleotides and various amounts of unlabeled poly dI-poly dC (10–500 ng, Pharmacia) in a final volume of 10–20 μl . Binding reactions were at 22°C for 30–45 min. Both the free peptide and the preformed zinc complex were used. The samples were loaded on a 1 mm-thick 5% polyacrylamide gel (29:1 acrylamide to bisacrylamide) in 90 mM Tris-borate buffer, pH 8.3, and subjected to electrophoresis at 125–150 V (8–10 V/cm) for 2.5 to 3.5 h. The gels were pre-electrophoresed at 150 V for 1 h prior to electrophoresis. The labeled DNA was visualized by autoradiography and the peptide-bound and free DNA was quantitated by liquid scintillation counting of gel slices obtained with the aid of the autoradiogram.

2.4. NMR measurements

Zinc-free Xfin-31 was prepared as a 4 mM solution in 90% H₂O/10% ²H₂O and in ²H₂O at pH 4.15 and ²H₂O at pH 4.4. Care was taken to prevent oxidation and disulfide bridge formation while preparing the sample for the NMR experiments. All solvents were purged with argon and all transfers were carried out under argon. The ¹H NMR spectra were recorded at 278 K using a Bruker AM500 spectrometer. Data processing was carried out on CONVEX C120 and C240 computers using software provided by Dr Dennis Hare.

All of the NMR data described here refers to the zinc-free Xfin-31 peptide (see [10,11] for NMR studies of the zinc complex). Two-dimensional NMR spectra were recorded in the phase-sensitive mode with quadrature detection in the ω_1 dimension using time-proportional phase incrementation [21,22]. Spectra were recorded with the carrier placed on the ¹H₂O or HOD resonances. Double quantum filtered COSY (DQF-COSY) spectra were acquired using the standard pulse sequence and phase cycling [23]. Phase-sensitive NOESY spectra were acquired using standard methods [24] with 300 and 500 ms mixing periods.

Spectra were Fourier-transformed using a Lorentzian to Gaussian weighting function in the ω_2 dimension and a shifted sine bell weighting function in ω_1 . Baseline-correction [25] and t_1 ridge suppression [26] were used for cosine modulated experiments. For most experiments, the final spectrum contained 2048 real points in both dimensions

3. RESULTS AND DISCUSSION

Standard sequential assignment procedures [27] were used to obtain specific assignments for the zinc-free Xfin-31 peptide. The $d_{\text{NN}}(i,i+1)$ and $d_{\text{NN}}(i,i+1)$ sequential NOE enhancements observed for zinc-free Xfin-31 are summarized in Fig. 1 along with a representative region of the NOESY spectrum. A few very weak $d_{\text{NN}}(i,i+2)$ and $d_{\text{NN}}(i,i+3)$ NOE's are observed in NOESY spectra recorded at very long mixing times ($\tau_m = 300$ and 500 ms). These are indicative of a small population of helical and turn-like conformations in the zinc-free peptide. However, the weakness of these NOE's and the dominance of $d_{\text{NN}}(i,i+1)$ sequential NOE connectivities shows that the peptide is largely unfolded in the absence of Zn²⁺.

Secondary structure prediction [28] for Xfin-31 peptide shows strong propensity for helical conformation for residues Cys-6 through Gln-20. Overall, the secondary structure prediction correlates well with the experimental observation of weak $d_{\text{NN}}(i,i+3)$ medium-range NOE's which indicate the presence of a small population of helical conformers between Glu-7 and Gln-20 in the zinc-free peptide (Fig. 2). The $d_{\text{NN}}(i,i+1)$ sequential NOE's become weaker relative to the $d_{\text{NN}}(i,i+1)$ NOE's at both ends of the peptide, suggesting that the helix is frayed and more disordered toward both termini.

The NOE connectivities observed for the zinc-complexed peptide [11] are notably different from those of the zinc-free peptide. The medium-range NOE's defining the helix are very much stronger in the presence of Zn²⁺, are more extensive, and occur in a different region of the sequence (Fig. 2). In contrast to the free peptide, the helix extends from residues 12–24 in the zinc complex. In the absence of Zn²⁺ the $d_{\text{NN}}(i,i+1)$ NOE's are weaker than the $d_{\text{NN}}(i,i+1)$ NOE's throughout the sequence (Fig. 1), consistent with a low population of helical conformers; for the zinc-complexed peptide the $d_{\text{NN}}(i,i+1)$ NOE's are stronger than the $d_{\text{NN}}(i,i+1)$ NOE's (see Fig. 2 in ref. 11) in the helical region. Circular dichroism measurements on related finger peptides confirm the presence of helix in the presence of Zn²⁺ but fail to detect helix in the zinc-free peptides (unpublished observations on TFIIF finger 5; see also [6,7]). Finally, many long-range NOE's are observed for the zinc-complexed peptide between residues distant in the polypeptide chain [10,11]; no long-range NOE's are observed for the zinc-free peptide, even at a mixing time of 500 ms.

Substantial changes occur in the NMR spectrum of the Xfin-31 peptide upon binding of Zn²⁺. In the absence of Zn²⁺, ¹H and ¹³C chemical shifts are very close to 'random coil' values (Lee, Palmer and Wright, manuscript in preparation). The dispersion of the amide and the C α H proton resonances increases considerably when Zn²⁺ is bound and several methyl,

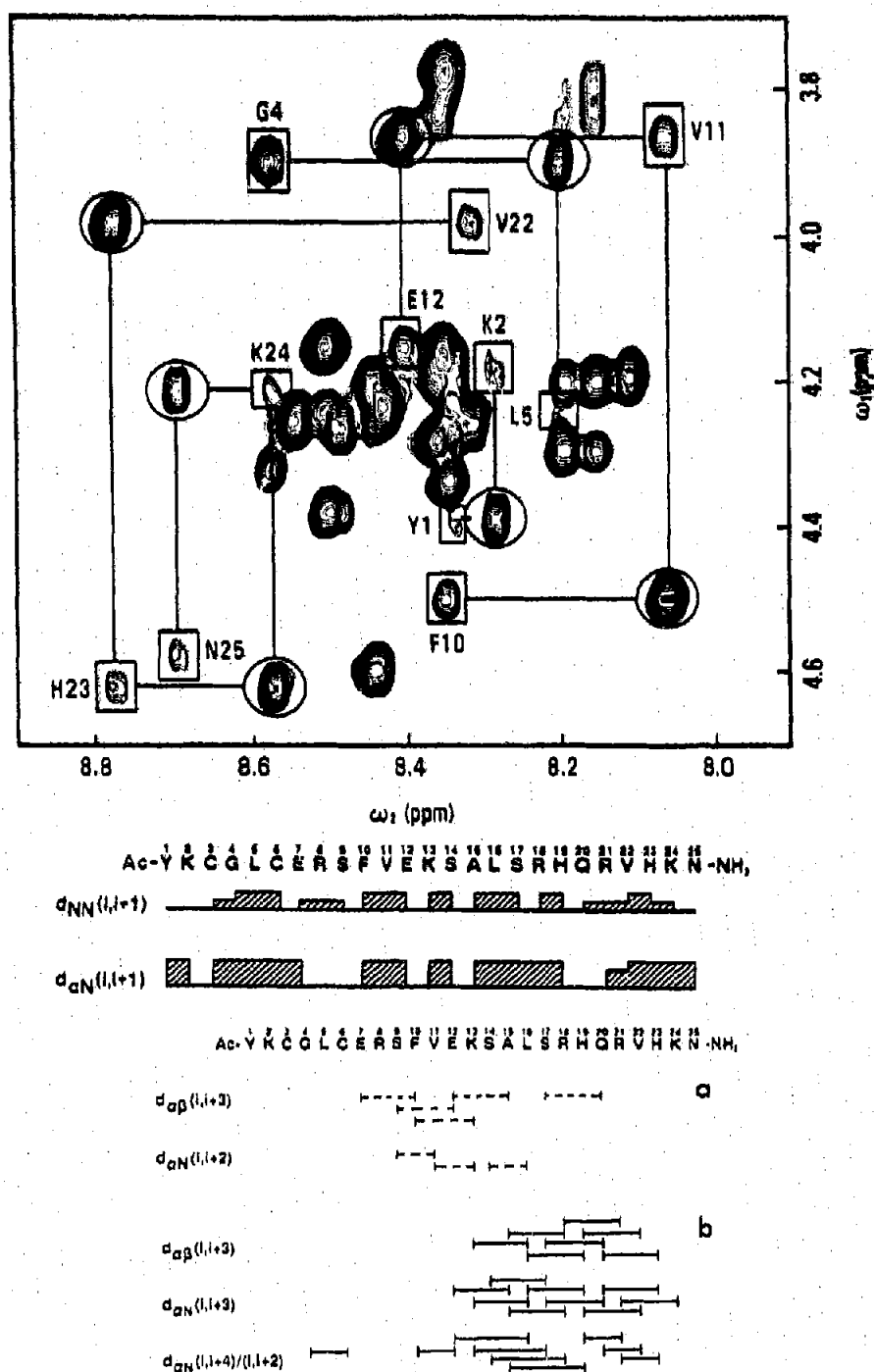


Fig. 1. Finger print region of NOESY spectrum of zinc-free Xfin-31 peptide in 90% H_2O /10% 2H_2O with summary of sequential NOE connectivities. Sequential (i,i+1) NOE's are represented as hatched blocks. The height of the block is a qualitative measure of NOE intensity in the $\tau_m = 300$ ms NOESY spectrum at 278K.

aromatic, and amide proton resonances are shifted significantly relative to their positions in the zinc-free peptide. These characteristic resonance shifts were observed for both Xfin-31 and finger 5 of TFIIIA [11] and are similar to those described by Kleivit and co-workers for a synthetic zinc finger from ADR1 [7-9]

and Neuhaus and co-workers for fingers from SW15 [12]. In all cases, the finger peptides appear to fold only in the presence of Zn^{2+} .

To investigate further the role of Zn^{2+} , DNA binding assays for Xfin-31 peptide in the presence and absence of Zn^{2+} were carried out. Gel electrophoresis of labeled

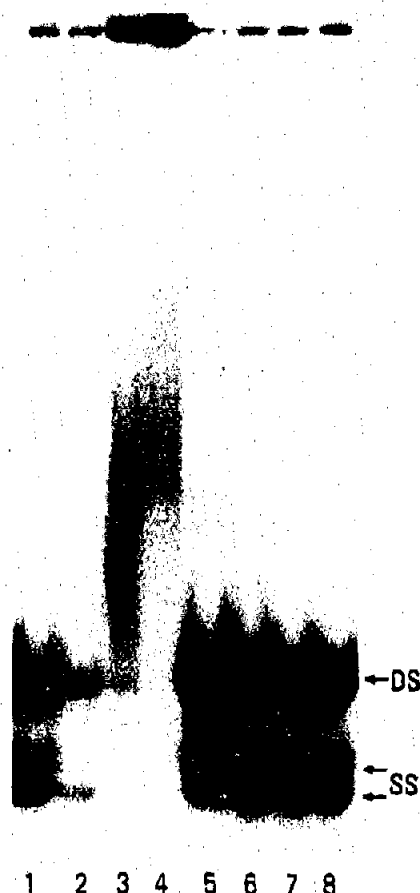


Fig. 2. Comparison of medium range NOE's of (a) zinc-free Xfin-31 and (b) zinc-complexed Xfin-31 at 278K. The NOESY spectra of the zinc-free Xfin-31 peptide were measured with mixing times of 300 and 500 ms. The NOESY spectra of zinc-complexed Xfin-31 were measured with mixing times of 150 and 200 ms. The dashed lines for the medium-range NOE's for zinc-free Xfin-31 indicate the much weaker intensities observed relative to the zinc-complexed Xfin-31. Even at the longer mixing times used, the medium range NOE intensities are much weaker for the zinc-free peptide.

DNA fragments under non-denaturing conditions [29] was used to monitor binding of the Zn^{2+} finger peptides to small double stranded deoxyoligonucleotides. Since the binding site for Xfin is unknown [16], we used two oligonucleotides available in our laboratory. One of these corresponds to the major binding site for the RNA polymerase III transcription factor TFIIC (B-block oligo) [30] and the other corresponds to part of the binding site for the 5 S gene-specific transcription factor TFIIA [4]. As TFIIA is the prototype zinc finger protein [5] and TFIIC is also likely to be a zinc finger protein since metal chelation with *o*-phenanthroline inhibits TFIIC binding (E.P. Gelduschek, personal communication), these oligonucleotides seemed to be appropriate for assays of Xfin-31 peptide binding. Fig. 3 shows the results of a titration of the B-block oligonucleotide with Xfin-31 peptide in the presence (lanes 1 to 4) and absence (lanes 5 to 8) of Zn^{2+} . With

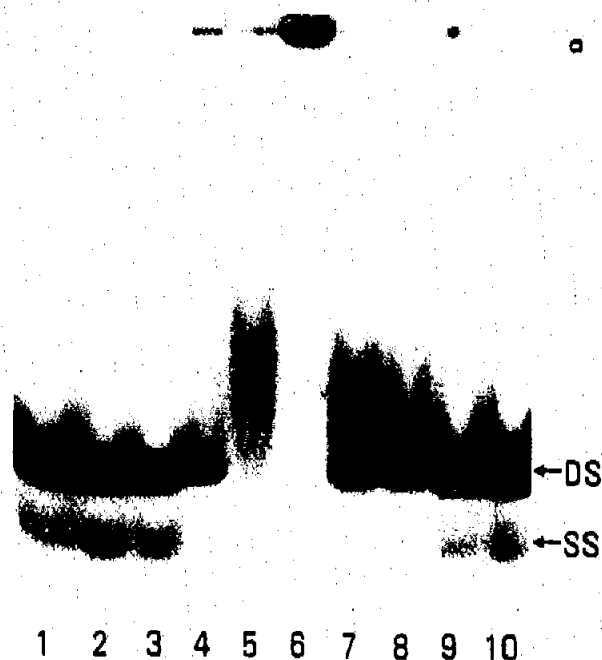


Fig. 3. Non-denaturing gel electrophoresis showing Xfin-31 peptide binding to a double stranded deoxyoligonucleotide. Various amounts of Xfin-31 peptide were mixed with 10 ng of the labeled B-block oligonucleotide in a final volume of 14 μ l. Data for the Zn^{2+} -containing (lanes 1-4) and Zn^{2+} -free peptide (lanes 5-8) are shown. The ratios of n. of peptide per mol of DNA base pairs were: lane 1, 0:1; lane 2, 4:1; lane 3, 12:1; lane 4, 40:1; lane 5, 0:1; lane 6, 0.3:1; lane 7, 4:1; lane 8, 40:1. The autoradiogram of the gel is shown.

increasing amounts of Zn^{2+} finger peptide, the labeled oligonucleotide is retarded in its migration in the gel, indicating an interaction with the peptide. Binding assays were performed, giving an association constant of $1.5 \times 10^6 M^{-1}$. The diffuse nature of the retarded species and the absence of specific retarded bands suggests a non-specific interaction of the peptide and the labeled DNA. Similar results were obtained for the TFIIC oligonucleotide and for a non-specific restriction fragment from pUC19 (data not shown). In the absence of Zn^{2+} (Fig. 3, lanes 5 to 8), no retardation of the oligonucleotide is observed, even with 40-fold excess of peptide to DNA base pairs suggesting that Zn^{2+} is required for DNA binding. It is clear from Fig. 3 that the zinc finger peptide binds to both single stranded and double stranded DNA; this is not surprising since intact TFIIA is also known to bind single stranded DNA [31].

In order to determine whether the binding of the Xfin peptide exhibits any sequence specificity, binding to the labeled oligonucleotide was challenged by the inclusion of various amounts of unlabeled poly dI-poly dC as a competitor. Fig. 4 shows that poly dI-poly dC competes effectively with the labeled oligonucleotide for peptide binding. From the competition experiments, the apparent association constant for Xfin-31 peptide binding to poly dI-poly dC is $9.4 \times 10^5 M^{-1}$ and the active pep-

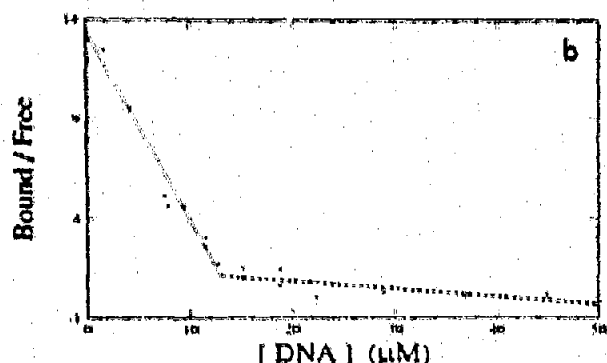


Fig. 4. Poly dI-poly dC competition for Xfin-31 peptide binding to a labeled double stranded deoxyoligonucleotide. (a) In lanes 1 to 5, 1 ng (16 pmol base pairs) of labeled B-block oligonucleotide was mixed with increasing amounts of zinc-containing Xfin-31 peptide while in lanes 6 to 10, a constant amount of peptide (0.6 nmol), 1 ng of labeled DNA (16 pmol) and various amounts of unlabeled poly dI-poly dC were analyzed by non-denaturing gel electrophoresis. The ratios of mol of peptide to total mol of DNA base pairs were: lane 1, 0:1; lane 2, 0.26:1; lane 3, 0.8:1; lane 4, 5:1; lane 5, 17:1; lane 6, 36:1; lane 7, 6:1; lane 8, 3:1; lane 9, 1.5:1; lane 10, 0.75:1. The autoradiogram of the gel is shown. (b) A plot of the ratio of bound to free labeled oligonucleotide versus total input DNA concentration. Data were obtained with a constant amount of peptide (55 μ M concentration), 1 ng (16 pmol) labeled DNA and increasing amounts of unlabeled poly dI-poly dC. Data are included from three separate experiments. The apparent association constant for the DNA-peptide complex is calculated from the slope and the active peptide concentration from the x-intercept.

tide concentration is $14.0 \pm 1.5 \mu$ M, assuming that the binding site for the peptide is 1 base pair (as the competitor concentration is given in molar base pairs). The actual peptide concentration used in this experiment was 55 μ M. Previous NMR studies [10,11] indicated that the Xfin-31 peptide is structurally homogeneous and have inferred that essentially all the peptide is active in binding DNA. Using this peptide concentration, the interaction of Xfin-31 with DNA was determined to be 3.9 ± 0.4 base pairs (55 μ M / $14 \pm 1.5 \mu$ M). Within experimental uncertainty, these data suggest that the Xfin-31 peptide interacts with about 4 bp of DNA.

There have been other reports of DNA binding by single zinc finger peptides. The second zinc finger from TFIIIA appears to bind to DNA with some sequence specificity; however, binding showed no clear dependence on Zn^{2+} [6]. (Our own experiments with a zinc finger peptide corresponding to finger 5 of TFIIIA fail to detect any specificity in binding to an oligonucleotide containing the correct recognition site). A single zinc finger from ADR1 binds non-specifically to DNA; the affinity is increased in the presence of Zn^{2+} [7]. In both cases, folding of the peptide is dependent on the presence of Zn^{2+} . However, a zinc finger motif from *mKr2* was reported recently to adopt a stable tertiary structure in aqueous solution in the absence of Zn^{2+} [15]. Although both zinc-free Xfin-31 and de-metallated SWI5 do not show any long-range

NOE's, the free *mKr2* peptide has been reported to show a number of 3 range NOE's indicative of folded structure. In contrast, the chemical shifts reported for *mKr2* closely resemble those of a 'random coil' [32]; in fact, the chemical shift dispersion of the fingerprint region of the NOESY spectrum of *mKr2* [15] closely resembles that of the unfolded zinc-free Xfin-31 (Fig. 1).

4. CONCLUSION

For a single synthetic zinc finger of the TFIIIA type, Xfin-31, NMR experiments show unequivocally that *zinc is required* for correct folding and that the zinc-free peptide is not folded in a stable tertiary conformation. Since the peptide is largely unfolded in the absence of Zn^{2+} , the metal appears to play a key role in stabilization of the folded structure. Concordantly, DNA binding assays clearly indicate that the Xfin-31 peptide binds to DNA (non-specifically) only in the presence of Zn^{2+} . These assays further support the NMR evidence for the correct folding of the Xfin-31 peptide in the presence of Zn^{2+} .

One of the more interesting results of the DNA binding experiments is the apparent number of base pairs that interact with the Xfin-31 peptide. Our experimental data suggest that about 4 bp of DNA interact with the folded Xfin zinc finger. We note that ~ 5 base pairs per finger are implicated in binding of TFIIIA and SWI5 to DNA [1,33,34], while binding of Sp1 fingers is believed to involve ~ 3 base pairs per finger [35]. Thus there may be differences in the mode of interaction of zinc fingers with DNA, depending on whether the binding is specific or non-specific and upon the exact nature of the zinc fingers and the target DNA. Elucidation of these interactions will require direct structural studies of DNA-zinc finger complexes, which are currently in progress in this laboratory.

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