Spectroscopic studies of wild-type and mutant “zinc finger” peptides: Determinants of domain folding and structure
(nuclear magnetic resonance/transcription factor ADR1)

G. Párraga*, S. Horvath†, L. Hood‡, E. T. Young*, and R. E. Klevit*

*Department of Biochemistry, SI-70, University of Washington, Seattle, WA 98195; and †Division of Biology, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT  The “zinc finger” model [Miller, J. M., McLachlan, A. D. & Klug, A. (1985) EMBO J. 4, 1609–1614; Brown, R. S., Sander, C., & Argos, P. (1985) FEBs Lett. 186, 271–274] makes both specific structural and specific functional predictions about zinc finger consensus sequences that can be tested with a combination of genetic, molecular biological, and biophysical techniques. The yeast transcription factor ADR1 contains two adjacent zinc finger domains; genetic and deletion analyses showed that amino acid substitutions and deletions in the zinc finger domains resulted in the loss of protein activity. To test the structural and functional predictions of the zinc finger model, peptides encompassing each of the ADR1 fingers were synthesized (ADR1α and ADR1β) as well as a mutant finger peptide (del138) deleted for a single amino acid residue. The folding and metal-binding characteristics of these peptides were assessed by 1H nuclear magnetic resonance (NMR) and visible spectroscopy. While a single unique conformational species was detected for the two wild-type peptides upon tetrahedral binding of zinc, the deletion peptide did not bind zinc with tetrahedral geometry, nor did it fold into a zinc finger domain. The metal-binding and folding results found with the mutant peptide were similar to those obtained when thiol alkylation or imidazole protonation of the wild-type peptides was performed. These data indicate that ligand spacing and both thiol and imidazole participation in zinc binding are specific and necessary requirements for zinc finger folding, which provides direct support for the initial predictions of the model.

Subsequent to the proposal of the “zinc finger” model (1, 2), amino acid sequences similar to those found in the Xenopus transcription factor TFIIIA have been found in a variety of putative and demonstrated eukaryotic nucleic acid binding proteins. The zinc finger domains have been implicated as containing the residues responsible for sequence-specific DNA binding, though it appears that for proteins containing many fingers, multiple finger contacts are required for base-specific recognition and binding (3–5). ADR1 is a transcription factor that activates expression of the glucose-repressed alcohol dehydrogenase 2 (ADH2) gene in Saccharomyces. The transcription factor contains two adjacent zinc finger motifs between residues 102 and 159. An ADR1-β-galactosidase fusion protein containing residues 17–229 binds specifically to a 22-base-pair dyad in the ADH2 promoter, whereas a deletion fusion protein containing residues 17–150 could not (6). This type of deletion analysis as well as genetic analyses and site-directed mutagenesis of some of the zinc finger proteins (3, 7, 8) strongly supported the zinc finger model, as did preliminary circular dichroism and NMR spectroscopic studies of a single zinc finger peptide (9, 10).

To solve the structure of a zinc finger domain, it was first necessary to ascertain whether peptides corresponding to zinc finger sequences fold in vitro into a single conformational form upon the addition of metal ions. Once the reproducible folding and unfolding of single-finger peptides could be achieved and monitored by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy (9, 10) several issues could be addressed. Do different single-finger peptides fold into similar or identical structures? What elements of the primary sequences of the peptides are absolutely required for proper folding into a domain? To address these questions, three different zinc finger peptides, ADR1α, ADR1β, and del138, were synthesized (Fig. 1), and their metal-binding geometry and folding were studied. A low-resolution model of a single-finger peptide, ADR1α (10), and a higher-resolution distance geometry structure of another, ADR1β (11), have been determined and are reported elsewhere. The wild-type zinc finger structures that we have determined consist of an N-terminal loop involving the two conserved cysteine residues, a C-terminal amphiphilic α-helix, and a tightly packed hydrophobic core. The structure of a 25-residue peptide corresponding to 1 of the 37 zinc finger domains from the Xenopus protein Xfin (Xfin-31) was reported (12); this peptide has structural features similar to those described for the ADR1 fingers.

In this report, we present the metal-binding and folding properties of a deletion peptide in which spacing between pairs of ligands has been altered. To ascertain the fundamental components of the primary sequence necessary for domain folding, we have also studied the effect of chemical modification of ligand residues on wild-type zinc finger folding.

MATERIALS AND METHODS

Materials. Peptide synthetic reagents were obtained from Applied Biosystems. Vinylpyridine was obtained from Aldrich. Iodoacetamide and all other reagents and buffers were from Sigma. Deuterated Tris buffer was from MSD Isotopes and all other deuterated solvents were from Sigma.

Peptide Synthesis. Peptides (ADR1α, ADR1β, del138) were synthesized by the stepwise solid-phase technique (13, 14). They were purified by preparative reverse-phase HPLC, and sequences and composition were confirmed by amino acid compositional analysis, peptide sequencing, and mass spectrometry.

Peptide Folding. Subsequent to purification and lyophilization, peptides were dissolved in 50 mM Tris acetate or deuterated Tris acetate buffer containing either 1 mM EDTA or a stoichiometric amount of ZnCl2 at pH 7.5. The samples were warmed to 70°C, allowed to cool to room temperature, and stored at 4°C. To prevent oxidation, samples were stored lyophilized and/or purged with argon.

Thiol Alkylation. Thiol groups were alkylated with iodoacetamide as previously described (15). Alkylated peptide

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Abbreviation: 1D, one-dimensional.
One-Dimensional (1D) $^1$H NMR Spectroscopy. Proton NMR spectra of $^2$H$_2$O solutions were obtained on a Bruker AM500 at 25°C with a spectral width of 6410 Hz and a relaxation delay of 1.5 s. For pH titrations, peptide concentrations were 1 mM in 50 mM deuterated Tris acetate/25 mM deuterated acetic acid, and 2 μl of 4% $^2$HCl solution was added for each titration point over the range of pH 7.8–3.0.

**CD Spectroscopy.** CD experiments were conducted at 21°C on an Online Systems (Jefferson, GA) modified Cary 61 spectropolarimeter. Peptide concentrations were 0.25 mM in 50 mM Tris and spectra were obtained with a 0.05-cm path length.

**Cobalt Visible Spectrophotometry.** Ultraviolet and visible light spectra of cobalt-peptide adducts were obtained on a Beckman DU50 UV–Visible spectrophotometer at 21°C. The spectrum was scanned at 500 nm/min from 800 to 200 nm. Peptide concentrations were 0.25 mM in 50 mM Tris and spectra were obtained with a 1-cm path length.

**RESULTS**

**Role of Cysteine Ligands in Folding.** It has been clearly shown for ADR1a (10) as well as another zinc finger peptide (9) that Zn$^{2+}$ is tetrahedrally bound. In the case of the zinc finger protein TFIIIA (17), extended x-ray absorption fine structure spectroscopy (EXAFS) has shown that cysteine and histidine residues are the ligands that bind zinc with tetrahedral geometry. To ascertain whether cysteine residue binding of Zn$^{2+}$ is absolutely required for the finger peptide to fold, the thiol moieties were blocked by alkylation. In Fig. 2, the 1D $^1$H NMR spectra of ADR1balk is shown in the presence (Fig. 2, spectrum c) and absence (Fig. 2, spectrum b) of Zn$^{2+}$. For comparison, the spectrum of unmodified ADR1b is shown in Fig. 2, spectrum a. As with ADR1a (10), when ADR1b is reconstituted in the presence of Zn$^{2+}$ (Fig. 2, spectrum a), C-2 and C-4 proton lines of the three histidine residues have shifted from their positions when the peptide is reconstituted in the presence of EDTA (11). There is as well considerable rearrangement of other aromatic resonances, C$^\alpha$ proton resonances, and methyl proton resonances, indicating that the peptide is folded. In Fig. 2, spectrum b, sharp lines and the lack of any upfield-shifted methyl peaks or downfield-shifted C$^\alpha$ proton peaks indicate that ADR1balk is not folded when reconstituted in the absence of zinc. Upon the addition of a stoichiometric amount of Zn$^{2+}$ (Fig. 2, spectrum c), the spectrum still shows no evidence of folding. Rather, the entire spectrum broadens, which could be a consequence of exchange broadening (Zn$^{2+}$–peptide exchange rate is increased relative to the slow exchange behavior observed when thioles are not alkylated), or of peptide aggregation mediated by Zn$^{2+}$ binding to histidine residues. Upon the addition of excess Zn$^{2+}$, the spectrum broadens further (data not shown). We have performed Zn$^{2+}$ titrations on another thiol-alkylated peptide (ADR1aalk) with similar results.

CD spectroscopy confirms that Zn$^{2+}$ addition to ADR1balk was not coincident with secondary structural changes in the peptide (data not shown). This is in contrast to TFIIIA-2 (9),

![Fig. 1. Amino acid sequence (standard one-letter symbols) of zinc fingers in ADR1 and the sequences of the single-finger peptides between residues 102 and 159 in the ADR1 protein. Sequences of the peptides ADR1a and ADR1b are bracketed and del138 (ADR1a lacking N-138) is denoted by an unfilled arrow. Filled arrows point to single-finger missense mutant peptides. Circles indicate invariant and highly conserved residues.](image)

![Fig. 2. 500-MHz $^1$H NMR spectra indicating the effect of cysteine residue alkylation on folding. One-dimensional NMR spectra ($^2$H$_2$O solvent) are shown of 1 mM unmodified ADR1b reconstituted in 1.1 mM ZnCl$_2$ and 50 mM Tris acetate, pH 7.5 (a), 1 mM ADR1balk reconstituted in 50 mM Tris acetate, pH 7.5 (b), and 1 mM ADR1balk reconstituted in 1.0 mM ZnCl$_2$ and 50 mM Tris acetate (c). Histidine C-2 protons are indicated by * in spectrum a.](image)
ADR1a (10), and ADR1b (data not shown), where the addition of Zn$^{2+}$ resulted in a significant increase in α-helical content in the peptide. In the case of unmodified ADR1b and ADR1a, two-dimensional NMR nuclear Overhauser enhancement spectroscopy experiments confirmed the existence of an α-helix in both peptides (10, 11) and identified the residues involved in the helix, which includes both conserved histidine residues. Thus, it appears that cysteine residue alkylation abolishes the ability of the α-helix of zinc finger peptides to form even when histidine residues are free to participate in Zn$^{2+}$ binding.

**Role of Histidine Ligands in Folding.** To study the role of the His residues in the zinc-mediated folding of these peptides, advantage was taken of the well-resolved His C-2 proton peaks in the 1D $^1$H NMR spectrum of ADR1a. The ADR1a peptide contains two His residues. When folded in the presence of Zn$^{2+}$, the imidazole C-2 and C-4 proton resonances from His-150 and His-155 shift and occupy unique chemical shift positions (ref. 10 and Fig. 3). The C$^\alpha$ proton and methyl proton peaks which have shifted relative to the zinc-free spectrum also confirm that ADR1a is in a folded state. These proton peaks can be followed as a function of pH as is shown in Fig. 3. As the pH of the solution is decreased, the imidazole C-2 and C-4 proton resonances do not shift. Instead, as the pH is lowered below 5.5, proton peaks begin to decrease in intensity, while peaks at the chemical shift positions of the "unfolded" form increase in intensity (shown by the filled arrows). Thus, under these conditions, the peptide exists in two states, unfolded and folded, that are in slow exchange (interconversion between folded and unfolded states is slow on the chemical shift time scale). The fact that the imidazole resonances do not shift as a function of pH indicates that a species in which protonated imidazoles are bound to the zinc ion is short-lived and/or is populated at a low level. That is, protons appear to be competing with Zn$^{2+}$ for imidazole, indicating that only the unprotonated imidazole groups serve as ligands for Zn$^{2+}$ in the peptide. Once protonated (Fig. 3, spectra d–f), the imidazole peaks shift with decreasing pH (protonated and unprotonated imidazole in fast exchange or interconversion between protonated and unprotonated forms is fast on the chemical shift time scale) in the same manner as unfolded ADR1a (i.e., with a pK$_a$ of 6.2; data not shown).

Since the states of ADR1a are in slow exchange, the peak intensities for each of the forms are a direct measurement of the fractional populations in each state. A plot of C-2 proton peak intensity vs. pH yields an apparent pK of 5.2. This apparent pK is the midpoint value for the overall pH-dependent unfolding process—i.e.,

$$P^\alpha\text{-His}_{2}Zn^{2+} + 2H^+ \Rightarrow P^\alpha\text{-His}_{2}2H^+ + Zn^{2+}$$

where $P^\alpha$ and $P^f$ represent the folded and unfolded species, respectively. The process can be described as a competition between Zn$^{2+}$ and H$^+$ for the free imidazole groups and the observed or apparent pK of 5.2 is the pH at which half the population is zinc bound (and folded) and half the population is protonated (and unfolded).

The fractional populations of the two states can also be plotted in a Hill plot. Hill plots for the C-2 proton peaks of the two histidines gave Hill coefficients of 2, indicating that the pH-dependent unfolding is a cooperative process (18). The simplest interpretation is that once one of the imidazole groups is protonated, the other becomes much easier to protonate.

As illustrated in Fig. 3, the histidine resonances are not the only ones to decrease in intensity with decreasing pH; the same behavior is observed for the downfield-shifted C$^\alpha$ protons and the upfield-shifted methyl protons (shown by the

![Fig. 3. 500-MHz $^1$H NMR spectra of pH titrations of ADR1a. ADR1a was reconstituted at 1 mM in the presence of 1.1 mM ZnCl$_2$, 50 mM Tris acetate, and 25 mM acetic acid (deuterated buffers) at the indicated pH values. Filled arrows point to the chemical shift positions of the titrating "unfolded" C-2 proton resonances. Unfilled arrows point to "folded" methyl proton peaks. His C-2 protons are indicated by + in spectrum a.](image-url)
provide enough space for a loop or turn between the Cys and His residues. The folding of del138 was monitored by 1D $^1$H NMR and CD spectroscopy, while its metal-binding properties were assayed by visible spectrophotometry. Fig. 4 shows visible spectra of cobalt-peptide adducts of ADR1a, ADR1b, and del138. The spectra of both ADR1a and ADR1b contain an absorbance maximum centered at 635 nm which is characteristic of tetrahedral coordination of Co$^{2+}$ (19). In contrast, when del138 is reconstituted with CoCl$_2$ (Fig. 4), it does not bind the metal with tetrahedral geometry; the visible spectrum lacks the absorbance maxima at 635 nm characteristic of $d-d$ transitions in the cobalt electron orbitals. Though Cys and His ligands are present and chemically able to participate in metal binding, tetrahedral binding of Co$^{2+}$ does not occur, which strongly suggests that tetrahedral coordination of Zn$^{2+}$ does not occur either.

Because it was clear that del138 was incapable of binding Co$^{2+}$ tetrahedrally under conditions that allowed the two wild-type peptides to do so, we were interested in assessing what conformational changes, if any, occurred when del138 was reconstituted in the presence of other metals, regardless of the exact geometry of metal binding. Fig. 5 shows 1D $^1$H NMR spectra of a zinc titration experiment with del138. The deletion peptide was reconstituted in the absence of Zn$^{2+}$ (Fig. 5, spectrum a), the presence of 0.25 equiv of Zn$^{2+}$ (Fig. 5, spectrum b), and the presence of excess Zn$^{2+}$ (Fig. 5, spectrum c). In the absence of Zn$^{2+}$, the spectrum exhibits sharp lines—in particular, the C-2 and C-4 imidazole proton lines are sharp, and both His residues occupy the same C-2 $^1$H and C-4 $^1$H chemical shift positions, indicating that the peptide is unfolded and not aggregated due to oxidation. When 0.25 equiv of Zn$^{2+}$ is added, the entire spectrum broadens. This is most obvious in the aromatic region of the spectrum, especially at the imidazole chemical shift positions. When excess Zn$^{2+}$ is added, the spectrum broadens even further (Fig. 5, spectrum c). This titration experiment was repeated with del138 concentrations of 0.25 mM and 5 mM at 25°C and 35°C. The results were the same in all cases. The broad 1D $^1$H NMR spectra suggest that, as was the case with ADR1alk, the addition of Zn$^{2+}$ to del138 does not drive the folding of the peptide to a single conformation. Rather, zinc addition mediates an exchange process between the peptide and zinc (intermediate zinc–peptide exchange) and/or aggregation of the peptide, either of which would result in broadened proton resonance lines. CD spectroscopy strengthens the notion that stable domains are not being formed. When Zn$^{2+}$, Cd$^{2+}$, and Co$^{2+}$ titrations of del138 were monitored by CD spectroscopy, no secondary structural changes of the type detected for both ADR1a (10) and ADR1b (data not shown) were observed. Therefore visible, NMR, and CD spectroscopies confirm that del138 does not bind metals with tetrahedral geometry nor does it fold into a stable domain structure.

**DISCUSSION**

The work of Frankel et al. (9) and our own work (10) indicated that tetrahedral metal binding by zinc finger peptides was concomitant with secondary and tertiary structural changes, suggesting that metal binding closely parallels the folding of the peptides. There were no previous indications that 30-residue peptides (corresponding to interior sequences of proteins) could be folded into stable proteins between the domains suitable for structural studies. Because of the extremely compact and stable nature of the domains, we were interested in identifying the features of the primary sequence required for independent folding.

If Zn$^{2+}$ binding could direct the folding of zinc finger domains, then modification of ligands should abrogate the ability to fold and/or effectuate the unfolding of the peptides. In this report, we have shown that when imidazole nitrogen atoms are protonated or thiol is carboxymethylated, the zinc finger sequences unfold and are incapable of folding, respectively. Thus, the folded solution structures that have been solved by NMR spectroscopy (10–12) absolutely depend on the presence of reduced unblocked thiols and unprotonated imidazole nitrogen atoms.

A major component of the folded structure of ADR1a and ADR1b is the C-terminal α-helix (10, 11), which can be easily monitored by CD spectroscopy. We can conclude from CD and NMR spectra of the carboxymethylated peptides ADR1balk and ADR1aalk that the C-terminal α-helix does not form upon the addition of zinc, even though histidine residues are capable of binding zinc. Similarly, when histidine residues are protonated, NMR spectra indicate that the entire domain unfolds even though the cysteine residues are still capable of binding zinc. Taken together, these findings indicate that under experimental conditions there is no evidence of partially folded domains, suggesting that the folding process involves a two-state cooperative transition.

Although it is generally acknowledged that the nature of metal ligands is conserved among zinc fingers, it is often overlooked that spacing between ligands is also conserved. The distance between the pair of Cys ligands is 2–4 residues and between the pair of His ligands is 3 or 4 residues (but never fewer than 3 residues, possibly to accommodate the α-helix and/or bulky His side chains). Although there is some flexibility in the spacing requirements between the pair of Cys ligands and between the pair of His ligands, the distance between the most C-terminal Cys and N-terminal His residues is absolutely conserved at 12 residues. The possible importance of this sequence conservation is demonstrated by our results with the deletion peptide del138. Under conditions where both wild-type zinc finger peptides bound zinc and folded into domains, the deletion peptide was neither capable of binding zinc tetrahedrally nor capable of stable folding.
del138 contains five possible Zn$^{2+}$ ligands (Fig. 1). In addition to altering conserved spacing between ligands, the deletion of Asn-138 alters the wild-type sequence as well, bringing a third possible thiol ligand (Cys-140) into the proximity of Cys-137 and Cys-134. In the NMR solution structure of ADR1a (10) the thiol of Cys-140 is directed out into the solvent, well away from Cys-134 and Cys-137 and the zinc metal ion. The sequential proximity of three possible thiol ligands at the N terminus of the domain may act to complicate the folding “choices” of the peptide, rendering tetrahedral liganding and ultimately proper folding impossible under these conditions. This possibility is consistent with the visible spectroscopy data indicating that del138 does not bind metal ions tetrahedrally. Another explanation for these results is that we have altered the apposition of the conserved Phe and Leu residues as well as other residues that are interacting in the central hydrophobic pocket observed in the structure of the wild-type peptides ADR1a (10) and ADR1b (11). This could have the effect of decreasing the stabilization of the structure by the hydrophobic core.

It should be informative to assess the metal-binding and folding characteristics as well as the structures of other mutant (missense) single-finger peptides from ADR1. No missense point mutants containing alterations of conserved aromatic and aliphatic residues (Tyr, Phe, Leu) could be isolated in a preliminary genetic analysis of ADR1 (3). However, site-directed mutagenesis of these residues should allow us to assess the structural and functional significance of the hydrophobic core in the folded zinc finger structure. In conjunction with functional assays of the mutant proteins in vivo and in vitro this type of analysis should afford us a better understanding of the determinants of both zinc finger folding and DNA binding.

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