Toward Ligand Identification within a CCHHC Zinc-Binding Domain from the NZF/MyT1 Family

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A family of proteins that contain presumed zinc-binding domains with the consensus sequence Cys−X_4−Cys-X_4−His-X_7−His-X_5−Cys has recently been identified, but the metal binding and structural properties of these domains have not been investigated. This consensus is striking because of the presence of *five* conserved potential zinc-binding residues. A peptide corresponding to the third putative zinc-binding domain from the transcription factor NZF-1 (hereafter NZF-13) has been synthesized and characterized. The UV−visible absorption spectroscopic properties of the cobalt(II) complex of this peptide demonstrate that metal binding is tetrahedral, and the position of the visible absorption bands suggests coordination by three cysteinates and one histidine. To identify which of the two conserved histidine residues acts a metal-binding residue, two histidine to alanine variant peptides were also synthesized. Both variant peptides bound cobalt(II) in a tetrahedral fashion; replacement of the first of the two histidines has a somewhat larger effect on the detailed shape of the absorption spectral features than does replacement of the second histidine. These results suggest that the metal-coordinating residues (italicized) are *Cys*−X_4−*Cys*−X_4−*His*−X_7−His−X_5−*Cys*. However, simultaneous substitution of both histidine residues with alanine generated a peptide with much more dramatically affected metal binding properties. These observations suggests that the relatively modest effects observed for the singly substituted peptides may be due to metal interactions involving the remaining histidine. Because of these phenomena, further studies will be required to establish more conclusively the roles of the two histidine residues in metal binding and the potential significance of the apparent alternative histidine coordination.

Introduction

The number of putative zinc-binding domains continues to expand as DNA sequences from different organisms are analyzed [2]. These domains are often recognizable at the level of amino acid sequence because of the presence of patterns of cysteine and histidine residues that are directly involved in zinc binding. For example, the Cys_2His_2 zinc finger proteins, originally identified in TFIIIA, include sequences of the form Cys−$X_{2,4}$−Cys−X_{12}−His−X_{3-5}−His, where X indicates a relatively variable amino acid.[3,4] The role of the cysteine and histidine residues in zinc binding was proposed on the basis of the TFIIIA sequence and subsequently found to be correct from experimental studies. Another type of zinc-binding domain, found in the steroid hormone receptor superfamily, is characterized by the presence of nine conserved cysteine residues.[5] Structural studies revealed that eight of these are involved in zinc binding within two distinct Cys_4 zinc-binding domains while the function of the remaining conserved cysteine remains to be well defined.[6−8] The difficulty in deducing structures unambiguously from amino acid sequence information alone is revealed from analysis of the family of proteins typified by GAL4. These proteins contain sequences of the form Cys−X_2−Cys−X_6−Cys−X_6−Cys−X_2−Cys−X_6−Cys. It was proposed that four of these cysteine residues bound one zinc;[9] subsequent experimentation revealed that all six cysteine participate in binding two zinc ions with two of the cysteines acting as bridging ligands.[10−12]

A novel family of zinc-binding domain has recently been identified in proteins involved in the neuronal development. This class of zinc binding domain has been referred to as the NZF/MyT1 family. These sequences have been found in deduced sequences from human,[13−15] rat,[15,16] mouse,[14,17] *C. elegans*,[18] and *Xenopus*.[18] No such sequences are detectable in the yeast genomic sequence. In each putative zinc-binding sequence, the

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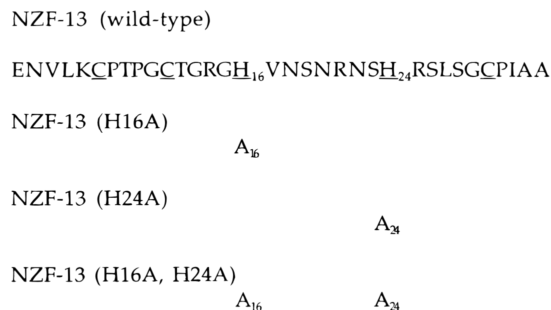

Figure 1. Amino acid sequences of the peptide NZF-13 and its variants.

motif sequence Cys−X_4−Cys−X_4−His−X_7−His−X_5−Cys, with five conserved potential metal-binding Cys and His residues, is present. On the basis of similarities in spacing between the metal-binding residues in the TFIIIA-type zinc-binding domains and four of the residues within the NZF/MyT1 domain, it has been proposed the italicized residues with the sequence *Cys*−X_4−*Cys*−X_4−His−X_7−*His*−X_5−*Cys* directly interact with zinc.[13] However, many other binding schemes can be conceived. Initial metal-binding studies have been performed on fragments containing two tandem domains of NZF-1.[19]

We present herein experiments directed toward determination of the residues involved in metal binding within a NZF-MyT1 domain. A 34-residue peptide, corresponding to residues 543−577 of the NZF-1 protein, was synthesized and characterized. The amino acid sequence of this peptide, hereafter referred to as NZF-13, is shown in Figure 1. The cobalt(II) and zinc(II) binding properties of this peptide were investigated. Three additional peptides corresponding to alanine substitutions at one or both histidine positions were also prepared and characterized. The spectra of the cobalt(II) complexes of these peptides indicate that the metal-binding site involves three cysteines and one histidine residue. Studies of the peptides with alanine substituted for histidine are most consistent with the histidine in position 16, not position 24, acting as a metal-binding residue in the wild-type sequence. This suggests that the metal-binding residues are *Cys*−X_4−*Cys*−X_4−*His*−X_7−His−X_5−*Cys*. However, alternate coordination by His_{24} appears to occur in the peptide for which His_{16} has been replaced by alanine. This plasticity as well as conformational disorder manifest in NMR spectra of this isolated domain makes completely unambiguous determination of the metal-coordinating residues impossible without additional studies on other protein fragments. Such fragments have been identified, and studies are underway.[19]

Experimental Section

Peptide Synthesis and Purification. All peptides were synthesized with use of a Milligen/Biosearch 9050 peptide synthesizer using Fmoc chemistry with TBTU activation. The peptides were cleaved from the resin by treatment with reagent B (88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) for 3 h. After precipitation in ether, the peptides were washed five times with cold ether to remove any remaining scavengers. The crude peptides were reduced prior to purification by incubating at 55 °C for 2 h in the presence of 5 equiv of DTT/cysteine residue in 100 mM Tris, pH 8.0. The peptides were purified on a Rainin C18 reversed-phase HPLC column with an acetonitrile gradient containing 0.1% TFA. Collected fractions were dried under a 95% nitrogen/5% hydrogen atmosphere in a Savant SpeedVac concentrator. All peptide manipulations were performed in this atmosphere to prevent cysteine oxidation. All buffers were degassed extensively with helium prior to use. Peptide identities were confirmed by mass spectrometry.

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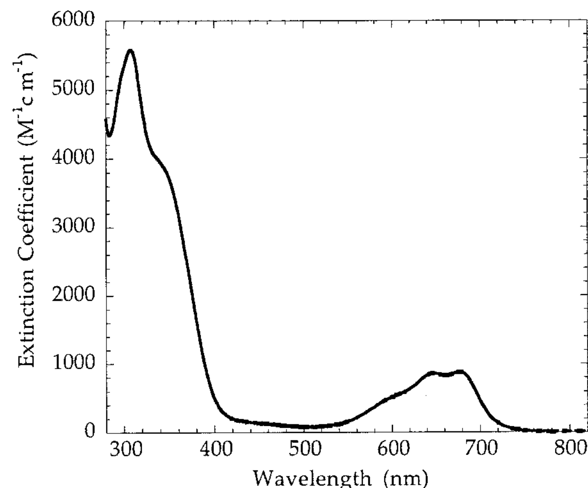

Figure 2. Absorption spectrum of the cobalt(II) complex of NZF-13.

UV/Visible Spectroscopy. Spectroscopic studies were performed on a Perkin-Elmer Lambda 9 spectrophotometer at room temperature (23 °C). Teflon-stoppered quartz cuvettes were used to minimize sample exposure to oxygen. Peptide concentrations were determined by absorbance at 679 nm in the presence of excess cobalt(II). The concentration of reduced cysteine residues was periodically confirmed by reaction with DTNB. The cobalt(II) titrations were performed in a 100 mM HEPES/50 mM NaCl buffer at pH 7.0 with peptide concentrations at approximately 100 μM. Binding constants for cobalt(II) and zinc(II) binding were determined using nonlinear least-squares methods. Titrations of the mutant peptides with β-mercaptoethanol were performed in 200 mM Tris/50 mM NaCl buffer at pH 8.0 with peptide concentrations of approximately 100 mM in 1 mL of buffer. The peptide was saturated with 1.2 equiv of cobalt(II) before prior to the addition of β-mercaptoethanol.

NMR Studies. NMR studies were preformed on a Varian Unity Plus spectrometer at a proton frequency of 500 MHz in D_2O.

Results

A peptide, NZF-13, corresponding to a putative zinc-binding domain from the transcription factor NZF has been prepared and characterized. Metal binding has been probed through the use of the zinc(II) substitute cobalt(II).[20] The absorption spectrum of the cobalt(II) complex of NZF-13 is shown in Figure 2. The intensity of the bands in the visible region with extinction coefficients of >800 M^{-1} cm^{-1} clearly indicate tetrahedral coordination, and the intense charge-transfer bands in the UV region are consistent with coordination by cysteine thiolate ligands. Titration studies reveal a stoichiometry of one cobalt(II) bound per peptide with an apparent dissociation constant of $5(\pm1) \times 10^{-6}$ M for cobalt(II). Competition studies with zinc(II)[21] indicate that zinc(II) binds more strongly than does cobalt(II) with a dissociation constant of $2(\pm1) \times 10^{-9}$ M for the zinc(II) complex. These values are comparable to those for other naturally occurring zinc binding domains.[21−26] Comparison of this spectrum with those of other cobalt(II) complexes of peptides with mixed cysteine/histidine coordination reveals

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that the spectrum is highly suggestive of coordination by three cysteines and one histidine.[27] The absorption features are too red shifted to be due to coordination by two cysteines and two histidine ligands. Thus, studies of the wild-type peptide confirm that these domains do, indeed, bind metal ions and strongly suggest coordination by the three conserved cysteine residues and one of the two conserved histidine residues.

The remaining studies were directed toward determining which of the two conserved histidine residues is directly coordinated to the bound metal ion. Three additional peptides were synthesized: two peptides with each histidine changed to alanine and one peptide with both histidines simultaneously changed to alanine (Figure 1). Titrations with cobalt(II) were performed as shown in Figure 3. The spectra for the cobalt(II) complexes of the wild-type and the two singly changed peptides are remarkably similar. Detailed examination reveals that the shape of the spectrum for the NZF-13(H24A) complex is more similar to that for the wild-type than that for the NZF-13(H16A) complex, as shown in Figure 4. The close similarity is also seen in the dissociation constants for cobalt(II) and zinc(II) summarized in Table 1. In both cases, the singly mutated peptides appear to bind cobalt(II) slightly less tightly than does the wild-type peptide. However, these effects are quite modest and do not readily distinguish the two singly mutated peptides from one another. In contrast, the spectrum of the complex of the doubly mutated peptide is clearly quite different from those from the other three peptides. Furthermore, titration with cobalt(II) revealed that the metal binding is approximately 2 orders of magnitude weaker than that for the wild-type or singly mutated peptides.

The two singly mutated peptides were further probed for their ability to bind the exogenous ligand β-mercaptoethanol. This approach has been used to examined a TFIIIA-like peptide from which one of the histidine ligands was deleted.[28] The NZF peptides were saturated with cobalt(II), and up to 110 equiv of β-mercaptoethanol was added at pH 8.0. No significant spectral changes were observed for any of the peptides. This indicates that either the exogneous ligand was not able to compete with a protein-derived ligand or that a solvent-occupied site was inaccessible.

As an additional structural probe, NMR studies of these peptides were attempted. In the absence of added metal, sharp spectra indicative of unfolded peptides were observed for all peptides. Upon the addition of zinc(II), significant changes in line position and substantial line broadening occurred (data not shown). However, in all cases, more resonances were observed than could be accounted for by a single conformation. Many attempts were made to improve the appearance of these spectra through variation in concentration and temperature. However, spectra of sufficient quality to address the questions related to histidine coordination could not be obtained.

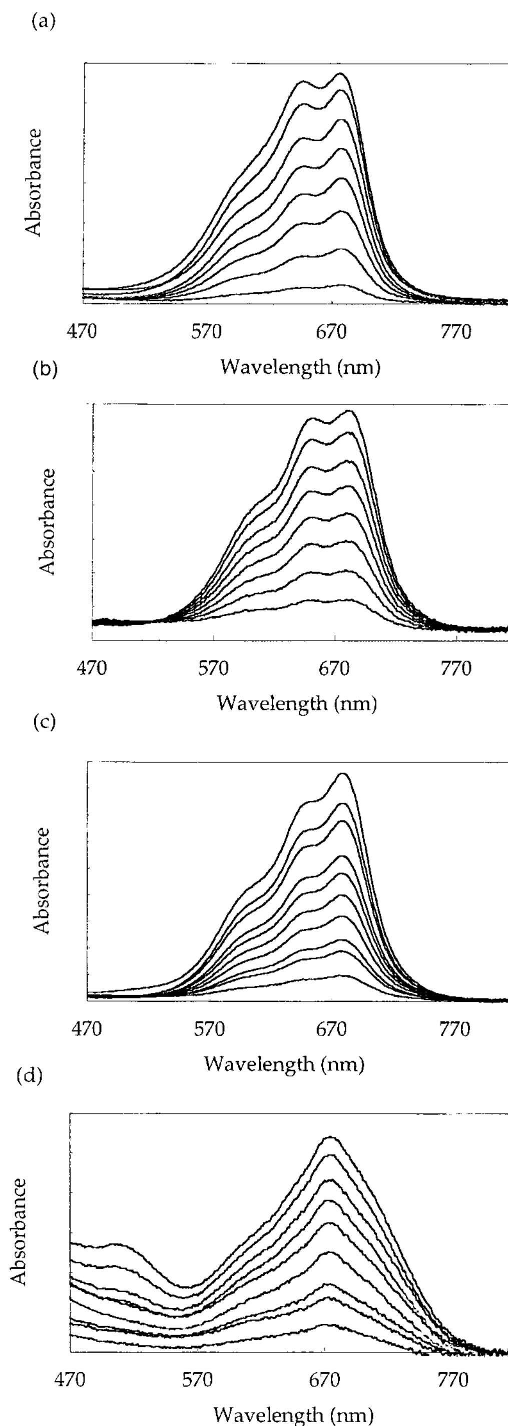

Figure 3. Optically monitored titrations of (a) NZF-13, (b) NZF-13-(H16A), (c) NZF-13(H24A), and (d) NZF(H16A, H24A) with cobalt-(II). The intensities of the bands in the visible region are essentially the same for (a)−(c) while those for the doubly modified peptide in (d) are less intense by more than a factor of 2 and have been normalized for display.

Discussion

Studies of the wild-type NZF-13 peptide demonstrate that a NZF-MyT1 domain peptide binds cobalt(II) and, presumably, zinc(II) in a tetrahedral site with metal ion affinities comparable to those observed for other zinc-binding domains. The absorption spectrum of the cobalt(II) complex strongly suggests that all three of the conserved cysteine residues and one of the two conserved histidine residues are involved in metal binding. The roles of the two histidine residues in metal binding were examined through the preparation of peptides in which one or both of the histidine residues were substituted with alanine. The properties of the peptide in which both histidines had been replaced by alanine were dramatically affected, indicating that at least one histidine is required for high-affinity metal binding. Each of the singly mutated peptides formed cobalt(II) complexes with spectra that were quite similar to that of the wild-type peptide and had similar metal ion affinities. Previous studies had revealed that removal of a metal binding histidine residue

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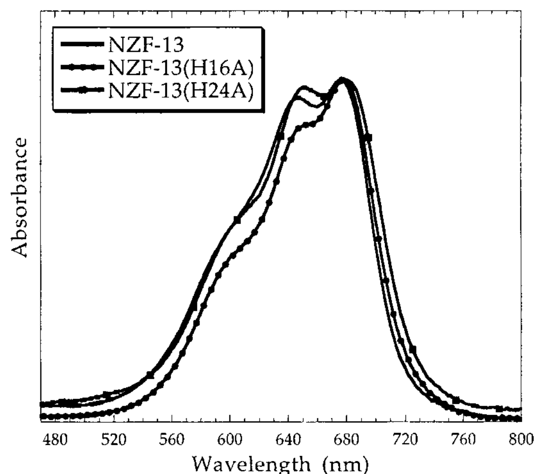

Figure 4. Comparison of the normalized absorption spectra of NZF-13, NZF-13(H16A), and NZF-13(H24A).

Table 1. Dissociation Constants for NZF-13 and Its Variants[a]

	K_d^{Co} (M)	K_d^{Zn} (M)
NZF-13	$5(\pm1) \times 10^{-6}$	$2(\pm1) \times 10^{-9}$
NZF-13(H16A)	$2(\pm1) \times 10^{-5}$	$6(\pm3) \times 10^{-9}$
NZF-13(H24A)	$9(\pm2) \times 10^{-6}$	$2(\pm1) \times 10^{-9}$
NZF-13(H16A,H24A)	$9(\pm4) \times 10^{-4}$	nd

[a] Each value is the average of at least three experiments. Estimated standard deviations are given. nd = not determined (due to weak metal binding).

from a TFIIIA-type peptide resulted in a peptide that still bound cobalt(II) in a tetrahedral fashion but with an accessible coordination site that could be occupied by solvent or added exogenous ligands.[28] The absorption spectra of the native TFIIIA-like peptide with coordinated histidine and the modified peptide with coordinated water were only slightly different. Thus, the similarity of the spectra for wild-type NZF-13 and the mutated peptides is not highly informative. The binding of an additional thiolate ligand from β-mercaptoethanol is expected to lead to substantial spectra changes, based on previous studies. However, the addition of large exesses of β-mercaptoethanol to the cobalt(II) complexes of either peptide did not lead to an appreciable spectra change. This suggests that the cobalt(II) complexes of each of the peptides have four peptide-derived ligands.

One possible model that would account for these observations is replacement of the metal-binding histidine by alanine leads to recruitment of the remaining histidine for metal coordination. A similar phenomenon has been demonstrated for the adenovirus E1A protein where mutation of a metal-coordinating cysteine results in coordination of two histidine residues not normally involved in metal coordination as well as loss of coordination by an additional cysteine residue.[29] In the present case a less substantial rearrangement would be required. Thus, our observations are most consistent with the model shown in Figure 5. In the wild-type peptide, the metal is coordinated by the three cysteines and by histidine 16. Upon replacement of histidine 16 with alanine, the structure rearranges somewhat so that histidine 24 now binds the metal ion.

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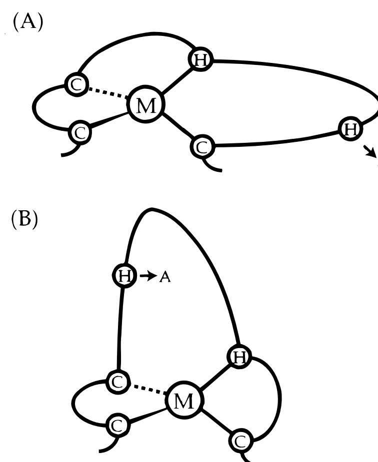

Figure 5. Model for metal coordination by NZF-13 and the effect of replacement of histidine 16 by alanine: (A) metal coordination for wild-type NZF-13 and NZF-13(H24A) peptides; (B) metal coordination for the NZF-13(H16A) peptide.

These studies have been performed on peptides that extend five and four residues before and after the first and last metal-coordinating cysteine residue, respectively. In parallel, we have initiated studies on larger fragments of NZF-1 with boundaries defined by limited proteolysis.[19] A peptide with two, tandem NZF-MyT1 domains binds cobalt(II) with an apparent dissociation constant of 1×10^{-7} M at pH 7. Future experiments will be required to compare the effects of domain boundaries on metal coordination and the relative stabilities of different forms of the NZF-MyT1 metal-binding domain. These studies should help clarify whether the plasticity observed for the metal-binding peptides studies herein is an intrinsic property of these domains, important for biological function, or it is unique to the particular peptides studied.

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