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Forum

Site Selection in Tandem Arrays of Metal-Binding Domains

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The metal binding properties of peptides corresponding to metal-binding sites spanning regions that normally function as linkers in tandem arrays of metal-binding domain-containing proteins were examined. For a peptide with two His residues from one TFIIIA-like zinc finger domain, a canonical TFIIIA-like linker, and two Cys residues from an adjacent zinc domain, the dissociation constant for the 1:1 peptide to cobalt(II) was found to be 15 ± 10 μM, compared with 60 nM for the corresponding zinc finger domains themselves. Peptides overlapping two sets of metal-binding domains from human TRAF (tumor necrosis factor receptor-associated factor) proteins were examined. In one case, the affinity of the presumed metal-binding domain and that for the linker region were comparable, while in the second case, the affinity of the linker peptide was higher than that for the corresponding presumed metal-binding domain peptide. These studies revealed that cobalt(II) affinities in the micromolar range can occur even for peptides that do not correspond to natural zinc-binding domains and that the degree of distinction between authentic metal-binding domains and the corresponding linker-spanning peptides may be modest, at least for single domain peptide models.

Introduction

In recent years, a large number of metal-binding protein domains have been discovered and characterized.[1−5] These domains are relatively short stretches of polypeptide, typically 20−50 amino acids in length, that coordinate one or more metal ions, usually through cysteine and histidine residues. The prototypical family of proteins containing these domains is the TFIIIA-like C_2H_2 zinc finger protein family. The founding member of this family, transcription factor IIIA (TFIIIA), was discovered to contain nine tandem sequences that approximated the form Cys-$X_{2,4}$-Cys-X_3-Phe-X_5-Leu-X_2-His-$X_{3,4}$-His.[6,7] It was proposed,[6] and subsequently confirmed, that the four Cys and His residues tetrahedrally coordinate a zinc ion to form stable structural domains. These domains are separated from one another by short linker sequences that are usually seven amino acids in length.[8]

Shortly after the discovery of these sequences in TFIIIA, other protein sequences, deduced from cDNA clones, were found to contain variable numbers of the similar sequences.[9−13] In many cases, the zinc-binding domain sequences were in tandem sets separated by linker sequences that approached

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the sequence ThrGlyGluLysProTyrX.[8] Indeed, this linker sequence was often the most highly conserved portion of the protein sequences. In a limited number of other cases, the presumed zinc-binding domain sequences were separated from one another by much longer linker sequences. With the availability of complete genomic sequences,[14,15] a more comprehensive analysis of these sequences can be performed. Remarkably, the TFIIIA-like zinc finger domain appears to be the most frequently encoded domain in the human genome with more than 5000 domains encoded in more than 700 proteins. In addition, more than 15 other classes of zinc-binding domains have been characterized in eukaryotes.[1−5] These domains show a range of structures and patterns of metal-binding residues but have the common property of stabilization by zinc ions bound to tetrahedral sites with ligands derived from cysteine and histidine. Metal-binding studies have been reported peptides derived from many of these protein classes.[16−23]

Peptides corresponding to single TFIIIA-like zinc-binding domains have proven to be useful for studies of protein folding.[3,24−27] The utility of these peptides is due to the fact that they are largely or completely unfolded in the absence of bound metal ions yet fold into well-defined structures upon metal binding. Thus, the thermodynamics of domain folding is directly reflected in the thermodynamics of metal binding. Metal-induced domain folding has been monitored optically using cobalt(II) as a probe and, more recently, using isothermal titration calorimetry. These studies have revealed thermodynamic features responsible for metal-induced protein folding as well as general characteristics such as β-sheet propensities and the energetics of salt bridge formation.

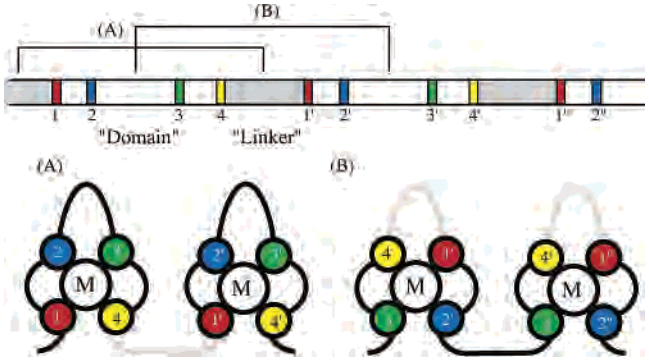

Figure 1. Coordination schemes for a tandem set of metal-binding domains. In case A, ligands 1, 2, 3, and 4 coordinate one metal ion while ligands 1′, 2′, 3′, and 4′ coordinate a second ion. In case B, ligands 3, 4, 1′, and 2′ coordinate one metal ion while ligands 3′, 4′, 1″, and 2″ coordinate a second. These schemes may not be readily distinguishable on the basis of sequence information alone.

A protein with a set of tandem potential metal-binding domains presents an additional challenge from an inorganic chemistry perspective, namely, that alternative coordination schemes are possible (Figure 1). In case A, each metal ion is coordinated by four ligands within each "domain" with each pair of presumed domains connected by a "linker". In case B, each metal ion is coordinated by the carboxyl-terminal two liganding amino acids from one "domain" and the amino-terminal two liganding amino acids from the adjacent "domain". In this case, the "linker" residues form the body of the metal-binding domain. These alternative structures can be viewed from two perspectives. First, distinguishing the "domain" from the "linker" from sequence information alone is not always straightforward. In the case of TFIIIA-like proteins, the correct assignment was hypothesized at the time of discovery and supported by a variety of observations. However, for other proteins such as the TRAF (tumor necrosis factor receptor-associated factor) family[28,29] to be discussed subsequently, such an assignment was not entirely clear. Second, alternative structures could still form depending on the relative stability and kinetics of formation of the various structures. It has been assumed that the "correct" structure is uniquely formed, but the degree of

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Table 1. Metal-Binding Peptide Sequences[a]

P-HHLCC	LV**H**QRT**H**TGEKPYK**C**PE**C**GKS
P-TRAF2-A	YPLT**C**DG**C**GKKKIPRE*KFQD**H**VKT**C**GG*
P-TRAF2-B	Y*KFQD**H**VKT**C**GG*CRVP**C**RFHAIG**C**LET
P-TRAF4-A	SVY**C**ENK**C**GARMMRRLL*AQ**H**ATSE**C**PKRT*
P-TRAF4-B	*AQ**H**ATSE**C**PKRT*QP**C**TY**C**TKE

[a] Amino acid sequences are shown in one-letter code. Presumed metal-binding residues are shown in bold. The regions of overlap between the peptides from TRAF2 and TRAF4 are shown in italic.

preference of the correct structure over alternatives does not appear to have been examined prior to this work.

Here, we examine the metal-binding properties of peptides derived from two systems. The first case involves a peptide derived from the linker between two TFIIIA-like zinc finger domains. In this case, the metal ion affinities of the TFIIIA-like domains themselves have been extensively studied,[30−33] and the biologically relevant coordination scheme is not in doubt, but the affinity of alternative coordination schemes has not been examined. A preference of approximately 2 orders of magnitude for the canonical coordination scheme appears to apply. The second case involves peptides derived from two members of the TRAF family. In this case, no studies of metal ion binding to peptides related to any coordination scheme have been reported, and only a modest amount of structural information[34] is available. Here, the affinities of peptides relevant to the two possible coordination schemes are comparable. These observations suggest either that the two alternative structures do, indeed, have similar stabilities or that the assumption that single domain peptides accurately reproduce the metal-binding properties of a protein with a set of tandem metal-binding domains is not applicable to these proteins.

Results

TFIIIA-like zinc finger protein-derived domains are the most well characterized class of zinc-binding domain. Peptides related to both natural zinc finger protein sequences and a designed consensus zinc finger sequence have been used for metal-binding studies. To examine the possibility that metal ions could bind using an alternative coordination scheme, the peptide LV**H**QRT**H**TGEKPYK**C**PE**C**GKS (hereafter referred to as P-HHLCC) was prepared. P-HHLCC corresponds to the carboxyl-terminal half of one zinc finger domain (with two His residues in bold), the linker sequence (underlined), and the amino-terminal half of the adjacent zinc finger domain (with two Cys residues in bold) (see also Table 1). The metal-binding properties of this peptide were examined using cobalt(II) as a probe. This metal ion offers several advantages over zinc(II). First, this ion has spectroscopic properties that change dramatically upon peptide binding due to the change in coordination geometry from octahedral in the absence of peptide to tetrahedral when bound to many metal-binding domain peptides. Specifically, the extinction coefficients for the d−d transition in the visible region are substantially larger for tetrahedral complexes than they are for octahedral complexes. Extinction coefficients greater than 300 M^{-1} cm^{-1} are characteristic of tetrahedral complexes. For a purely tetrahedral complex, the visible band is due to the $^4A_2$ to $^4T_2(P)$ transition. This transition is split into three components in a less symmetrical environment. The position of these bands and their splitting are sensitive to the ligands coordinated to the cobalt(II) center. In particular, complexes with Cys_2His_2, Cys_3His, and Cys_4 coordination units can generally be distinguished.[32]

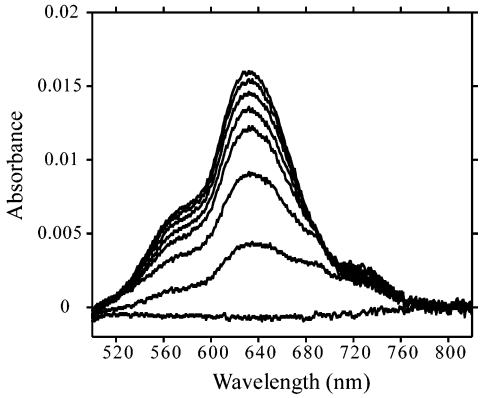

Figure 2. Cobalt(II)-binding by a TFIIIA-like linker peptide. The results of a titration of P-HHLCC with cobalt(II) monitored in the visible region.

The results of titration of P-HHLCC with cobalt(II) are shown in Figure 2. Addition of cobalt(II) results in the appearance of a spectrum due to tetrahedral complexes of cobalt(II) with a maximum absorbance near 635 nm. Initial spectra (where the sample has a high peptide to metal ion ratio) have a shoulder near 740 nm that grows in and then disappears as the titration continues. This behavior has been observed previously and is suggestive of the formation of 2:1 peptide to cobalt(II) complexes with coordination exclusively through cysteinate residues.[35] Curve fitting with these and other data with this peptide reveals a dissociation constant of 15 ± 10 μM for the 1:1 P-HHLCC/cobalt(II) complex.

The second class of zinc-binding domains that were studied is from the TRAF proteins.[29] Six members of this family are encoded in the human genome. These proteins associate with membrane-bound tumor necrosis factor α receptors. The TRAF proteins contain at least two classes of zinc-binding domain that appear to play structural roles or to mediate protein−protein interactions. They are unlikely to participate in interactions with nucleic acids. A RING finger motif[36] is found near the amino-terminus of each TRAF protein. These were not studied here and will not be discussed further. Adjacent to the RING finger motif is a tandem set of sequences that approximate the form Cys-X_{2-6}-Cys-$X_{11,12}$-His-$X_{3,4}$-Cys-X_6. Using an alternative coordination

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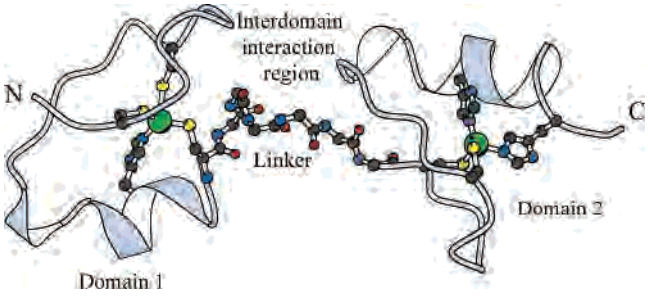

Figure 3. Structure of a tandem set of two TRAF-like zinc-binding domains from Siah.

scheme (see Figure 1), these sequences could be described as His-$X_{3,4}$-Cys-X_6-Cys-X_{2-6}-Cys-$X_{11,12}$. When this project was initiated, the nature of the natural coordination structure was unknown. Furthermore, no direct structural information on the TRAF proteins themselves is yet available. However, a crystal structure of a fragment of a related protein, Siah (seven in absentia homolog), has since been reported.[34] This structure reveals the presence of two TRAF-like zinc-binding domains of the form Cys-X_6-Cys-X_{11}-His-$X_{3,4}$-(Cys or His) joined by a six amino acid linker (Figure 3). The overall structure of these domains is quite similar to that for the TFIIIA-like zinc finger domains although the TRAF-like domains lack the conserved hydrophobic core that is characteristic of TFIIIA-like zinc fingers. There is significant interaction between the two domains due, in part, to the fact that the linker (of six amino acids) is one amino acid shorter than the typical TFIIIA-like zinc finger protein linker, drawing the domains closer together.

To examine the relative stability of the alternative coordination structures of the TRAF domains, peptides derived from two different TRAF proteins were investigated. These four peptides are from the fourth zinc-binding domain from human TRAF2, the linker spanning the fourth and fifth domains from TRAF2, the fourth zinc-binding domain from human TRAF4, and the linker spanning the fourth and fifth domains from TRAF4. These domains appear to be representative of the TRAF-like domains based on sequence features. The sequences of these peptides are shown in Table 1. Solutions of each of these peptides were titrated with cobalt(II) with the titrations monitored by absorption spectroscopy. In each case, spectra consistent with tetrahedral cobalt(II) complexes with Cys_3His coordination were observed with three partially resolved transitions in the 500−800 nm region of the spectrum. The spectra are shown in Figure 4, and a representative fit is shown in Figure 5. In each case, no significant changes in spectral shape were observed over the course of the titrations suggesting that only 1:1 cobalt(II) to peptide complexes were formed to a significant degree. The shapes of the spectral envelopes for the two peptides that correspond to the Cys-$X_{2,3}$-Cys-$X_{11,12}$-His-$X_{3,4}$-Cys metal-binding domains (P-TRAF2-A and P-TRAF4-A) were similar to one another and distinct from the two His-$X_{3,4}$-Cys-X_6-Cys-$X_{2,6}$-Cys linker peptides (P-TRAF2-B and P-TRAF4-B). The titration data were fit to yield dissociation constants for the four peptide−cobalt(II) complexes. The results are summarized in Table 2.

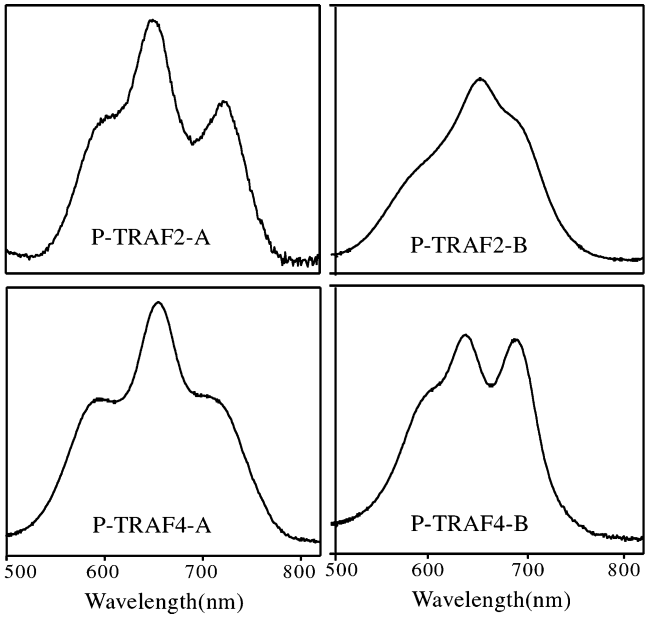

Figure 4. Absorption spectra of TRAF-derived peptide−cobalt(II) complexes. These spectra are on approximately the same scale with maximum extinction coefficients between 700 and 900 M^{-1} cm^{-1}.

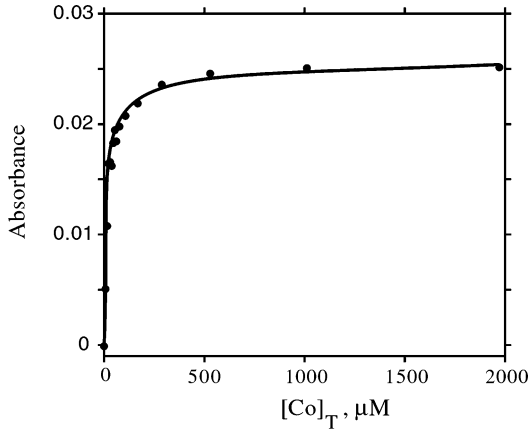

Figure 5. Cobalt binding by TRAF2-A. Absorbance values at 635 nM for 7 μM were fit to reveal a dissociation constant of 13 ± 3 μM.

Table 2. Dissociation Constants for Peptide−Cobalt(II) Complexes

P-HHLCC	15 ± 10 μM
P-TRAF2-A	13 ± 3 μM
P-TRAF2-B	13 ± 5 μM
P-TRAF4-A	70 ± 20 μM
P-TRAF4-B	3 ± 2 μM

Discussion

For TFIIIA-like zinc finger proteins, a wide range of studies support the notion that these proteins consist of tandem arrays of Cys-$X_{2,4}$-Cys-X_{12}-His-$X_{3,4}$-His metal-binding domains joined by relatively flexible linkers that are very often seven amino acids in length. Metal-binding studies have revealed that single TFIIIA-like zinc finger peptides bind metal ions tightly with affinities for cobalt(II) ranging from 60 nM to 10 μM at pH 7.0 and affinities for zinc(II) that are 3−4 orders of magnitude more tightly.[16,30,32,37]

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The potential to form structures based on alternative coordination schemes has not been extensively examined. Certain TFIIIA-like peptides, particularly those containing significant mutations, have been shown to form 2:1 peptide to metal complexes with coordination through the Cys residues.[35,38,39] Here, a different possibility has been examined, namely the formation of a 1:1 complex, but with the metal-binding unit spanning the linker region. P-HHLCC, a peptide corresponding to the carboxyl-terminal portion of one consensus zinc finger domain, a canonical linker, and the amino-terminal portion of a second consensus zinc finger domain, binds cobalt(II) in a tetrahedral Cys_2His_2 environment at appropriate peptide to cobalt(II) ratios. The dissociation constant for this complex is 15 ± 10 μM. This binding is approximately 200-fold weaker than that observed for the consensus zinc finger peptide itself,[30,32] although it is close to affinities for other natural-sequence zinc finger peptides.[16,37] Thus, the canonical binding mode is favored by TFIIIA-like zinc finger domains by approximately 2 orders of magnitude. The relative instability of the P-HHLCC complex is supported by two other observations. First, the peptide does show a tendency to form a 2:1 peptide to cobalt(II) complex at high peptide to cobalt(II) ratios. Second, the binding of zinc(II) to P-CCLHH does not result in the formation of a structurally well-defined complex as determined by NMR methods (data not shown).

Peptides corresponding to two representatives of the TRAF family were also examined. For peptides derived from TRAF2, no significant difference was observed in cobalt(II) affinity for peptides designed to allow binding in coordination scheme A or coordination scheme B. The peptide P-TRAF2-A likely corresponds to the natural binding scheme, based on analogy with the structure observed in Siah. Each TRAF2-derived peptide binds cobalt(II) to form a complex with a dissociation constant near 15 μM with no evidence for the formation of complexes other than the 1:1 complex. For the peptides derived from TRAF4, there is a substantial difference in affinity between the two peptides corresponding to different coordination schemes. Remarkably, however, the peptide that corresponds to coordination scheme B shows a higher affinity for cobalt(II) by a factor of approximately 20. This is not the anticipated form based on analogy with Siah.

Comparison of the absorption spectra of the four complexes reveals an additional correlation. The features in the absorption spectrum of P-TRAF2-A-cobalt(II) are somewhat sharper than those of P-TRAF4-A-cobalt(II), and P-TRAF2-A has a higher affinity (13 ± 3 μM) than does P-TRAF4-A (70 ± 20 μM). Similarly, the features in the absorption spectrum of P-TRAF4-B-cobalt(II) are substantially sharper than those of P-TRAF2-B-cobalt(II), and P-TRAF4-B has a higher affinity (3 ± 2 μM) than does P-TRAF2-B (13 ± 5 μM). Thus, sharper features (and, hence, a presumably more well-defined and less dynamic structure) appear to correlate with higher metal ion affinity.

How can we account for the comparable metal ion affinities of peptides that correspond to natural and unnatural binding sites for the TRAF domains? The most likely explanation is that full metal-binding domain stability requires tandem arrays of TRAF-like metal-binding domains supported by the sorts of interdomain interactions observed in the Siah structure.[34] In the absence of these interactions, single domain peptides may show decreased metal affinity and may not fold into well-defined structures. This suggests that different metal-binding properties may be observed for tandem arrays of TRAF-like zinc-binding domains including metal-binding cooperativity. If such cooperativity were demonstrated, it would stand in contrast to results for arrays of TFIIIA-like zinc finger domains joined by canonical seven amino acid linkers where no significant metal-binding cooperativity was observed.[40] This must await further investigations.

In summary, these results demonstrate that metal binding to sites including linker regions from sets of tandem zinc-binding domains may be of comparable or even higher affinities to those to sites that correspond to the functional metal-binding domains. This competition is intrinsic to the nature of tandem arrays of metal-binding domains. These studies lay the groundwork for further studies to probe the chemical and biologically evolved characteristics of metal binding and metal binding-induced domain folding reactions.

Materials and Methods

Peptide synthesis and Purification. Peptides were either synthesized by solid phase synthesis on a MilliGen/Biosearch 9050 Peptide Synthesizer as described previously[32] or purchased from Bio-Synthesis. All peptides were purified by reversed-phase HPLC with a Vydac C-18 column using gradients of 10−45% acetonitrile. Peaks were collected, and peptide identities were confirmed by MALDI-TOF mass spectrometry. Collected samples were transferred into a 2−4% dihydrogen in dinitrogen environment and lyophilized. All manipulations with purified peptides were performed in an anaerobic environment to prevent oxidation of the cysteine residues.

Cobalt Titrations. All spectroscopic studies were done using a Perkin-Elmer Lambda 9 spectrophotometer. The peptides were suspended in distilled, deioninzed water. A 200 mM Hepes, 50 mM sodium chloride buffer at pH 7 was used as the titration buffer. All solutions were sterile filtered and degassed before use. Cobalt chloride solutions in the same buffer were titrated into cuvettes containing peptide solutions. The absorption spectra of the solutions were monitored between 450 and 820 nm. Titration data were analyzed by nonlinear least-squares analysis of the concentration of cobalt versus the absorbance at the maximum absorbance wavelength using KaleidaGraph (Synergy software) as described previously.[32] Specifically, the absorption at the absorption maximum was fit to the equation $A = \epsilon[\mathrm{PM}]$ where $[\mathrm{PM}] = (1/2)((P_T + M_T + K_d) - \sqrt{((P_T + M_T + K_d)^2 - 4P_TM_T)})$ where ϵ is the extinction coefficient, P_T is the total concentration of active peptide, M_T is the total concentration of cobalt, and K_d is the dissociation constant. Variables fit include ϵ, P_T, and K_d. Total peptide concentrations ranged from 6 to 17 μM.

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