Metal binding properties of single amino acid deletion mutants of zinc finger peptides: Studies using cobalt(II) as a spectroscopic probe

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ABSTRACT Peptides corresponding to Cys_2His_2 zinc finger domains from which one amino acid has been deleted have been synthesized and their metal-binding properties characterized. In contrast to earlier reports (Párraga, G., S. Horvath, L. Hood, E. T. Young, and R. E. Klevit. 1990. *Proc. Natl. Acad. Sci. USA.* 87:137–141.), such peptides do bind metal ions such as cobalt(II). A peptide with the sequence ProTyrLys**Cys**ProGlu**Cys**LysSerPheSerGlnLysSerAspLeuValLys**His**GlnArgThr**His**ThrGly (which corresponds to a previously characterized consensus zinc finger sequence from which a Gly residue immediately following the second Cys residue has been deleted) was found to form a 1:1 peptide to cobalt(II) complex with an absorption spectrum quite similar to those previously observed for zinc finger peptide-cobalt(II) complexes. The dissociation constant for this complex is 6×10^{-6} M, a factor of 100 times higher than that for the parent peptide. A peptide with the sequence LysProTyrPro**Cys**GlyLeu**Cys**ArgCysPheThrArgArgAspLeuLeuIleArg**His**AlaGlnLysIle**His**SerGlyAsnLeu corresponding to a similar mutation of the peptide ADR1 was also characterized. Spectroscopic studies with cobalt(II) revealed that this peptide forms both 1:1 and 2:1 peptide to cobalt(II) complexes. The absorption spectra of the two forms and the dissociation constants were determined via deconvolution methods. In contrast, the parent peptide ADR1a was found to form only a 1:1 complex under comparable conditions and this 1:1 complex was found to be more stable than that for the mutant. These results reveal that deletion mutations do adversely affect the stability of zinc finger peptide-metal complexes but that the effects are not as drastic as had been previously described.

INTRODUCTION

The development of site-directed mutagenesis methods have allowed investigation of the effects of variation of amino acid sequences on protein structure, function, and stability (2, 3). Proteins have been demonstrated to be remarkably plastic. For many positions, a wide variety of amino acid substitutions are tolerated without drastic effects. Furthermore, the effects of single and double amino acid insertion mutations have also been probed (4, 5). Again, these changes are often accommodated with relatively small losses in stability. Perhaps the mutations that are expected to have the largest effects are deletions. A striking example of such a mutation was reported by Párraga et al. (1). They reported that a single amino acid deletion within a peptide corresponding to one of the zinc finger domains from the yeast transcription factor ADR1 resulted in the loss of detectable metal binding activity. ADR1 belongs to the Cys_2His_2 class of zinc finger proteins (6). These are characterized by the presence of one or more sequences of the form (Tyr,Phe)-X-Cys-$X_{2,4}$-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His. The Cys and His residues are involved in chelating a zinc ion. Note that with the exception of one of the domains from the human oncogene GLI[7] and its homologs (8), the spacing between the second of the Cys residues and the first of the His residue is conserved at exactly 12 amino acids.

The peptide studied was based on the sequence of ADR1a (1, 9). This peptide has the sequence LysProTyrProCysGlyLeuCysAsnArgCysPheThrArgArgAspLeuLeuIleArg**His**AlaGlnLysIle**His**SerGlyAsnLeu. A deletion mutant (hereafter referred to as ADR1adel138) was synthesized that involved removal of the asparagine residue that immediately follows the second metal-coordinating cysteine (underlined above) (1). Based on optical studies with cobalt(II) and ^1H nuclear magnetic resonance (NMR) studies with zinc(II), it was concluded that this peptide did not bind metal ions with affinities remotely comparable to those of the unaltered zinc finger peptide. Given the striking conservation of the metal binding residue spacing among the zinc finger domains, this observation seemed plausible. However, since zinc finger peptides bind zinc with dissociation constants in the nanomolar to picomolar range (10, 11), this lack of appreciable binding of metal ions at millimolar concentrations indicates a loss of up to 8 to 12 kcal/mol in the stability of the peptide-metal complexes. Because of the apparent size of this destabilization and because corresponding mutants of minimalist zinc finger peptides containing mostly alanine residues still bound metal ions (12), we chose to reinvestigate these observations.

Spectroscopic probes have played extremely important roles in characterizing biological macromolecules. A useful probe should combine reasonable sensitivity, so that it can be observed at low to moderate concentrations, with variability in properties so that the spectroscopic properties of the probe provide information about the structural and/or dynamical properties of the probe attachment site. The nitroxide spin labels (developed by McConnell and coworkers) represent one class of probes

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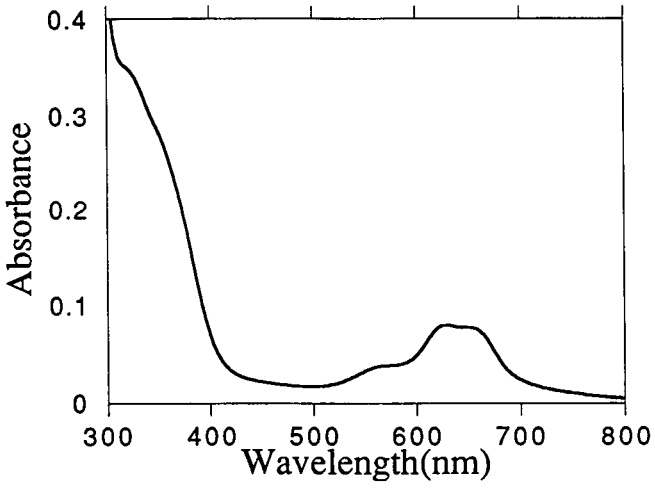

FIGURE 1 The absorption spectrum of ADR1del138 in the presence of saturating levels of cobalt(II). The peptide concentration was 128 micromolar and 5 equivalents of cobalt(II) had been added.

that meets these conditions (13). For probing zinc(II) binding sites in proteins, cobalt(II) has many desirable properties (14). Among these properties are the fact that the extinction coefficients of the ligand field transitions in the visible region depend on coordination number. For octahedral complexes such as those formed by cobalt(II) in aqueous solution, the extinction coefficients are approximately 5–10 M^{-1} cm^{-1} whereas for tetrahedral sites such as those in the zinc finger domains, the extinction coefficients are approximately 100-fold higher. Five coordinate complexes show intermediate values. In addition, the precise energy and the shape of the absorption envelope are sensitive to the details of the coordination environment. For example, for tetrahedral sites containing a mixture of cysteine and histidine ligands, the visible bands shift to lower energies as the number of thiolate ligands increases (15). Because of these properties, cobalt(II) has been used extensively to probe zinc sites in nucleic acid-binding and gene regulatory proteins (16–18). Here, we use optical studies with cobalt(II) to demonstrate that a peptide with the sequence attributed to ADR1-del138 does, indeed, bind metal ions with reasonable affinity. Furthermore, these studies revealed that it forms a metal-bridged two peptide complex under some conditions. In addition, we have synthesized and characterized a corresponding deletion mutant of the zinc finger consensus peptide CP-1[11]. This peptide forms a 1:1 complex with cobalt(II) with a dissociation constant increased by a factor of approximately 100 over the parent peptide. These observations reveal that deletions are tolerated with only moderate loss in stability.

MATERIALS AND METHODS

Peptides were synthesized, purified, and characterized using methods described previously (11). The identity of all peptides was confirmed by amino acid composition. Furthermore, the identify and purity of the deletion mutant peptides was confirmed by mass spectrometry. In both cases, the parent ion peak was observed within 1 mass units of the expected value. Methods for analysis of the formation of the 1:1 and 2:1 peptide to metal complexes were adapted from other studies (12).

RESULTS AND DISCUSSION

The absorption spectrum of a solution of our ADR1del138 peptide in the presence of an excess of cobalt(II) is shown in Fig. 1. The presence of absorption bands in the visible region stands in sharp contrast to the results reported previously (1). This spectrum is similar to those observed for the cobalt(II) complexes of other zinc finger peptides.

In order to more quantitatively interpret the metal-binding properties of this peptide, a cobalt(II) titration was performed. The results are shown in Fig. 2. Early in the titration when the peptide to cobalt(II) molar ratio is high, the absorption envelope in the visible region shows a red-shifted feature not present in the spectrum of the peptide-cobalt(II) complex observed at higher levels of cobalt(II). As the addition of cobalt(II) was continued, this feature decreased in intensity and the spectrum saturated to that shown in Fig. 1. This behavior is reminiscent of that observed for a minimalist zinc finger peptide, which was shown to be due to formation of both 1:1 and 2:1 peptide to cobalt(II) complexes (12). The data shown in Fig. 2 were fit to this model. This involves deconvoluting the spectra into two components based on the initial and final spectra, and fitting the intermediate spectra, using an equilibrium model involving 1:1 and 2:1 peptide-to-metal complexes. This analysis results in estimates for the dissociation constants for the two species as well as generation of the absorption spec-

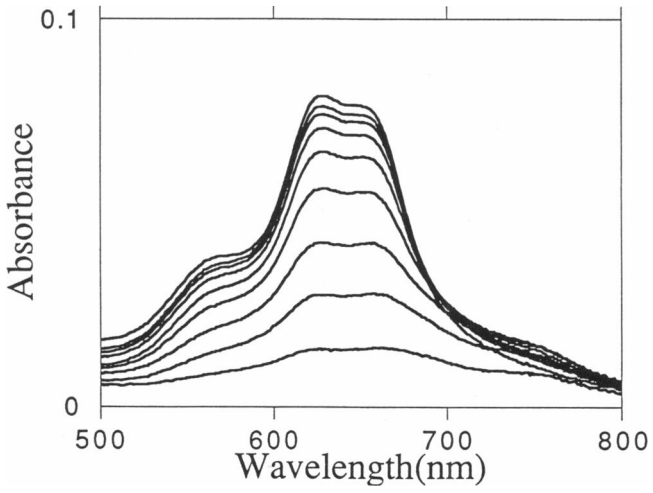

FIGURE 2 An optically monitored titration of ADR1adel138 with cobalt(II). Note the absorption around 750 nm, which first appeared then faded as the amount of cobalt(II) added was increased.

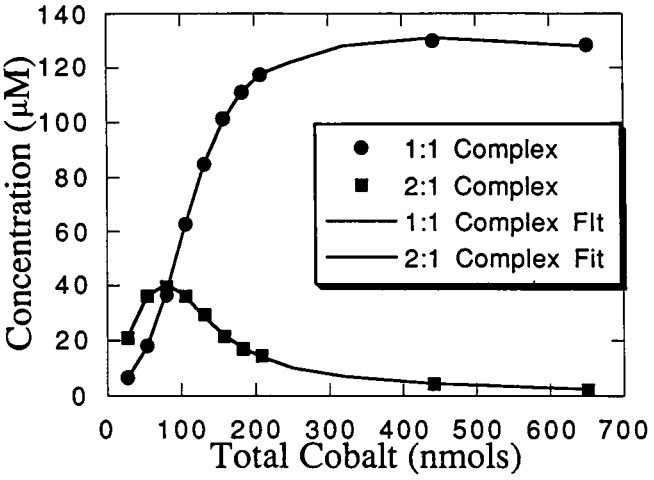

FIGURE 3 A plot of the concentrations of the 1:1 and 2:1 ADR1adel138 to cobalt(II) complexes as a function of added cobalt(II). Both the experimental data points and the fit are shown.

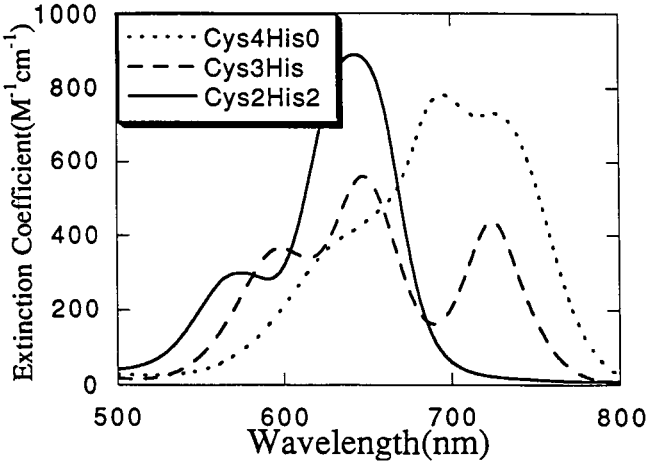

FIGURE 5 The absorption spectra of cobalt(II) complexes of zinc finger peptides with Cys_4, Cys_3His, and Cys_2His_2 coordination sites. These peptides (15) are derived from CP-1 (11) with two, one, or none of the histidines replaced with cysteine. In all cases, the peptides appear to adopt reasonable well-folded metal complexes.

tra for the two complexes in pure form as linear combinations of the initial and final spectra. The results are shown in Figs. 3 and 4. The data shown in Fig. 3 reveals that a good fit can be obtained, assuming the formation of 1:1 and 2:1 complexes. From the fits, the dissociation constant for the 1:1 complex is approximately 2×10^{-6} M, whereas the dissociation constant for the 2:1 complex is approximately 5×10^{-11} M^2. These values are not precisely determined due to parameter correlation. We estimate the uncertainties to be 1×10^{-6} M and 4×10^{-11} M^2, respectively. The absorption spectra for the 1:1 and 2:1 complexes generated from the analysis are shown in Fig. 4. The spectra for cobalt(II) complexes of peptides with Cys_nHis_{4-n} (n = 2–4) coordination sets are shown in Fig. 5 (15). Comparison reveals that the spectrum of the 1:1 complex of ADR1del138 resembles that for the Cys_2His_2 complex, whereas that for the 2:1 complex resembles the Cys_3His_1 complex spectrum. The points of comparison include both overall peak position as well as the shape of the absorption envelope. The spectra of both complexes of the deletion mutant are somewhat broader and have slightly lower extinction coefficients than do the model peptide complexes. The results for the 1:1 species are consistent with the formation of a complex with the expected coordination by two cysteinates and two histidines. The spectrum of the 2:1 complex suggests that an asymmetrical complex with one peptide binding the metal via two cysteinates with the other using one cysteinate and one histidine. This differs from previously observed 2:1 zinc finger peptide-cobalt(II) complexes, which have been shown via similar methods to involve cysteinate-only coordination (12). The reasons for this difference are unclear and will require additional study.

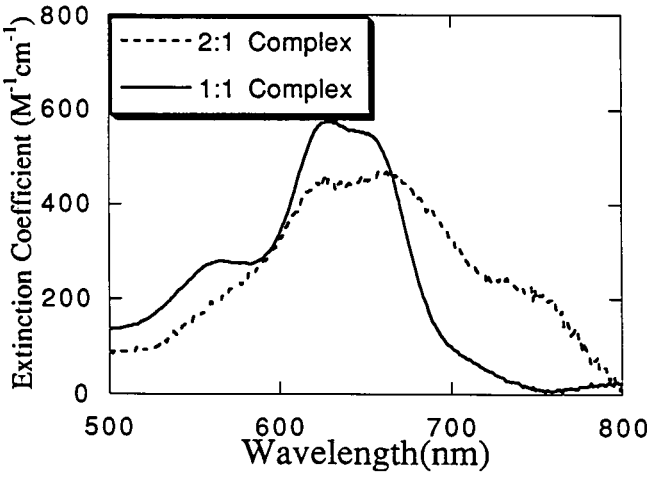

FIGURE 4 The absorption spectra for the 1:1 and 2:1 ADR1adel138 to cobalt(II) complexes deduced from the fit shown in Fig. 3.

Two additional peptides were synthesized and characterized for comparison with these results. The first is ADR1a itself. A titration of this peptide with cobalt(II) is shown in Fig. 6. No evidence of the formation of more than one species was observed. The spectrum closely matches those of other zinc finger peptide-cobalt(II) complexes in position and intensity. These data could be fit to yield a dissociation constant of $7 \pm 2 \times 10^{-7}$ M.

The second is a deletion mutant of the consensus peptide CP-1. The deletion mutant has the sequence ProTyrLysCysProGluCysLysSerPheSerGlnLysSerAsp-LeuValLysHisGlnArgThrHisThrGly. A Gly residue that follows immediately after the second cysteine of CP-1 has been deleted. Titration of this peptide with cobalt(II) produced results shown in Fig. 7. Only a very

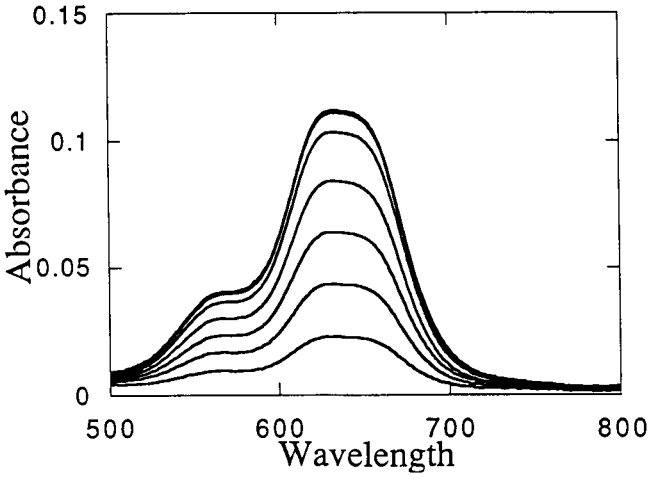

FIGURE 6 A titration of ADR1a with cobalt(II). Note the absence of any features between 750 and 800 nm. These data could be fit with a dissociation constant of $7 \pm 2 \times 10^{-7}$ M.

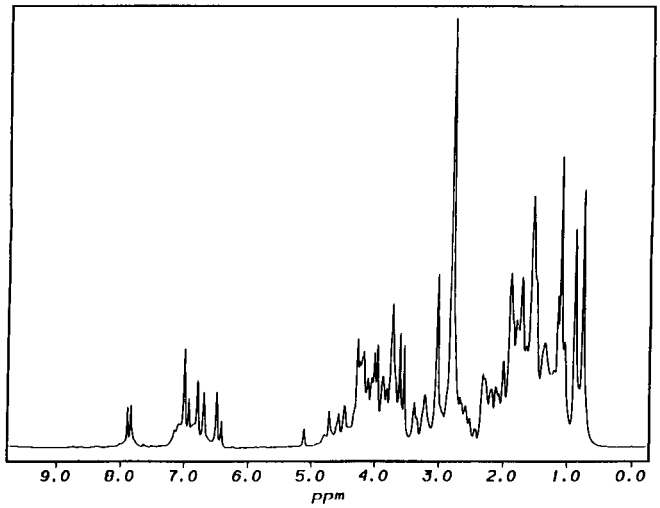

FIGURE 8 A ^{1}H NMR spectrum of the zinc(II) complex of the deletion mutant of CP-1 in D_2O. This spectrum was recorded at 600 MHz with 2.5 mM peptide, 7.5 mM zinc(II), 75 mM perdeuterated Tris-HCl, pD = 6.2, temperature = 290 K.

small amount of 2:1 complex appears to be present for this peptide. The data could be fit, using a dissociation constant of $6 \pm 2 \times 10^{-6}$ M for the 1:1 complex. This is a factor of 100 larger than that for the parent peptide CP-1. Initial studies of the zinc(II) complex of this peptide have been performed using NMR methods. An ^{1}H NMR spectrum of this complex in D_2O is shown in Fig. 8. The dispersion of this spectrum indicates that this peptide is at least partially folded. More detailed structural studies are underway and will be reported in due course.

These studies have revealed that deletion of a single amino acid within the large loop of Cys_2His_2 zinc finger peptides does have significant effects of peptide-metal complex stability. These effects, however, are much more modest than had previously been reported. Through the use of cobalt(II) as a probe, dissociation constants for a number of species have been obtained. Furthermore, some evidence for the formation of a asymmetrical 2:1 peptide-cobalt(II) complex has been seen. Additional studies will be necessary to more fully characterize the structures of the complexes of the deletion mutant peptides. Nonetheless, the properties elucidated to date reveal that even a small and apparently highly constrained peptide like a zinc finger-metal complex can accommodate deletion of a residue without complete loss of its metal binding properties. Additional studies will be required to determine if zinc fingers containing such deletions are functional within the context of an array of zinc finger domains.

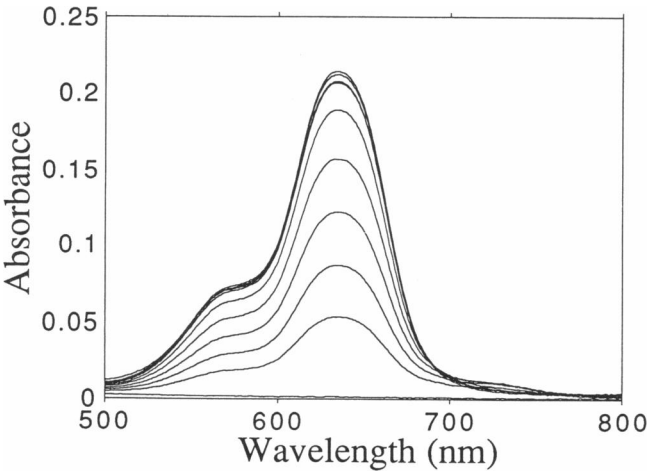

FIGURE 7 A titration of the deletion mutant of CP-1 with cobalt(II). Only a small amount of absorption between 750 and 800 nm is observed. These data could be fit with a dissociation constant of $6 \pm 2 \times 10^{-6}$ M.

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