

Investigation of Zinc Chelation in Zinc-Finger Arrays by Electrospray Mass Spectrometry

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The chelation of zinc by consensus zinc-finger arrays of the CCCC, CCHC, and CCHH type has been investigated by electrospray ionization mass spectrometry. Accurate mass measurements of the most abundant isotopic species have demonstrated that two protons are lost for each Zn(II) ion chelated. Methylation of zinc-finger peptides has revealed that two thiolate anions from cysteine side-chains are necessary to maintain chelation. The other cysteine(s) retain the thiol proton(s) and can be methylated without loss of chelating ability.

Introduction

At levels of 2–3 g in adult humans, zinc is one of the most prevalent “trace” elements.^{1,2} Over 300 proteins have been characterized so far which contain zinc in a structural or catalytic role. In addition, clusters have been found associated with proteins regulating zinc homeostasis and participating in signal transduction.²

There are at least ten classes of zinc-based domains³ whose common feature is tetrahedral Zn-binding sites involving four ligands, typically provided by side chains of Cys, His, Asp, and Glu and by external ligands (e.g. water). In the structural arrays termed zinc-fingers, divalent zinc is bound by different combinations of cysteine thiol groups and histidine imidazole groups. Arrays of this kind have been found in a wide variety of nucleic acid binding proteins,⁴ in the steroid/thyroid hormone receptor superfamily,⁵ and in the RING finger protein family.⁶

Understanding the structural and chemical properties of these metal clusters is essential for understanding their biological function. The structures of these arrays have been successfully resolved by nuclear magnetic resonance (NMR) spectroscopy⁷ and X-ray crystallography.⁸ One question remaining is how many of the thiol groups in the coordination sphere are deprotonated. For lack of sufficient resolution or because of rapid exchange with the solvent, the presence of thiol protons has been difficult to characterize by either X-ray crystallography or NMR. By bringing the clusters into the gas phase, electrospray ionization mass spectrometry (ESI-MS)^{9,10} freezes the exchange and allows for an accurate proton count.

The present work evaluates the hypothesis that in all three types of zinc-finger arrays, CCCC, CCHC, and CCHH (no-

menclature of Berg^{11,12}) two thiolate groups must be present to maintain chelation of divalent zinc. Other cysteine(s) in the array retain thiol proton(s). The presence of thiol protons in model zinc-fingers is probed by accurate mass measurements and correlated with the ability of methylthiol ethers to participate in the ligand field.

Experimental Section

Consensus zinc-finger peptides of the CCCC, CCHC, and CCHH types, whose sequences have been recently described by Berg and colleagues,^{11,12} were obtained from the Biopolymers Laboratory of Johns Hopkins University (Baltimore, MD), purified by reversed phase HPLC, reconstituted as described by Winge,¹³ and dialyzed overnight against 10 mM NH₄HCO₃, pH 7.5, across a Spectra/Por CE (cellulose ester) membrane from Spectrum, Inc. (Houston, TX).

Methylation with *p*-nitrobenzenesulfonic acid methyl ester (PNBS) from Sigma (St. Louis, MO) was performed on holo-zinc fingers using a modified procedure of Henrikson.^{14,15} PNBS was dissolved in a very small volume of acetonitrile and aliquots were added to 900 μ M solutions of holo-zinc fingers in 10 mM NH₄HCO₃, pH 7.5. For CCHH and CCHC a molar ratio of 1:2 (cysteine to PNBS) was used. For CCCC a 1:1 ratio was used. Care was taken in order to keep the pH at near neutral conditions to avoid denaturation of the folded peptides. After 2 h reaction, the solutions were again dialyzed overnight to improve the spectral quality.

Electrospray ionization mass spectra were acquired using the first mass spectrometer of a JEOL (Tokyo, Japan) HX110/HX110 four-sector mass spectrometer fitted with an Analytica of Branford (Branford, CT) thermally assisted electrospray source. Analyte solutions were injected through a loop injector system into the source at 1 μ L/min using a syringe pump. To improve spray conditions and avoid denaturation, a stable sheath flow of methanol was sprayed coaxially to the analyte solutions at 1 μ L/min. The spray needle was kept at ground potential and the counter-electrode at 4 kV. The interfacing capillary was heated to 120 °C.

Linear scans were performed over the appropriate scan range with a typical duty cycle of 9–12 s. To resolve the isotopic envelope for accurate mass measurements, the resolution was set to 3000 (10%

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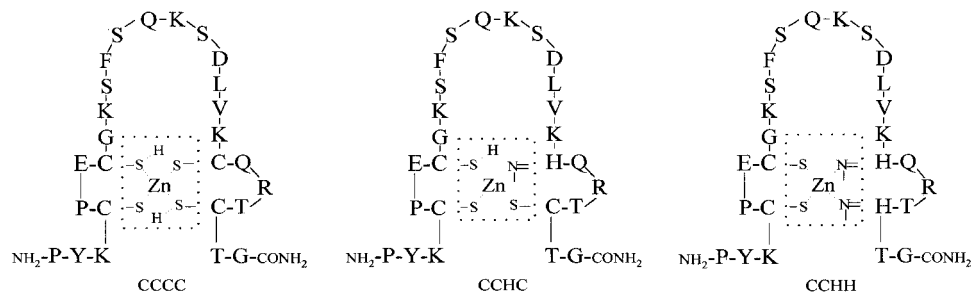


Figure 1. Consensus zinc-finger arrays used in the study

valley) by adjusting the slit width. For the detection of methylated products the resolution was set to 500 (10% valley). Each spectrum was the averaged profile of 20–50 scans recorded by the JEOL MP7000 data system.

Accurate mass measurements were obtained at high resolution and using the external standard method. Briefly, analyte and external standards were introduced into the source by consecutive injections through a loop-injection system, without mixing and without stopping the data acquisition. Mass assignments were made by manually interpolating the target analyte peak from reference ions bracketing the unknown from both sides. Optimally, external standards should provide ions of similar charge state and fall within 500 m/z units. In the present study, the 2+ charge state was measured directly, with no computer-assisted deconvolution. For the determination of holo-zinc-finger peptides, mass accuracy of up to 15 ppm has been obtained by this method.¹⁶

Masses for the different species were calculated from the elemental compositions using the atomic masses of the most abundant isotopes ^{12}C , ^1H , ^{14}N , ^{16}O . For Zn(II) the mass of ^{64}Zn (63.92915 Da, which is the lightest of the five isotopes and is also the most abundant, with 48.89% of the total natural abundance) was used. The calculated monoisotopic mass was compared with the experimental mass obtained from the ^{12}C peak of resolved isotopic envelopes. The experimental isotopic distributions were compared with the theoretical patterns generated for holo-peptides containing two thiolate anions.

For accurate mass measurements, holo-zinc fingers were analyzed directly in 10 mM NH_4HCO_3 , pH 7.5, while apo-peptides were incubated for several minutes in 1 mM HCl, pH 0.5, at room temperature, and analyzed in the same solution, without further purification.

Products of the methylation reactions were analyzed at lower resolution (≈ 500 res.), which allowed methyl groups to be counted in the holo-peptides. All holo-peptides, before and after methylation, were analyzed in 10 mM NH_4HCO_3 , pH 7.5.

Results and Discussion

Accurate Mass Measurements. The structures of the zinc-finger peptides studied in this investigation are represented in Figure 1. These sequences for arrays termed here CCCC, CCHC, and CCHH are consensus sequences obtained from a large number of known zinc-finger peptides, as published by Berg.^{11,12} These structures constitute good models for the three types of zinc-finger arrays and have been successfully used for metal binding studies.^{11,12}

To obtain accurate mass measurements, the isotopic distribution was resolved for both apo- and holo-peptides. Figure 2 shows the electrospray ionization mass spectra for doubly charged holo-peptides. Mass assignments for the most abundant isotopic species of CCCC, CCHC, and CCHH zinc fingers are reported in Table 1. The experimental masses for the fully reduced apo-peptides, with all cysteines present as $-\text{SH}$ groups,

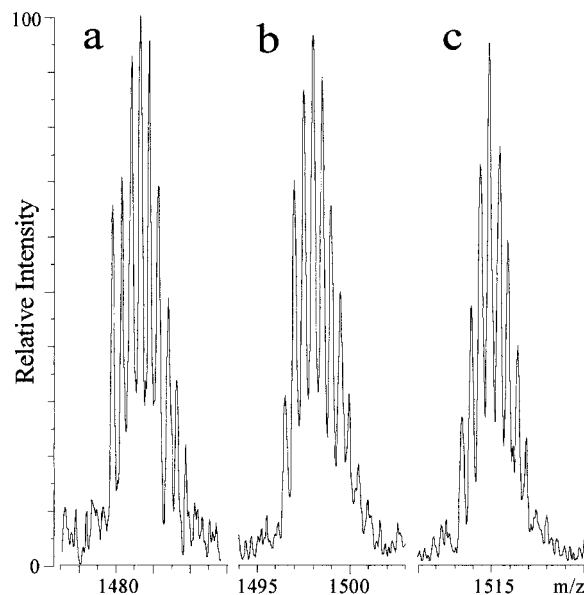


Figure 2. High-resolution electrospray ionization mass spectra of (a) CCCC, (b) CCHC, and (c) CCHH zinc-finger array. Isotopic envelopes for the doubly charged peptides are resolved. See the Experimental Section for conditions.

Table 1. Accurate Mass Measurements of the Most Abundant Isotopic Species of Apo- and Holo-Zinc-Finger Peptides^a

peptide	formula	calculated ^b (Da)	observed ^c (Da)
apo-CCCC	$\text{C}_{121}\text{H}_{198}\text{N}_{36}\text{O}_{38}\text{S}_4$	2891.4	2891.2 ± 0.2
holo-CCCC ^d	$\text{C}_{121}\text{H}_{196}\text{N}_{36}\text{O}_{38}\text{S}_4\text{Zn}$	2953.3	2953.4 ± 0.2
apo-CCHC	$\text{C}_{124}\text{H}_{200}\text{N}_{38}\text{O}_{38}\text{S}_3$	2925.4	2925.5 ± 0.1
holo-CCHC ^d	$\text{C}_{124}\text{H}_{198}\text{N}_{38}\text{O}_{38}\text{S}_3\text{Zn}$	2987.3	2987.3 ± 0.1
apo-CCHH	$\text{C}_{127}\text{H}_{202}\text{N}_{40}\text{O}_{38}\text{S}_2$	2959.5	2959.6 ± 0.1
holo-CCHH ^d	$\text{C}_{127}\text{H}_{200}\text{N}_{40}\text{O}_{38}\text{S}_2\text{Zn}$	3021.4	3021.2 ± 0.1

^a Isotopic resolution (≈ 3000 res.) was used for these measurements. Conditions are described in the Experimental Section. ^b The monoisotopic masses are calculated using the most abundant isotope of Zn (^{64}Zn , 63.92915 Da). ^c Standard deviations are obtained from three consecutive experiments. ^d Masses were calculated assuming the loss of two protons from the mass of the fully reduced apo-peptides.

analyzed at low pH are in good agreement with molecular masses calculated from the sequences. The accuracy of these measurements is 0.2 Da or better, based on standard deviations, $n = 3$. The molecular masses observed after binding of zinc indicate that two protons are lost from each peptide in the ligand field. This is consistent with results obtained from larger zinc-proteins, for example HIV-1 NCP7 (a 6 kDa protein containing two arrays of CCHC type) and rabbit liver metallothionein 2a (a 6 kDa protein containing CCCC clusters of non-zinc-finger type).¹⁶

The possibility of artifactual reprotonation in the electrospray process seems unlikely, given the range of pI values of the peptides and proteins that have been studied here and in earlier

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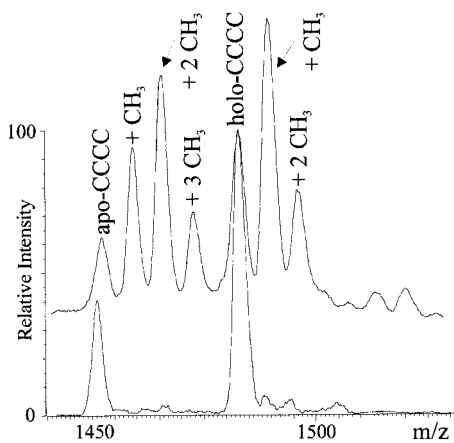


Figure 3. Electrospray mass spectra of a holo-CCCC peptide before (lower track) and after (upper track) methylation with *p*-nitrobenzenesulfonic acid methyl ester (PNBS). See the Experimental Section for conditions.

work¹⁶ and the precise and consistent stoichiometry of proton losses even in metallothionein complexed with seven zinc cations.¹⁶

Methylation of the Zinc Arrays. To test further the finding that only two thiolate groups are present in holo-zinc-finger arrays, all three holo-peptides were methylated in 10 mM $\text{NH}_4\text{-HCO}_3$, pH 7.5, room temperature, using *p*-nitrobenzenesulfonic acid methyl ester (PNBS). This is reported to be a specific reagent for cysteine side chain groups.^{14–16} The ratios between substrate and methylating agent were controlled to avoid exhaustive methylation of all cysteines present and denaturation of the fingers. For the CCCC peptide a 1:1 ratio (cysteine to PNBS) was employed, for CCHC and CCHH peptides the ratio was 1:2. Reactions were performed for 2 h. The products were analyzed by electrospray mass spectrometry at near neutral pH (see Experimental Section). The low energy involved in the electrospray process and the native conditions used for the analysis facilitate the preservation of metal ion ligands.^{16,17}

Figure 3 shows lower resolution electrospray mass spectra of the CCCC finger measured before and after incubation with PNBS. The lower track represents the starting material containing both apo- and holo-CCCC. In the upper spectrum, zinc chelates are observed carrying both one and two methyl groups, and apo-peptide up to three methyl derivatives. The spectra suggest that two but not three methyl groups may be added to thiols in the ligand field before chelation is disrupted.

Analogous results are obtained for CCHC, as shown by the electrospray mass spectra measured before (Figure 4, lower track) and after (upper track) incubation with PNBS under the conditions described above. The monomethylated holo-peptide is the most abundant ion detected in the product spectrum. Little or no dimethylated holo-CCHC is observed; however, the apo-peptide is observed carrying one, two, and three methyl groups. This is consistent with the hypothesis that two thiolate ligands participate in zinc cation binding.

The electrospray mass spectra of CCHH obtained before and after reaction with PNBS are shown in Figure 5 (lower and upper track, respectively). In this case, the reaction mixture looks very similar to the starting material. Holo-CCHH is the most intense ion, as in the starting material, and no significant signals for methylated holo-peptides can be detected. Methylated species are observed only in the apo-form, again suggesting that two thiolate ligands are necessary for binding.

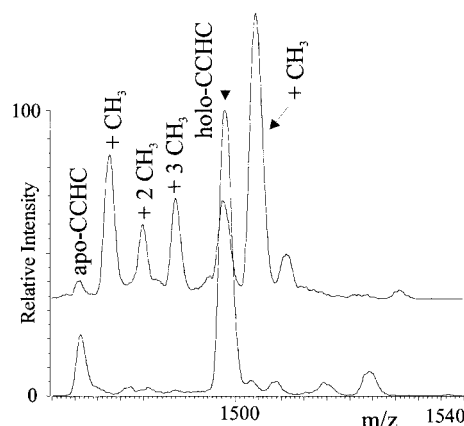


Figure 4. Electrospray mass spectra of a holo-CCHC peptide before (lower track) and after (upper track) methylation with PNBS.

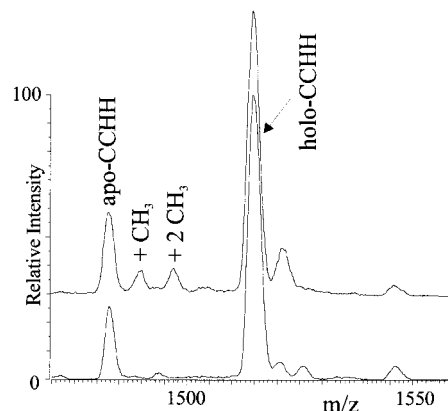


Figure 5. Electrospray mass spectra of a holo-CCHH peptide before (lower track) and after (upper track) methylation with PNBS.

A control reaction was performed on each model peptide to evaluate its susceptibility to methylation in absence of zinc. Aliquots of each apo-peptide, stored at low pH to avoid oxidation, were neutralized and treated with the same amounts of PNBS used for alkylating the correspondent holo-species, working at pH 7.5 in the presence of DTT. Electrospray mass spectrometry demonstrated that methylation of all the cysteines present (4 methyl groups for CCCC, 3 for CCHC, and 2 for CCHH, not shown) was readily achieved in 2 h in the absence of zinc.

These results indicate that the cysteines involved in zinc binding are less susceptible to alkylation than the cysteines in the apo-peptides. Assuming all groups are equally accessible, the reactivity of cysteine side chains with the methylating agent is determined by the nucleophilic character of the sulfur, which is significantly reduced by the presence of Zn^{2+} , a strong Lewis acid. The spectra in Figures 3–5 also show that zinc ions are no longer chelated when fewer than two thiolate groups remain.

Tandem mass spectrometry experiments with high energy collision-induced dissociation have been used to locate the sites of methylation on a related monomethylated CCHC.¹⁶ The analysis revealed methylation on either Cys-6 or Cys-16 in a 1.8:1 ratio, with no methylation detectable on Cys-3. This reactivity pattern could be caused by either different accessibility of the methylating agent to different thiol groups in the folded peptide, or by asymmetry in the electronic density over the CCHC array. The fact that at least two sites out of three can be methylated suggests that thiol protons may be delocalized over the array or shared between sulfur couples. A possibly similar situation, with sharing of one proton between two thiolates has

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been observed in thioredoxin and other redox proteins involved in disulfide bond turnover.¹⁸

Conclusions

In consensus peptides with zinc-finger arrays of the CCCC, CCHC, and CCHH type, two cysteine side chains are present

as thiolates and are effectively involved in chelating Zn(II) ions. The other cysteine(s) retain the thiol proton(s) and can be methylated without loss of chelating ability.

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