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Occupancy of a C_2-C_2 type 'Zinc-finger' protein domain by copper

Direct observation by electrospray ionization mass spectrometry**

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The metal ion specificity of most 'zinc-finger' metal binding domains is unknown. The human estrogen receptor protein contains two different C_2-C_2 type 'zinc-finger' sequences within its DNA-binding domain (ERDBD). Copper inhibits the function of this protein by mechanisms which remain unclear. We have used electrospray ionization mass spectrometry to evaluate directly the 71-residue ERDBD (K180–M250) in the absence and presence of Cu(II) ions. The ERDBD showed a high affinity for Cu and was completely occupied with 4 Cu bound; each Cu ion was evidently bound to only two ligand residues (net loss of only 2 Da per bound Cu). The Cu binding stoichiometry was confirmed by atomic absorption. These results (i) provide the first direct physical evidence for the ability of the estrogen receptor DNA-binding domain to bind Cu and (ii) document a twofold difference in the Zn- and Cu-binding capacity. Differences in the ERDBD domain structure with bound Zn and Cu are predicted. Given the relative intracellular contents of Zn and Cu, our findings demonstrate the need to investigate further the Cu occupancy of this and other zinc-finger domains both in vitro and in vivo.

Copper; Zinc; Mass spectrometry; Estrogen receptor; Metalloprotein; Zinc-finger peptide

1. INTRODUCTION

Several distinct 'zinc-finger' sequence patterns forming at least three different structural motifs have been identified in a wide variety of eukaryotic transcriptional regulatory proteins by cDNA sequence analyses [1–4]. Relatively few of the predicted Zn binding sites have been demonstrated to actually bind Zn ions; metal ion specificity is usually not evaluated. Furthermore, definitive structural information on these metal-binding domains is quite limited (see [2–4] for review).

The human estrogen receptor protein contains a highly conserved DNA-binding domain with two C_2-C_2 type zinc finger sequences (ERDBD) [5,6]. The function of this protein and of related hormone receptor proteins, both in vitro and in vivo, is altered in the presence of added Cu (e.g. [7–10]). The mechanism(s) by which Cu alters receptor structure and function remains unknown. We have reported previously that the estrogen receptor has a high affinity for immobolized Cu(II) ion affinity columns [11–14] and that this affinity is eliminated by proteolytic removal of a portion of the receptor that includes the DNA-binding domain [11,12]. Direct physical evidence for the coordinate covalent interaction of Cu with the DNA-binding domain, however, has not been presented.

We have recently reported the development of mass spectrometric techniques that enable accurate (0.01%) mass determinations of intact peptide–metal ion complexes using matrix-assisted UV laser desorption time-of-flight mass spectrometry (LDTOF) [15–19] and electrospray ionization mass spectrometry (ESI) [16,20]. In the case of the estrogen receptor DNA-binding domain, ESI has now been used to provide direct physical evidence of Zn bound in a probable tetra-coordinate covalent geometry [20]. In this report, we demonstrate by ESI that the two zinc binding sites within the DNA-binding domain of the human estrogen receptor protein are readily occupied with four bound Cu.

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2. EXPERIMENTAL

2.1. *Synthesis of the ERDBD peptide*

The 71-residue ERDBD peptide (K180–M250) was synthesized on an Applied Biosystems Model 430A automated peptide synthesizer using Fmoc/NMP(9-fluorenylmethyloxycarbonyl/*N*-methylpyrrolidone) chemistry (FastMoc, Applied Biosystems, Foster City, CA) and purified by reverse-phase HPLC on a Waters Delta-Pak C_{18} Prep

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Pak cartridge (25 mm i.d. × 100 mm; 15-μm particle size; 30-nm pore size). Mass and purity were evaluated by LDTOF [15,16,19] and ESI [16,20] as described previously. The amino acid sequence was verified with an Applied Biosystems Model 473A automated amino acid sequence analyzer.

2.2. *Electrospray ionization mass spectrometry*

Electrospray mass spectra were generated using a Vestec Model 201 single quadrupole mass spectrometer fitted with an electrospray ion source [21] (Vestec Corporation, Houston, TX) modified as described previously [16,20]. Aqueous solutions of the ERDBD peptide (2–8 μmol/l) were infused in the absence and presence of up to 100 μmol/l $CuSO_4$ or $CuCl_2$. Electrospray and data acquisition parameters, calibration, and data reduction were as described previously [20].

3. RESULTS AND DISCUSSION

The 71-residue amino acid sequence (K180–M250) that defines the DNA-binding domain of the human estrogen receptor protein [5] was chosen as a model for these investigations because it represents a C_2-C_2 type of zinc-finger sequence and structural motif that is common to several other transcription factors [2–4]. A recombinant ERDBD peptide [6] has recently been evaluated by 2D ^{1}H NMR techniques, however, direct evidence for bound Zn (or other bound metal ions) was not presented. Zn binding to an intact, fully reduced ERDBD has since been demonstrated by ESI [20]. During the course of these evaluations, however, it became apparent that metal ions other than Zn (i.e. contaminants) were capable of forming coordinate covalent bonds within the estrogen receptor DNA-binding domain. These observations, and our earlier investigations of estrogen receptor interactions with immobilized Cu(II) ions [11–13], suggested to us the need to better characterize the transition metal ion binding properties of the intact 71-residue ERDBD. Because estrogen receptor function can be inhibited by the presence of biologically relevant concentrations of Cu(II) ions (e.g. [7,8]), and because Cu is known to interact with the receptor surface at sites other than the hormone binding domain [14], we chose to evaluate directly the Cu-binding properties of the ERDBD. ESI was chosen for these studies for several reasons: (i) aqueous solutions of sample peptide can be introduced in the continued presence of up to 100 μmol/l $CuCl_2$ or $CuSO_4$ without loss of stable electrospray, (ii) apopeptide and peptides with 1 or more bound metal ions can be fully resolved in several different charge states to evaluate specific stoichiometries, (iii) accurate mass determinations from multiply charged species enable the coordinate covalent interaction geometry to be evaluated, (iv) relatively small amounts of sample peptide are required (pmol), and (v) highly reproducible and definitive data are acquired within minutes.

The calculated mass (8248.5 Da) and determined mass values of the fully reduced ERDBD apopeptide (9 Cys residues) were within 0.1 Da by both LDTOF and by ESI (8248.4 ± 0.4 Da). Fig. 1 shows a typical ESI spectrum for the fully reduced ERDBD apopeptide.

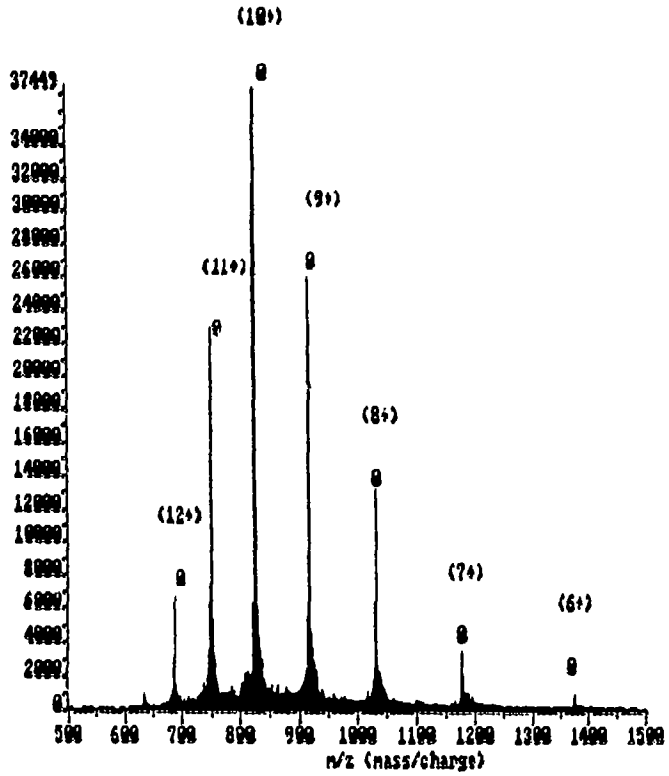

Fig. 1. Determined mass of the synthetic ERDBD apopeptide by electrospray ionization mass spectrometry. The full scan spectrum, displayed as relative signal intensity vs. mass/charge (m/z) from m/z values of 500–1500, shows the fully reduced ERDBD apopeptide. The charge states (6+) to (12+) are shown in parentheses. The assigned peak number (0) corresponds to the number of bound metal ions observed.

The presence of 9 reduced thiols per peptide was additionally verified by titration with 5,5-dithionitrobenzoic acid as described by Ellman [22].

The ERDBD with 0, 1, and 2 bound Zn has recently been observed by ESI [20] in the presence of 100 μmol/l $ZnSO_4$ (or $ZnCl_2$). The ERDBD with higher numbers of bound Zn (e.g. 3 or 4) was not observed. Furthermore, even though the ERDBD peptide was able to bind up to 2 Zn, under these conditions it was *never* observed to be completely occupied with 2 bound Zn. In contrast, electrospray ionization mass spectrometry of the ERDBD (8 μmol/l) performed in the presence of 100 μmol/l $CuSO_4$ (or $CuCl_2$) revealed ERDBD peptide completely occupied with 4 bound Cu (Fig. 2). Some ERDBD with 3 bound Cu was observed. Again, however, in contrast to ESI experiments performed with Zn [20], only a very small amount of ERDBD with 1 bound Cu was present and ERDBD with 2 bound Cu was never observed under these conditions. In every instance in which Cu was present, the most abundant ERDBD-Cu holopeptide form observed contained 4 bound Cu. The determined mass values for the ERDBD with 4 bound Cu (8494.5 ± 0.5 Da) was in agreement with the average chemical mass calculated for the ERDBD plus 4 bound Cu less 2 Da per bound Cu

(Table 1). In addition to the specific increase in mass observed, the ERDBD occupied with Cu provided qualitatively distinct ESI spectra. In contrast to the ESI spectra for the ERDBD apopeptide (Fig. 1 and 3, top) and the ERDBD-Zn holopeptide [20] where the 10⁺ charge state was typically most abundant, in the ERDBD-Cu holopeptide spectra (Fig. 2 and 3, bottom), the lower charge states (typically 8⁺) always represented the most abundant species. These observations are most likely related to the different conformational states of the apo- and holo-ERDBD peptides (manuscript in preparation). Regardless of charge state optimum, the striking 'phenotypic' differences in ESI profiles (charge envelope and ratio of ERDBD with 0 and 4 bound Cu), together with the observed difference in mass between the ERDBD apopeptide and the Cu holopeptide, were definitive evidence of 4 bound Cu (Fig. 3).

The ERDBD Cu binding stoichiometry was additionally verified by atomic absorption. Known quantities of the ERDBD peptide were exposed immediately to saturating amounts of Cu. After extensive dialysis (500 vols; 3–5 changes) over an 18-h period, or removal of unbound metal ions by gel filtration chromatography (Sephadex G-25), aliquots of the ERDBD peptide–Cu complex (along with appropriate positive and negative controls) were analyzed by atomic absorption spectroscopy. The metal-binding stoichiometry ranged from 3.86:1 to 4.2:1 Cu:ERDBD (n=8).

Full occupancy (i.e. saturation) of the two metal-binding sites with bound Zn was not observed by ESI [20]. The complete occupancy of all available ERDBD with 4 bound Cu under identical experimental conditions suggests that the ERDBD preferred bound Cu to bound Zn. If the affinity and capacity of the ERDBD peptide is indeed greater for Cu than for Zn, this finding may have important implications with respect to metal ion regulation of cell function, including genetic regulatory events.

Table I

Mass assignments for the ERDBD peptide[a] observed by ESI before and after Cu binding

Bound Cu ions (n)	Molecular mass (Da)	
	Calculated[b]	Observed[c]
0	8248.5	8248.4 ± 0.4[d]
1	8310.1	Not determined
2	8371.6	Not determined
3	8433.2	8431.2 ± 0.5
4	8494.7	8494.5 ± 0.5 (predominant ERDBD form observed)

[a]Mass values for the fully reduced 71-residue (K180–M250) ERDBD apopeptide ($C_{349}H_{533}N_{107}O_{104}S_{11}$) were derived from the published cDNA sequence [5].
[b]The mass of the ERDBD–Cu holopeptides were calculated from the chemical average mass of the fully reduced ERDBD apopeptide plus the weighted average mass of n bound Cu (63.55 U) and the decrease in net mass assumed for a bicoordinate covalent interaction (ERDBD + nCu − 2 nH).
[c]Observed mass values represent the average (± observed mass range) of 5 separate determinations.
[d]Taken from Fig. 1.

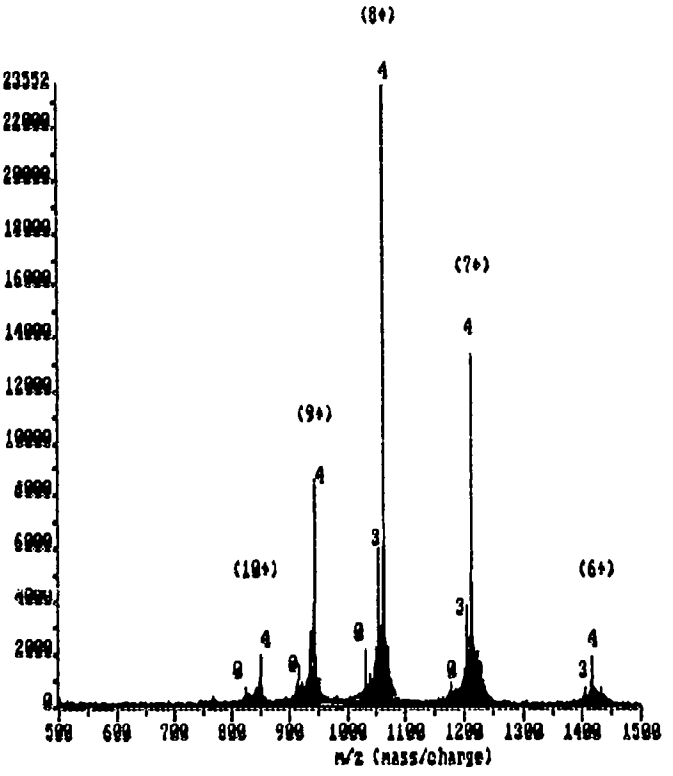

Fig. 2. Direct observation of the ERDBD peptide with bound copper. Electrospray ionization mass spectra of the ERDBD were obtained in the presence of 100 μM $CuSO_4$. The full scan spectrum, displayed as relative peak intensity vs. mass/charge (m/z) from m/z values of 500–1500, shows the ERDBD peptide with 0, 3, and 4 bound Cu. The charge states (6⁺) to (10⁺) are shown in parentheses. Assigned peak numbers correspond to the number of bound metal ions observed.

The intracellular contents of Zn and Cu in several different tissues, including estrogen target organs, indicate the possibility that Cu may occupy the ERDBD under conditions considered to be normal. Although the concentration of Zn exceeds that of Cu in most cells, published data on the tissue [23], cellular [24], and even intranuclear [25,26] levels of Zn and Cu demonstrate a maximum 4–25-fold difference in the relative amounts of these two transition metal ions. It is not presently known whether differences in the relative affinities of the ERDBD for Cu and Zn are greater or less than the differences in intracellular concentrations of these two metal ions. Hence, competitive binding experiments designed to quantitatively evaluate the relative affinities and interaction kinetics of the two different C_2-C_2 sites for Cu and Zn are needed. Furthermore, based on results we have presented here and elsewhere [20], a detailed investigation of the relative affinities and metal-binding specificities of the two separate C_2-C_2 sites within the intact 71-residue ERDBD peptide is needed.

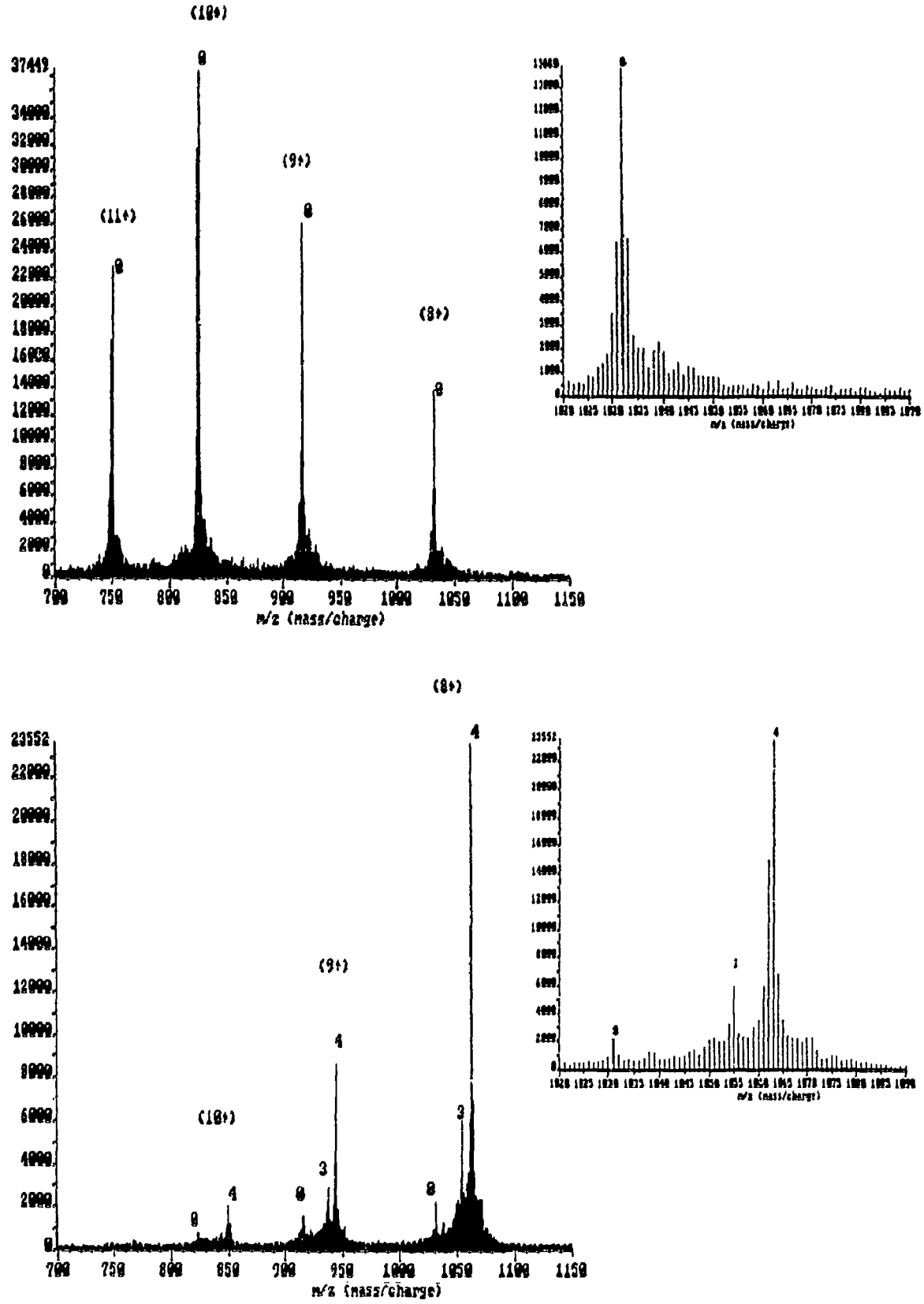

Fig. 3. Details of the ESI spectra showing the dramatic difference in optimum charge states and mass/charge (m/z) values for ERDBD before (top) and after (bottom) addition of $CuSO_4$. The m/z values from 700–1150 are shown. Insets show the (8°) charge state for the ERDBD apopeptide (top) and the ERDBD-Cu holopeptide (bottom).

If Cu is bound to the ERDBD, will receptor structure be altered significantly relative to the apo or Zn-occupied forms? Our ESI data demonstrate a probable tetra-coordinate interaction in the case of bound Zn (net loss of 4 Da per bound Zn) [20] but rather a bi-coordinate interaction with Cu (net loss of only 2 Da per bound Cu). A metal:peptide stoichiometry of 2:1 for Zn and 4:1 for Cu cannot be accommodated by any single ERDBD secondary or tertiary structure. Thus, significant differences in the ERDBD domain structure with bound Zn and Cu can be predicted based on the distinct chemical reaction mechanisms, stoichiometries, and observed differences in ESI charge states [27]. Metal ion-induced alterations in receptor structure may explain the negative effects of Cu on estrogen receptor function observed both in vitro and in vivo [7,8,10].

In summary, we believe that electrospray ionization mass spectrometry of intact peptide–metal ion complexes is important for investigators in the fields of molecular biology and bio-inorganic chemistry. The simultaneous determination of mass for peptides with various numbers of bound metal ions (varying stoichiometries) is possible. In the present case, ESI provided direct and unequivocal evidence for the ability of ERDBD to bind Cu. Our findings demonstrate the need to investigate further the occupancy of this and other 'zinc-finger' domains by the various transition metal ions normally found within cells.

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