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Mass Spectrometric Analysis of a Native Zinc-Finger Structure: The Glucocorticoid Receptor DNA Binding Domain

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Abstract: The DNA binding domain of the glucocorticoid receptor (GR DBD, recombinant, human amino acids 419–501) was analyzed *intact* under neutral conditions by electrospray ionization mass spectrometry (ESI MS). The Zn-containing GR DBD and its Cd-containing counterpart both showed stoichiometry of two metal atoms attached to a molecule of metalloprotein (molecular mass 9600 ± 2.1 Da (n = 4) and molecular mass 9693 ± 1.3 Da, respectively). GR DBD analyzed at low pH gave the molecular mass expected for the apoprotein: 9474 ± 1 Da (n = 4) (average M_r 9474.4). There was a difference in the distribution envelopes of molecular ions in ESI mass spectra of the Zn- and Cd-containing GR DBD's depending upon conditions of the ESI MS experiment. In acidic (denaturing) conditions, molecular ion envelopes moved toward lower m/z values, while at neutral pH in aqueous solvent, a characteristic low level of protonation was noted, the latter indicative of preservation of some higher-order structure during ESI MS analysis. For electrospray ionization mass spectrometric analysis, the native proteins in ammonium bicarbonate buffer were injected into a stream of 50 mM pyridine acetate (pH 5.9) and delivered to the VG BioQ instrument at a flow rate of 4 μL/min. Denatured proteins were analyzed either by injection into a stream of 50% acetonitrile/0.1% trifluoroacetic acid or on-line with HPLC using a reversed phase narrow bore PLRP-S column (1 × 50 mm).

Since the introduction of electrospray ionization mass spectrometry (ESI MS) as a technique for analyzing intact proteins,[1,2] several papers have discussed whether the higher-order structure they have in solution is likely to be retained during mass spectrometric analysis. Particular attention has been paid to proteins involved in noncovalent interactions.[3–7] These papers have spawned discussion on the importance of different factors that control protein conformation in solution and their relevance

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to the dynamic conditions of desolvation of macromolecular complexes in the gas phase.[8-11]

In this paper, we show that the DNA binding domain of the glucocorticoid receptor (GR DBD) which contains two zinc atoms bound to finger-like structures can be analyzed *intact* under neutral conditions by ESI MS. In common with other studies, we have shown that a low protonation found for the "native" *versus* "denatured" form implies that some higher-order structure characteristics of the native species have been retained during analysis.

Zinc fingers are present in many eukaryotic transcriptional regulatory proteins.[12] They exert their highly specific DNA recognition through loop structures whose common feature is a tetrahedrally coordinated Zn atom. GR DBD contains two zinc fingers which act in a cooperative manner and whose consensus sequences are $CX_2CX_{13}CX_2C$ and $CX_5CX_9CX_2C$, respectively.[13] The three-dimensional structure of GR DBD has been determined by NMR[14,15] and X-ray crystallography.[16]

Results

The Zn- and Cd-containing GR DBD's denatured in the process of delivery to the mass spectrometer gave the molecular mass expected for the apoprotein: 9474 ± 1 Da ($n = 4$) (average M_r 9474.4) (Figure 1). Thus, faithfulness of the expression/purification system was confirmed and it was demonstrated that no metal ions were bound in the presence of acid. However, the molecular mass of GR DBD that was electrosprayed from an ammonium bicarbonate solution measured 9600 ± 2.1 Da (in four independent experiments) (Figure 2). This result is consistent with full occupation of each of the two metal−ligand sites by one zinc atom, as expected on the basis of the GR DBD structure established for solution[14] and for crystal.[16] The same metal−protein stoichiometry has been previously shown by ESI MS for a synthetic 71-amino acid residue containing estrogen receptor DNA binding domain (ER DBD) studied by Hutchens *et al.*[17,18] However, contrary to our results, they observed a heterogeneous population of species carrying up to two zinc atoms rather than a single ER DBD species fully occupied with zinc. There is no indication for the presence in recombinant GR DBD of metal ions other than zinc (*in vitro* cadmium appears to have a higher affinity than zinc for GR DBD, as assessed by a DNA binding assay[13]). This result confirms the findings of Freedman *et al.*, who employed inductively coupled plasma mass spectrometry and found very little cadmium in native GR DBD purified from rat.[13] ESI MS of the cadmium-containing GR DBD (produced by stripping the original protein of zinc followed by its renaturation in the presence of cadmium chloride) showed M_r 9693 ± 1.4 (in six independent measurements). This molecular mass is consistent with a stoichiometry of two cadmium atoms for each GR DBD, as observed previously by Freedman *et al.*[13] The cadmium-containing DBD preparation was free of "zinc"−protein, thus proving that complete metal ion exchange can be achieved.

Relatively low precision for mass measurement (typically 0.02% in single analysis, and in series of repetitions) of the Zn-containing species does not allow us to conclude whether DBD−Zn formation results in a nominal loss of 4 or 2 au per zinc atom (measured 3 ± 2.1 Da), see the discussion in Hutchens *et al.*[18] The M_r measurement of Cd-containing GR DBD is again not conclusive regarding mass loss upon the Cd-finger formation since the measured value of 3 ± 1.4 au falls between the 2 or 4 au expected.

There is a noteworthy difference in distribution of molecular ions in ESI MS spectra of the Zn- and Cd-containing DNA-binding proteins depending upon conditions of the ESI MS experiment (Figure 3). The species analyzed in acidic (denaturing) conditions accommodate more protons, so their molecular ion envelopes are moved toward lower m/z values. The Zn- and Cd-finger mass spectra obtained *under the same instrumental conditions* from a solvent that would encourage preservation of native structure show identical molecular ion distributions with a characteristic low level of protonation. The major charge state in the "denatured" spectrum is nine *vs* six found in the "native" spectrum which suggests that "finger" formation disables use of some of the basic sites available for protonation. A similar trend of decreased protonation upon consecutive transformation of a metal-free apometallothionein to a fully reconstituted metal-containing species was noted by Yu *et al.*[19] Interestingly, Hutchens showed[17] that identical molecular ion envelopes were obtained for ER DBD analyzed under "native" conditions regardless of whether zinc was bound or not. This result might imply that protein conformation (as assessed by ESI MS) is not affected by metal coordination, contrary to the known characteristics of the native protein in solution. It is of interest that we have never observed full protonation of apo-GR DBD in ESI MS. In our hands, the apoprotein accommodates a maximum of 15 charges (protons) onto its 21 potentially available basic sites (7 Arg, 11 Lys, two His, and one amino terminus). This extent of protonation is observed at a relatively low extraction cone voltage of 60 V, and under these conditions, species with 12 and 13 charges are most abundant in the spectra (Figure 1, bottom). When higher nozzle to skimmer bias potentials are used, molecular ion distributions tend to move toward higher m/z values, likely due to preferential dissociation of the highly charged species upon collisional heating.[20] This phenomenon was clearly observed when the apo-GR DBD spectra were acquired at a high sampling cone voltage of 100 V (Figure 3, top).

Discussion

Distinguishing between specific and nonspecific complexes is hardly a trivial problem when interpreting ESI MS data. Experimental artifacts (*e.g.*, protein[21] and peptide[22] multimers,

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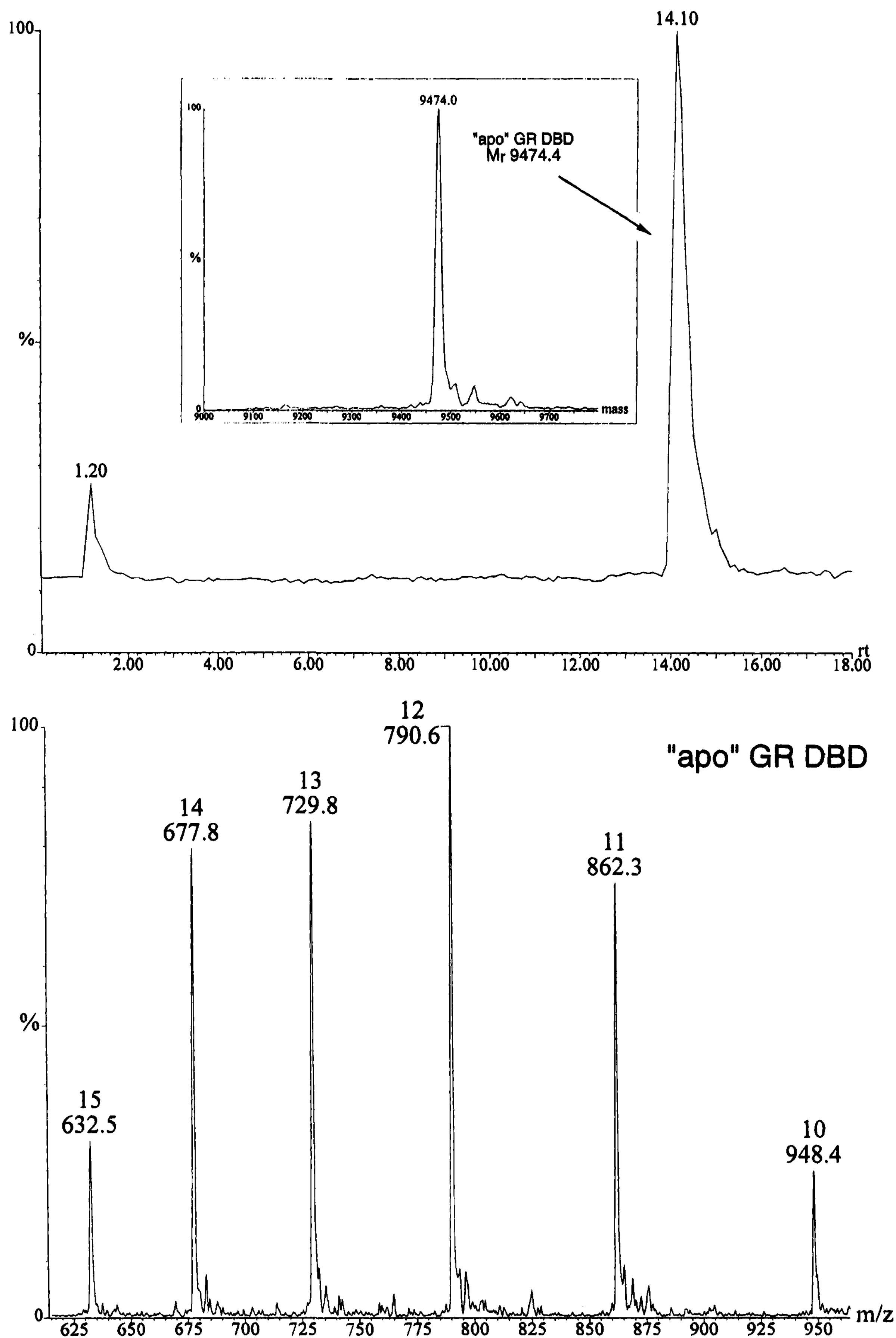

Figure 1. LC/ESI MS of GR DBD. Protein eluting from the reversed phase column was introduced directly to the mass spectrometer, and the signal derived from all detected molecular ions was plotted against time/scan number producing a total ion current chromatogram (TIC, top). Scans within a TIC peak of interest were combined to produce the mass spectrum (bottom) that shows an envelope of multiply charged molecular ions from which the molecular mass of a species was measured with high accuracy. The inset shows an ESI mass spectrum of the apo-GR DBD transformed to a real mass scale.

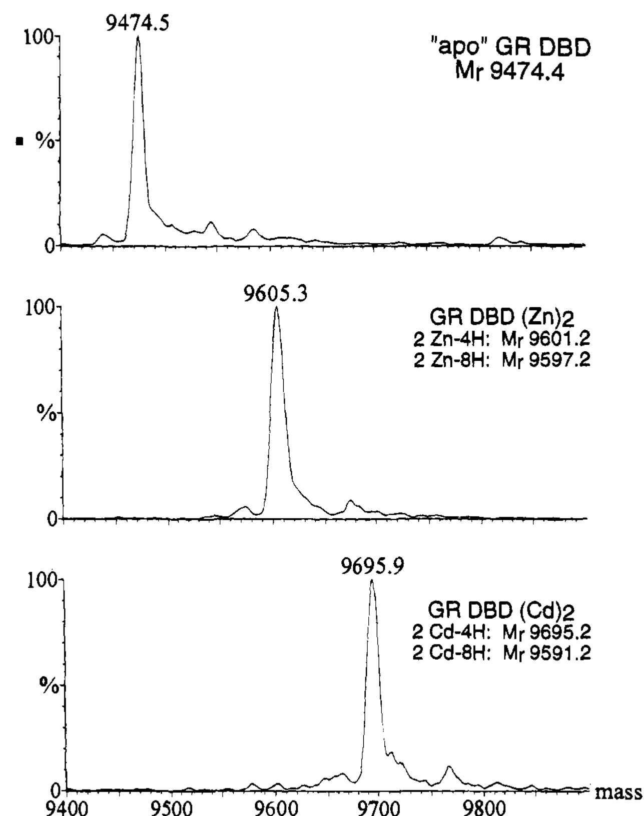

Figure 2. GR DBD $(Cd)_2$ and GR DBD $(Zn)_2$ mass spectra transformed to a real mass scale from the ESI MS data shown in Figure 3. The middle and bottom panel spectra were acquired for the Zn- and Cd-containing species electrosprayed from a neutral aqueous solution, while the top spectrum illustrates the Cd-containing species electrosprayed from the acidic 50% acetonitrile solution. Average M_r values of metalloproteins are the sums of average masses of the "apoprotein" and the metal atoms.

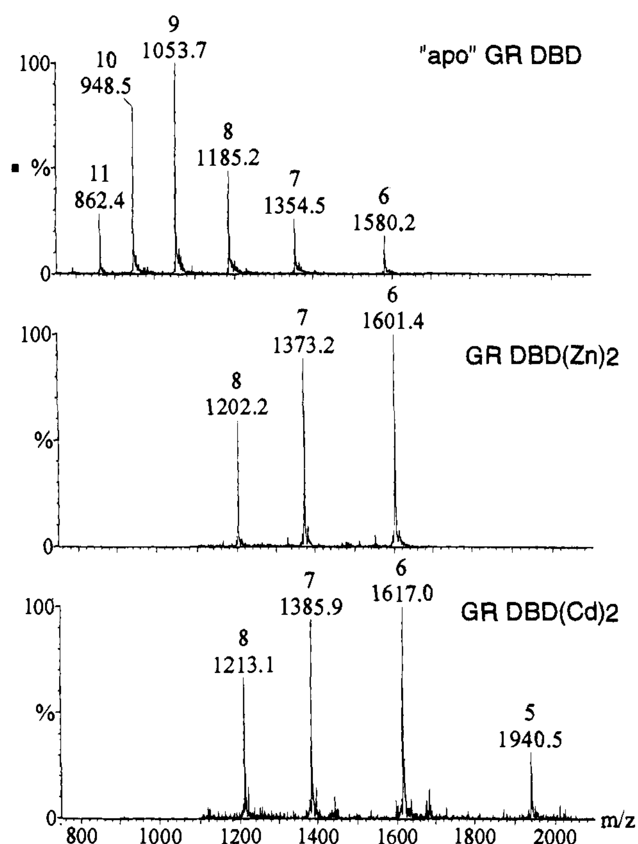

Figure 3. Molecular ion envelopes of GR DBD containing Zn and Cd ions (middle and bottom panels, respectively) electrosprayed from the neutral aqueous solution. The top panel shows an ESI mass spectrum of the Cd-containing GR DBD electrosprayed at acidic pH from a 50% acetonitrile solution; the Zn-containing GR DBD demonstrated analogous characteristics (not shown). All three spectra were acquired at the same nozzle to skimmer bias of 100 V.

sandwich alkali metal complexes,[23] and nonspecific oligonucleotide dimers[24]) have been observed on many occasions and their formation and abundance related to instrumental conditions. At the same time, many noncovalent interactions with characteristics similar to the known specific associations in solution have also been reported (*i.e.*, receptor–ligand,[4] enzyme–substrate/product[5]/inhibitor,[25] protein–prosthetic group,[6,26–28] protein–nucleotide,[7] protein–protein,[21,29] metallo-protein,[17–19,27,30,31] and oligonucleotide duplexes).[32] So far, no evidence of tightly bound water molecules within noncovalent complexes has been found, even in the case of the dimeric HIV-protease/inhibitor ternary complex, where a unique water molecule internal to the folded structure was detected by crystallography.[25] Smith and Light-Wahl proposed criteria which might constitute acceptable evidence for the solution specificity of the interactions observed by ESI MS.[9] One of these criteria is convincing stoichiometry at a reasonable relative intensity, and another is susceptibility to solvent conditions independently known to result in disruption of specific associations. The data we obtained comply with these criteria. The fact that the Zn- and Cd-containing GR DBD's are detected in ESI MS as single species of well-defined stoichiometry supports a supposition that indeed a structurally *specific* metal–protein interaction is observed in both cases. In contrast, nonspecific metal–protein aggregates tend to manifest themselves as a series of consecutive adducts spaced by the "*A* – 1 Da" intervals (*A* is a metal atomic mass). Transfer of the native protein to a 50% acetonitrile/0.1% TFA solution was expected to break the Zn (Cd)–sulfur ligand bonds, and formation of the apoprotein was repeatedly observed by ESI MS.

ESI MS manifestation of the solution phenomena (stability of noncovalent complexes, extent of protein folding, presence of protein conformers, etc.) is currently a matter of major interest and controversy. The hypothesis that distributions of molecular ions observed in ESI MS are fully representative of the aqueous solution chemistry of proteins[8] is difficult to reconcile with a dependence of spectrum appearance upon instrumental conditions.[11] However, the native and denatured proteins invariably show distinctly different ES mass spectra, as do proteins and peptides containing multiple disulfide bridges, depending upon

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their placement and extent of formation.[1,33-35] In addition, differently folded molecules of the same protein seem to demonstrate different reactivities when subjected to collisions or chemical reactions in the gas phase,[36-38] suggesting that they still might carry some higher-order structural information. Initial formation of highly solvated macromolecular ions that lose higher-order structure throughout *kinetically* controlled processes of desolvation was proposed by Smith and Light-Wahl.[9] This theory offers a plausible explanation for the puzzling observation that, while ESI MS seems to reflect some solution structure phenomena, the apparently stable macromolecular assemblages are devoid of any solvent molecules.

Given the complexity of the electrospray process, dissecting internal effects due to distribution of basic sites, the tertiary structure of protein, and its pK_a[39] and external effects of solution chemistry, conditions of generation of charged microdroplets, and gas-phase reactions[40] requires careful studies employing models. Zinc-finger structures constitute attractive model compounds since they have a highly organized structure in solution, contain many basic sites, and are available as the single point mutation analogs whose tertiary structure is disrupted due to the amino acid substitution. Given the difference in number of basic residues within the N-terminal (1 K, 1 H) and the C-terminal (4 R, 2 K) fingers, one might expect that species carrying only one of the two finger structures would have different molecular ion distribution depending upon which finger is formed. We plan to test this hypothesis by analyzing the GR DBD mutants that carry Cys → Ser replacements within the C-terminal finger structure.[41] These proteins do not bind DNA and, as compared with a wild-type protein, show considerable susceptibility to proteolysis suggestive of more open structure. We hypothesize that the molecular ion distribution of GR DBD's carrying only the N-terminal finger structure will be very close to the ion envelope observed for a denatured, metal-free form of the protein. The fact that fully denatured protein does not use all its potentially available sites for protonation, as observed by us for the GR DBD and by Hutchens and Allen for the ER DBD,[17,18,30] requires further examination and will also be addressed in our studies on mutant GR DBD proteins.

Inaccuracy in determination of molecular masses of the Zn- and Cd- containing proteins might partially be due to the broadening (about 30% at half-height) and shape distortion of the isotopic distribution peaks of the Zn_2- and Cd_2-GR DBD caused by the multiple isotopes present in significant abundance (Figure 4). Peak shape distortion is especially striking for the zinc derivative whose two major isotopic species have practically identical relative abundances, which results in a flattened isotopic distribution peak. Thus, a measurement of peak-top-determined average molecular mass becomes intrinsically difficult and is subject to high experimental error.

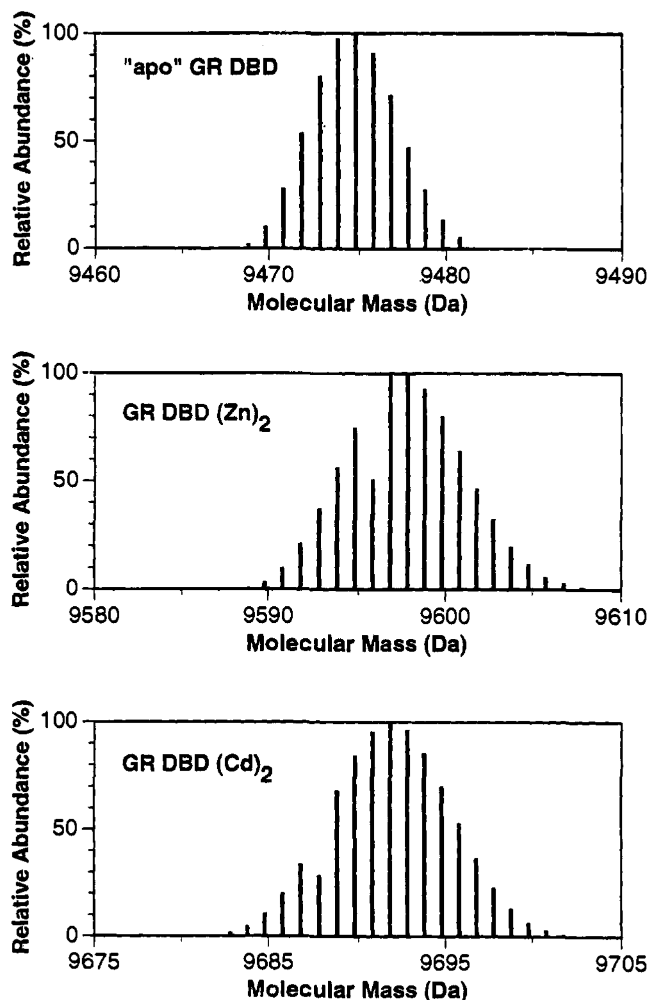

Figure 4. Approximation of theoretical isotopic distributions of the apo-GR DBD and its Cd- and Zn-containing derivatives. A loss of 4 Da per bound metal atom was assumed.

In conclusion, we offer this data on analysis of native GR DBD as further addition to the on-going debate on structural integrity of noncovalent complexes during mass spectrometric analysis. We also believe that our data will encourage the hormone receptor community to utilize the new mass spectrometric techniques in addition to the other physicochemical methods at their disposal.

Conclusions

Electrospray ionization mass spectrometry allows for accurate determination of the constitution and stoichiometry of protein–metal complexes, as shown for the GR DBD carrying two zinc-finger structures. The apparent "memory" effect demonstrated by macromolecules expelled from solvents of different chemistry can be explored by studying protein structure in solution. However, until more is known about the mechanism of macromolecular ion formation during the process of electrospray ionization and consequently their structure in the gas phase, no straightforward correlation between the solvent and ESI MS phenomena can be drawn.

Experimental Section

GR DBD Preparation. The human GR DBD domain whose sequence corresponds to amino acids 419–501 was expressed in *E.*

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coli, as described before.[42] Briefly, cell lysates were fractionated by ammonium sulfate and the protein was purified by a combination of ion exchange and gel filtration chromatography. The Zn-containing GR DBD protein solution was prepared for ESI MS analysis in nondenaturing conditions by dialyzing the original protein preparation (in 20 mM phosphate (pH 7.5), 150 mM NaCl, 1 mM DTT buffer, typical concentration 5 mg/mL) against 50 mM ammonium bicarbonate/1 mM DTT (pH 7.6) buffer (in cold, overnight). Exchange of Cd for Zn was accomplished by dialyzing the original Zn-containing protein (in a phosphate buffer specified above) against denaturing buffer (50 mM ammonium bicarbonate/10 mM EDTA/1 mM DTT/8 M urea (pH 7.6)) for 30 min at room temperature followed by a gradual protein renaturation to 50 mM ammonium bicarbonate/1 mM DTT/4 M urea (pH 7.6)/100 μM cadmium chloride (for 30 min, room temperature) to achieve full equilibration with a 50 mM ammonium bicarbonate/1 mM DTT/100 μM cadmium chloride buffer (pH 7.6) (in cold, overnight). The protein concentration after dialyses was not measured.

ESI MS Analysis of Native and Denatured GR DBD. All reported masses are the average masses and the isotopic distribution of GR DBD and metalloproteins was not resolved. ESI MS was performed on a VG BioQ triple quadrupole mass spectrometer interfaced with a Michrom BioResources narrow bore HPLC system. The extraction cone voltage was 55−65 V for electrospray of organic solvent-based solutions and 75−130 V for the water-based solutions. Comparison between molecular ion distribution of denatured and native proteins was performed using the same cone voltage 100 V. The source temperature was 60 °C. For mass analysis in denatured conditions, desalted and non-desalted protein samples were employed. In the former case, GR DBD (200 pmol−1 nmol) was applied to a reversed phase narrow bore PLRP-S column (1 × 50 mm, 4000 Å pore size, 8 μm particle size) and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 40 μL min^{-1}. A postcolumn flow split allowed 10% of the protein to be delivered to a mass spectrometer through a triaxial modified VG probe at a flow rate of 4 μL min^{-1}, while being mixed with 2 μL min^{-1} MeOH/0.1% TFA sheath liquid prior to nebulization.[43] The 6.9 s scans were acquired for a *m/z* range of 610−960. In later experiments, 10 μL aliquots of the 25 μM protein solution were injected via a loop injector into a stream of solvent (50% acetonitrile/0.1% TFA) at a flow rate of 4 μL min^{-1}. To achieve the desired final sample concentration, protein preparations in either phosphate or ammonium bicarbonate buffers (see above) were diluted with a 50% acetonitrile/0.1% TFA solvent (typically 20-fold). For ESI MS studies in nondenaturing conditions, 5 μL aliquots of 40−400 μM protein samples in 50 mM ammonium bicarbonate buffers (see above) were injected into a stream of solvent (50 mM pyridine acetate (pH 5.9)) at a flow rate 4 μL min^{-1}; when Cd-containing GR DBD was analyzed, 100 μM cadmium chloride was present.

Calculation of the Theoretical Isotopic Distribution of GR DBD. Approximation of the isotopic distribution of molecular ions of GR DBD containing Zn and Cd metal ions was made by calculating relative abundance of all species of the same mass derived from the various combinations of isotopic distributions of two Zn or two Cd atoms and an isotopic distribution of the apo-GR DBD, the former obtained using VG software supplied with a PDPII system and the latter using a MassBioSpec software (PE Sciex Instruments). A loss of 4 Da per one bound metal atom was assumed.

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