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Recognition of transition metal ions by peptides

Identification of specific metal-binding peptides in proteolytic digest maps by UV laser desorption time-of-flight mass spectrometry

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Metal-binding peptides in proteolytic digest maps have been identified by matrix-assisted UV laser desorption time-of-flight mass spectrometry (LDTOF-MS). The plasma and milk metal transport protein chosen to demonstrate this process, histidine-rich glycoprotein (HRG), was purified and then digested with trypsin; the cleavage products were analyzed by LDTOF-MS with dihydroxybenzoic acid as the matrix. The selective interaction of specific peptides with one or more Cu atoms was observed when Cu(II) ions were added to the digest mixture. At least one specific metal-binding peptide was identified by computerized sequence analysis using the molecular mass data and available cDNA sequence. These results demonstrate the first direct observation by mass spectrometry of differential peptide–metal ion interactions in protein digest maps. The ability to evaluate peptide–metal ion interactions, including stoichiometry, with less than 1 pmol of sample improves significantly our ability to identify metal binding domains in metal-binding proteins.

Mass spectrometry; Metalloprotein; Histidine-rich glycoprotein; Peptide; Digest; Metal ion

1. INTRODUCTION

The interaction of transition metal ions with intracellular proteins, via several different structural motifs, has been shown to be important in regulatory macromolecular recognition events [1]. Identification of protein surface metal-binding domains involved in metal ion transport also remains the subject of considerable interest [2–6]. Although we have evaluated immobilized metal ions for the identification and isolation of metal-binding peptides in proteolytic digests [7], there have not been any suitable methods to observe directly the interaction of one or more transition metal ions with a given peptide or peptides in a complex mixture.

Matrix-assisted UV laser desorption time-of-flight mass spectrometry (LDTOF-MS) has been used recently to determine the molecular weight of intact peptides and proteins with a high degree of sensitivity (<1 pmol), accuracy (ca. 0.01% at 20 kDa) and resolution (300–500 fwhm) [8,9]. We have recently demonstrated that specific peptide-metal complexes are stable during analysis by LDTOF-MS [10,11] and by electrospray ionization mass spectrometry [12]. We report here the use of LDTOF-MS to identify specific metal-binding peptides among the unfractionated proteolytic digestion products of a human plasma metal-binding transport protein known as histidine-rich glycoprotein (HRG).

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2. EXPERIMENTAL

2.1. *Purification of human plasma HRG by immobilized TED-Zn(II) ion affinity chromatography*

Human plasma was collected into tubes containing EDTA and protease inhibitors (soybean trypsin inhibitor, benzamidine, D-phenylalanyl-L-arginine chloromethyl ketone, N^{α}-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride). All plasma was dialyzed at 4°C against 50 mM EDTA, 20 mM sodium phosphate, pH 7.0, and then against 20 mM sodium phosphate, 5 mM imidazole and 0.5 M sodium chloride, pH 7.0. The synthesis of Tris(carboxymethyl)ethylenediamine-Sepharose 6B (TED) [13] and its use for the purification of HRG was as described [14]. The TED-Zn(II) column was equilibrated with 20 mM sodium phosphate containing 5 mM imidazole and 0.5 M NaCl, pH 7.0. Dialyzed plasma was applied; low affinity proteins were eluted with 0.1 M sodium acetate containing 0.5 M NaCl, pH 5.8. Purified HRG was eluted with 0.1 M sodium acetate containing 0.5 M NaCl, pH 3.8. After documentation of apparent homogeneity by SDS-PAGE (silver-stained) and high-performance reverse-phase chromatography, amino acid composition was determined by the picotag method [15]. The N-terminal amino acid sequence was determined by sequential Edman [16] degradation on an Applied Biosystems Model 473A automated peptide sequence analyzer (Applied Biosystems, Foster City, CA).

2.2. *UV laser desorption time-of-flight mass spectrometry*

Purified HRG was dialyzed against 50 mM EDTA, 20 mM sodium phosphate, pH 7.0, and then against 20 mM sodium phosphate, 0.15 M NaCl, pH 7.0. 2 μg of the dialyzed HRG was incubated with 0.04

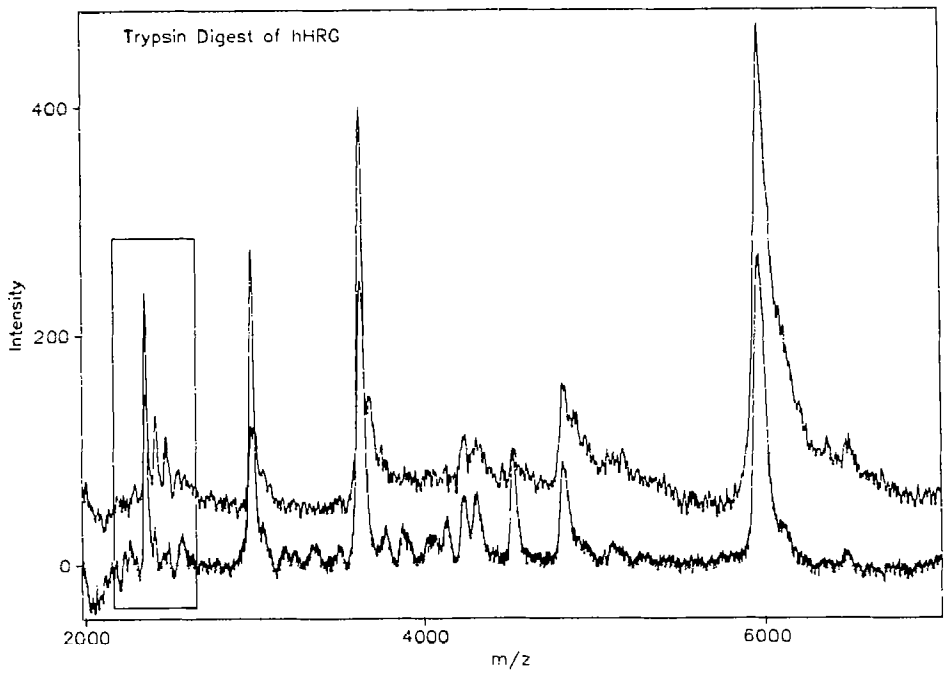

Fig. 1. Mass spectra of HRG tryptic digest products determined by LDTOF-MS before and after the addition of Cu(II) ions to the peptide digest. The portion of the spectrum outlined by the box is presented in Fig. 2 in more detail.

μg of trypsin (Promega) in 30 μl of 5 mM sodium phosphate (pH 7.8) at 37°C for 3.5 h; the protein samples digested were neither reduced nor denatured. Aliquots of the peptide digest were diluted to ca. 10 nmol/ml and mixed (1:1) with a saturated aqueous solution of 2,5-dihydroxybenzoic acid (154.12 Da); 2 μl of the digest/matrix mixture was applied to a stainless steel probe tip (2-mm diameter) and air-dried at room temperature. The air-dried peptide-matrix deposit on the probe tip, prepared with or without added copper sulfate (2 μl of a 20 mM solution), was rinsed gently with Milli-Q water to remove residual salts, and re-dried. Mass spectral analyses were performed on a Model 2000 laser desorption linear time-of-flight mass spectrometer (Vestec) using the frequency-tripled output from a Q-switched neodymium-yttrium aluminium garnet (Nd-YAG) pulsed laser (355 nm, 5 ns pulse, Lumonics HY400). Ions desorbed by pulsed laser irradiation were accelerated to an energy of 30 keV, allowed to drift along a 2-m flight path (maintained at 30 μPa), and detected with a 20-stage-focused mesh electron multiplier. A LeCroy model TR8828D transient recorder (5-ns time resolution) and LeCroy 6010 MAGIC controller were used for the real-time signal averaging of multiple (100) laser shots (recorded at 200 MHz). All calculations and time to m/z conversions were performed on PC-based software. Peptide sequences were identified from mol. wt. analyses by LDTOF-MS using the program PROCOMP (Phillip C. Andrews, University of Michigan).

3. RESULTS AND DISCUSSION

We are interested in the identification of protein surface metal-binding domains, the preservation of domain structure and metal-binding activity during protein degradation, and the generation (i.e exposure) of new metal-binding protein domains and fragments by proteolytic modification. There have been few techniques developed to identify specific peptide–metal ion interactions in complex mixtures, particularly in cases of relatively low affinity interactions involving low molecular mass peptides. The high-resolution separation of peptides typically requires experimental conditions incompatible with the retention of bound metal ions (e.g. high-performance reverse-phase liquid chromatography). The techniques currently available to investigate protein–metal ion interactions in complex mixtures include chromatography (e.g. size-exclusion, ion-exchange) or SDS-polyacrylamide gel electrophoresis followed by transfer of the proteins to a membrane for blotting (i.e. probing) with radioactive metal ions [17]; these procedures are not appropriate for the resolution of low mol. wt. peptides. Furthermore, these procedures are dependent on separate means of resolution and detection, resulting in low recovery and disruption of the peptide– and/or protein–metal ion interactions.

Our evaluation of synthetic metal-binding peptides by matrix-assisted LDTOF-MS has suggested to us that peptide-bound metal ions can be stable to the laser-induced desorption/ionization process [10–12]. To develop and extend the utility of this observation, we have investigated the suitability of LDTOF-MS as a technique to identify specific metal-binding peptides in complex mixtures without prior fractionation. Matrix-assisted LDTOF-MS is well-suited for the mass determination of peptide mixtures for several reasons. First, LDTOF-MS produces mass spectra that are, in contrast to spectra produced by other means of desorption/ionization, relatively free of artifacts (e.g. fragments, multiply-charged molecular ions). Second, the

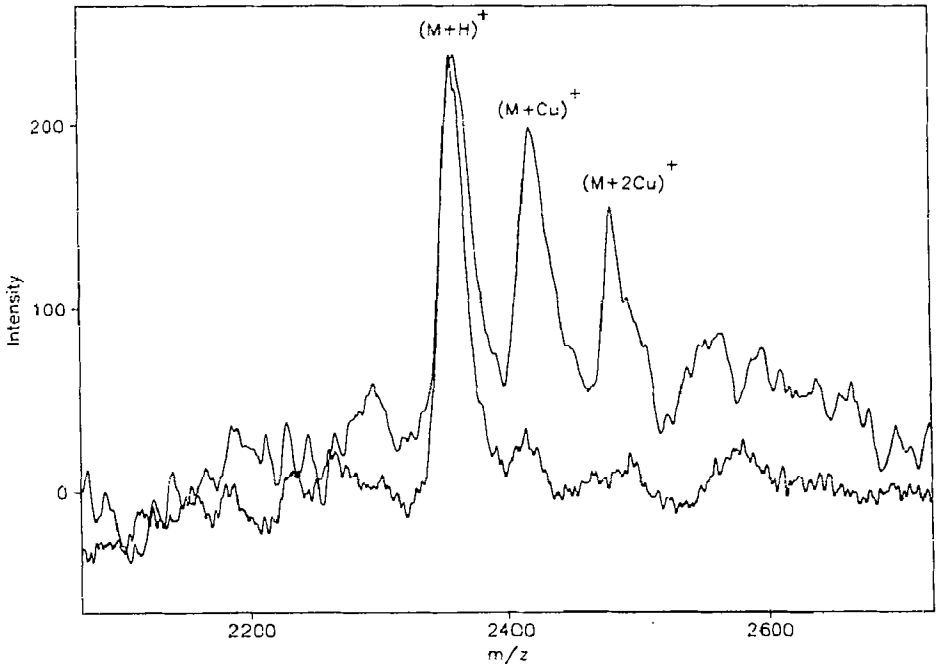

Fig. 2. A portion of the mass spectra revealing a 2356-Da metal-binding peptide $(M + H)^+$ in the HRG tryptic digest map. The spectra were determined by LDTOF-MS before and after the addition of Cu(II) ions to the peptide digest. The peptide with one and two bound Cu atoms is as indicated.

use of 2,5-dihydroxybenzoic acid as the matrix leaves the low mass region (<1000 Da) relatively free of matrix clusters. Finally, for proteins of known sequence, the accuracy of peptide mass determinations by LDTOF-MS allows each degradation product to be identified.

HRG was chosen as a model for these investigations because of its probable role in Cu(II) and Zn(II) ion transport in plasma [6,14]. During HRG-digestion with trypsin, the disulfide bonds were deliberately not reduced, nor was the protein denatured. Thus, elements of HRG protein secondary and tertiary structure were likely to be retained. The avoidance of reducing agents helped to preclude the artificial generation of free sulfhydryl groups; a potential major source of nonspecific metal ion binding sites otherwise unavailable in the non-reduced native protein.

Fig. 1 shows an overview of the HRG tryptic digest map observed by LDTOF-MS before and after Cu(II) ions were added to the peptide in solution with 2,5-hydroxybenzoic acid as matrix. Fig. 2 shows an example of one specific, high-definition region of the tryptic digest mass spectrum observed by LDTOF-MS before and after Cu(II) ions were added. In this portion of the protein digest mass spectrum, the major protonated molecular ion $(M + H)^+$ observed in the absence of metal ion had a mass of 2356 Da. In the presence of Cu(II), the protonated parent molecular ion $(M + H)^+$ was still observed; however, two additional peaks were generated, at 2419 and 2482 Da, with increments of 1× and 2× 63 Da (i.e. ^{63}Cu). To identify this particular metal-binding peptide, all possible trypsin cleavage sites in the HRG sequence [18] were identified. By computation of the mass for each of the several hundred possible partial and complete trypsin digest products (ca. 1650), the program PROCOMP was then used to generate a theoretical list of possible fragments and their molecular

Table I

Mass-dependent identification of one specific metal-binding peptide present in the HRG trypsin digest map evaluated by LDTOF-MS

$(M + H)^+$		Sequence	Position of sequence in HRG
Observed[a]	Calculated[b]		
2356	2355.8	KGEVLPLPEANFPSFPLPHHK	residues 448–468

[a] Observed $(M + H)^+$ value taken from the calibrated LDTOF-MS spectra shown in Fig. 2.
[b] The calculated value of $(M + H)^+$ shown here was selected from the list of calculated mass values generated for theoretical fragments predicted from the possible (partial and complete) trypsin digestion sites in HRG (see Experimental).

weights. Only 1 of the many possible proteolytic fragments had a calculated mass the same as that of the metal-binding peptide observed by LDTOF-MS; this peptide is identified in Table I. The specific amino acid sequence of the peptide identified by this procedure was predicted to bind Cu based on procedures that we have reported previously [7,19].

There are limits to the unequivocal identification of specific metal-binding peptides by this technique. For example, by calculation, the 507-residue HRG sequence also contains two other theoretical trypsin digest fragments of similar molecular mass (i.e. 2349.8 and 2350.5 Da) that would arise from partial digestion (i.e. each of these fragments contains an internal Lys or Arg residue). Based on their His residue content, each of these peptides (D267–R286 and G428–R447), if present in the trypsin digest mixture, would be expected to bind Cu atoms. Even with a demonstrated mass accuracy of 0.01% (up to 20 kDa), LDTOF-MS is currently unable to resolve molecular mass differences of less than 4–5 amu in this mass range. Thus, in this particular case, only the most probable identity of the specific Cu-binding peptide can be assigned.

Our results also demonstrate the presence of specific peptides with a portion of their metal-binding sites apparently occupied by endogenous metal ions (e.g. Fig. 2). Thus, interpretation of mass spectra obtained with relatively clean samples of peptides (synthetic peptides), simple peptide mixtures (e.g. peptide digests), and peptides in complex biological mixtures can be complicated by the presence of peptide-bound metal ions.

In conclusion, although we developed immobilized metal ion affinity chromatography as a method for the high-resolution separation of metal-binding peptides in proteolytic digest mixtures [7], LDTOF-MS appears to address this problem with far greater sensitivity and accuracy. Moreover, as we have demonstrated here and elsewhere with synthetic metal-binding peptides [10–12], it may also be possible to address the stoichiometry of metal ion-peptide interactions by LDTOF-MS. Together, immobilized metal ion interaction chromatography and LDTOF-MS can be used effectively to isolate and identify metal binding peptides in proteolytic digests.

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