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Novel method for $[M + Cu]^+$ ion formation by matrix-assisted laser desorption ionization

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Abstract

Direct deposition of a MALDI sample onto a copper sample stage and irradiation with UV light (337 nm) produces copper adduct ions of both the matrix and analyte molecules. This technique for introducing Cu^+ into the gas-phase avoids suppression of ion signal that accompanies addition of metal salts to the sample solution. We observe good correlation between the number of basic residues in peptides and the number of Cu^+ ions that add to the peptide. For example, the peptide KRQHPG contains three basic residues and forms ions with up to three Cu^+ adducts. Postsource decay experiments demonstrate that for arginine containing peptides, arginine anchors the Cu^+ ion. That is, all metastable ions contain the arginine complexed to Cu^+ and the only immonium ion observed is that of arginine–Cu^+. In addition, preliminary calculations indicate that guanidine has the highest Cu^+ ion affinity followed by histidine. (Int J Mass Spectrom 182/183 (1999) 185–195) © 1999 Elsevier Science B.V.

Keywords: MALDI; Cu^+; Peptides; Binding sites; PSD

1. Introduction

Gas-phase ionic complexes of alkali and alkaline-earth metals and small peptides have been extensively studied by a variety of mass spectrometry methods [1–9]. Transition metal ions complexed to amino acids and peptides have also been investigated by mass spectrometry [10–21]. In addition to peptides and proteins, transition metal ions are commonly used to ionize synthetic hydrocarbon polymers [22–27]. Structure determinations of transition metal ion complexes present unique challenges because the metal may have several stable oxidation states. For example, Cu(I) is considered a soft Lewis acid, whereas Cu(II) is considered borderline hard Lewis acid. In solution, Cu(I) and Cu(II) prefer different ligands; Cu(I) prefers sulphur and phosphorous ligands, whereas Cu(II) prefers nitrogen and oxygen ligands [28]. Cu(I) and Cu(II) also form different structures in biological systems. Generally, Cu(I) forms complexes of tetrahedral geometry, whereas Cu(II) forms square planar complexes [29].

There are two widely used methods for introducing metal ions into the gas phase. The most commonly used method is to add a metal salt such as NaCl, $Cu(So_4)$, or $AgNO_3$ to the fast-atom bombardment (FAB) or matrix-assisted laser desorption ionization (MALDI) matrix or to the solution used for electro-

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Dedicated to the memory of Ben Freiser, a friend for many years, but far too few!

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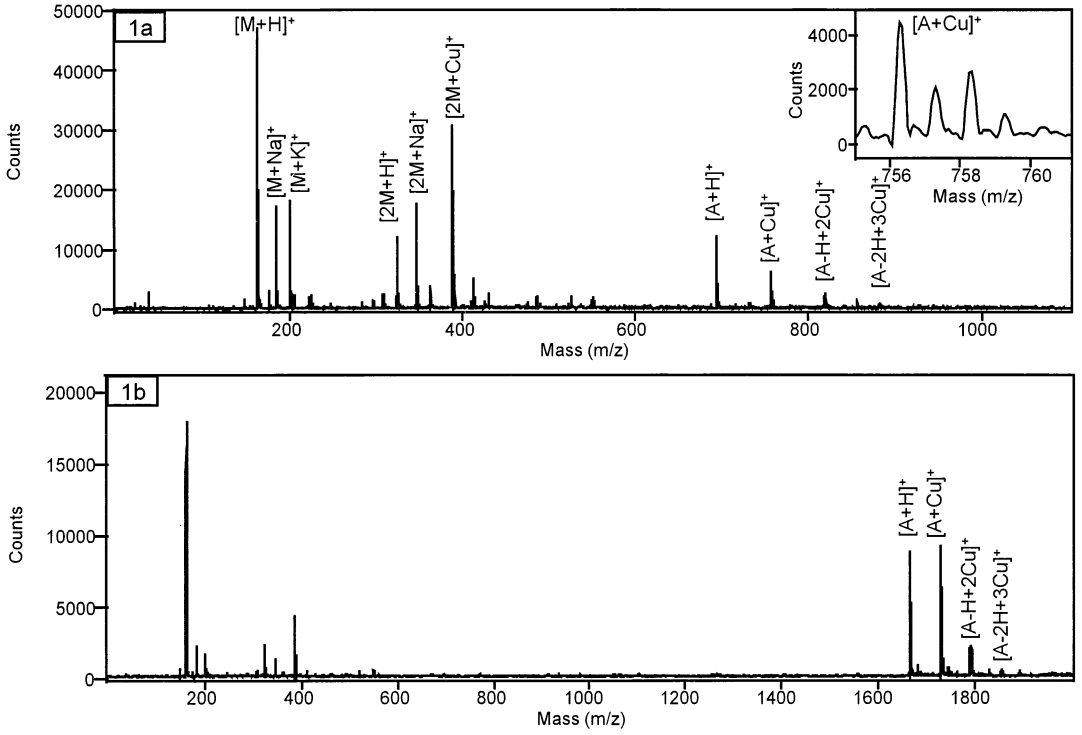

Fig. 1. Positive ion mass spectra of (a) RKEVY and (b) α-melanocyte STH desorbed from the copper sample stage. The matrix used for desorption is 7-hydroxycoumarin.

spray ionization (ESI) [1–20]. Alternatively, bare metal ions can be reacted with the analyte in the gas phase (e.g. chemical ionization [21] or by particle bombardment [kilo electron volt secondary ion mass spectrometry (keV SIMS)] of metal surfaces). However, the yields for $[\text{peptide} + \text{metal}]^+$ ions from keV energy SIMS is quite low [30,31]. This article introduces another technique for complexing transition metals, specifically copper, to biomolecules. Solutions containing the analyte and matrix are deposited onto a copper sample stage and the solid is irradiated with UV (337 nm) light of a nitrogen laser.

In this MALDI study we report copper complexation to the matrix and analyte by desorbing a matrix and analyte mixture deposited directly on a copper sample stage. This method of introducing copper to biomolecules is advantageous over the addition of metal salts because there are no counter ions to disturb the ionization process, which often leads to suppression of ion signal. Yalcin and coworkers [22] elaborate on the effects of such interferences in their studies using $Cu(NO_3)_2$ and $CuCl_2$ to ionize polyisoprene and polybutadiene. Using a copper sample stage for desorption eliminates the interference that salt addition to the matrix has on MALDI and offers sensitivity that is comparable to normal MALDI.

2. Experimental

The MALDI experiments described herein were performed on a Perseptive Biosystems Voyager Elite XL equipped with a single-stage reflectron time-of-flight (RTOF) mass spectrometer with a nitrogen laser (337 nm) for desorption/ionization [32]. All mass spectra are recorded in delayed extraction (DE)-RTOF mode unless otherwise noted. A copper sample stage was fabricated in our laboratory to accommodate the above instrument. All peptides and the matrix 3-hydroxypicolinic acid were purchased from Sigma

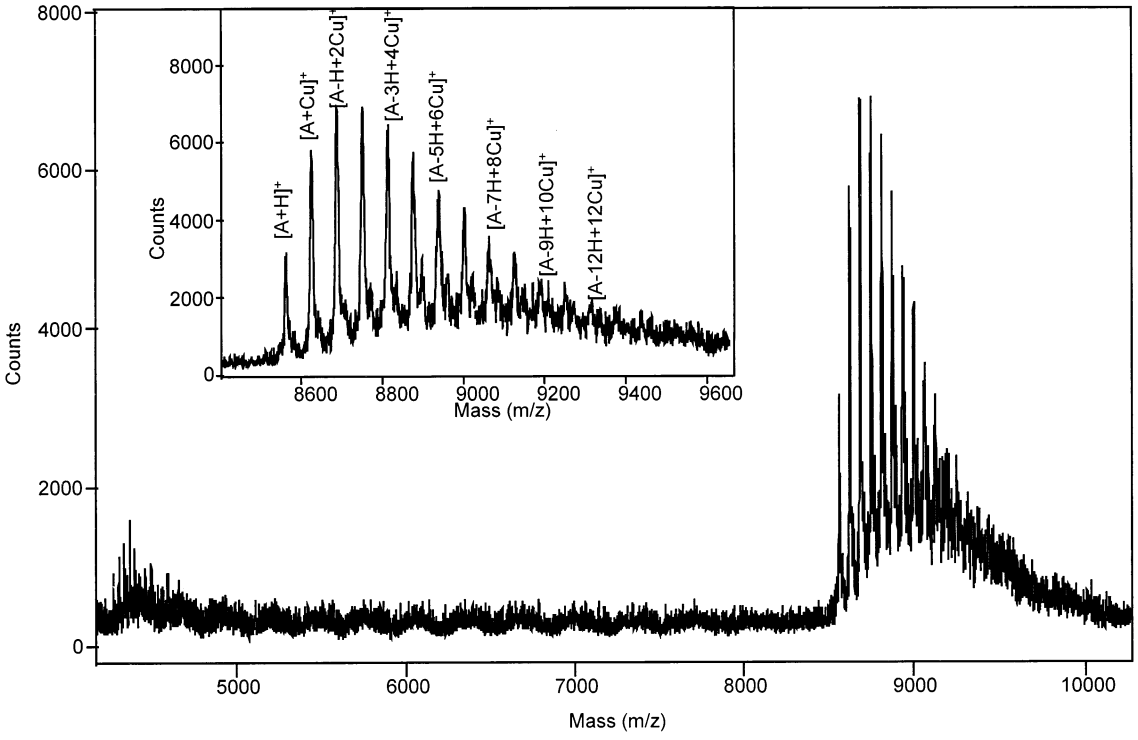

Fig. 2. A mass spectrum of Ubiquitin from the copper sample stage with ferulic acid as the matrix.

(St. Louis, MO) and used without further purification. Three matrices used in this study, α-cyano-4-hydroxycinnamic acid, 7-hydroxycoumarin, and ferulic acid were purchased from Aldrich (Milwaukee, WI) and used without further purification. Oligonucleotides were purchased from The Midland Certified Reagent Company (Midland, TX). A matrix:analyte molar ratio of 1000-2000:1 was used in every case with a total of 5 pmol of analyte deposited on the copper sample stage.

3. Results

Fig. 1 contains the positive ion MALDI mass spectra of a synthetic peptide, RKEVY [Fig. 1(a)], and α-melanocyte stimulating hormone [Fig. 1(b)] obtained from the copper sample stage. In both examples, abundant copper adduct ions of matrix and analyte are detected; however, no bare Cu^+ ions are detected. All matrix signals are denoted with the letter M and analyte signals are denoted with A. The inset of Fig. 1(a) shows the $[A + Cu]^+$ ion of RKEVY and is used here to illustrate the contribution of the ^{63}Cu (m/z 756) and ^{65}Cu (m/z 758) isotopes to the isotopic distribution of the peptide ion. Fig. 2 contains the positive ion mass spectrum of Ubiquitin desorbed from the copper stage. Table 1 lists the mass measurement data and relative abundances for the $[A + H]^+$ and $[A–(x - 1)H + xCu]^+$ ions of various peptides. The data in Table 1 are compiled from the average of 10 spectra with each spectrum containing a sum of 100 laser shots. The spectra were externally calibrated by using the $[A + H]^+$ ion of bovine insulin B-chain (m/z 3494.651) and the protonated matrix dimer (m/z 379.093). Fig. 3 contains the negative ion mass spectra of substance P–NH_2 [Fig. 3(a)] and α-melanocyte stimulating hormone [Fig. 3(b)] desorbed from the same copper sample stage.

Table 1
Mass measurement accuracies and relative abundances of the positive ions formed by MALDI off of a copper sample stage with the matrix 7-hydroxycoumarin

Peptide	Experimental mass (da)	Calculated mass (da)	Error (ppm)	Relative abundance (%)[a]
RKEVY				
$[A + H]^+$	694.384	694.389	−7.0	56.2
$[A + H + Cu(0)]^+$	**756.304**	**757.318**	**−1331**	**28.8**
$[A + Cu(I)]^+$	**756.304**	**756.311**	**−8.7**	**28.8**
$[A–H + Cu(II)]^+$	**756.304**	**755.303**	**1332**	**28.8**
$[A–H + 2Cu(I)]^+$	818.223	818.232	−11.8	10.9
$[A–2H + 3Cu(I)]^+$	880.149	880.154	−5.3	4.1
KRQHPG				
$[A + H]^+$	722.400	722.406	−8.3	34.7
$[A + Cu(I)]^+$	784.323	784.328	−5.9	50.1
$[A–H + 2Cu(I)]^+$	846.246	846.250	−4.1	11.5
$[A–2H + 3Cu(I)]^+$	908.172	908.171	0.8	3.7
Substance P				
$[A + H]^+$	1347.712	1347.736	−17.8	68.8
$[A + Cu(I)]^+$	1409.629	1409.658	−20.6	28.0
$[A–H + 2Cu(I)]^+$	1471.558	1471.580	−14.9	3.2
α-Melanocyte STH				
$[A + H]^+$	1664.793	1664.801	−4.7	46.6
$[A + Cu(I)]^+$	1726.724	1726.723	0.6	43.1
$[A–H + 2Cu(I)]^+$	1788.647	1788.644	1.5	8.0
$[A–2H + 3Cu(I)]^+$	1850.556	1850.566	−5.5	2.3
B-Chain of insulin				
$[A + H]^+$	3494.646	3494.651	−1.5	62.1
$[A + Cu(I)]^+$	3556.572	3556.573	−0.2	21.2
$[A–H + 2Cu(I)]^+$	3618.473	3618.495	−6.0	16.7

[a] $[A–(x - 1)H + xCu]^+/\Sigma[A–(x - 1)H + xCu]^+$.

Table 2 summarizes the mass assignments, mass measurement accuracies, and relative ion abundances of the $[A - H]^-$ and $[A–(x + 1)H + xCu]^-$ ions of various peptides.

Fig. 4 contains the metastable ion (MI) spectrum of the peptide RKEVY. Fig. 4(a) and 4(b) contain the MI mass spectrum of the $[A + H]^+$ ions and the $[A + Cu]^+$ ions, respectively. Fig. 5 contains MI spectra of substance P–NH_2, and sequence ions are labeled using the nomenclature proposed by Roepstorff and Fohlmann [33].

Fig. 6 contains the positive ion mass spectra of the oligonucleotide, 5′-pCTCTAGAG-3′, desorbed from the copper sample stage using ferulic acid as the matrix [Fig. 6(a)] and 3-hydroxypicolinic acid [Fig. 6(b)]. Both spectra were obtained in delayed extraction-linear TOF (DE-LTOF) mode.

4. Discussion

Complexation of metal ions to polar molecules is a commonly used technique to aid in ionization of labile molecules. This method dates back to early work in field desorption [34] and has been recently applied to analysis of synthetic polymers by MALDI [22–27]. In addition, the attachment of alkali metal cations to small peptides has been used extensively to probe the gas-phase structure of peptide ions. A number of studies on the binding of copper cations to α-amino acids, peptides, and proteins have also been reported [10,17–19,21,35–39]. For these studies the copper ion is derived from Cu^{+2} salts, which are mixed with the organic analyte. This article demonstrates the utility of direct deposition and desorption of the matrix and analyte from a copper sample stage as a method for

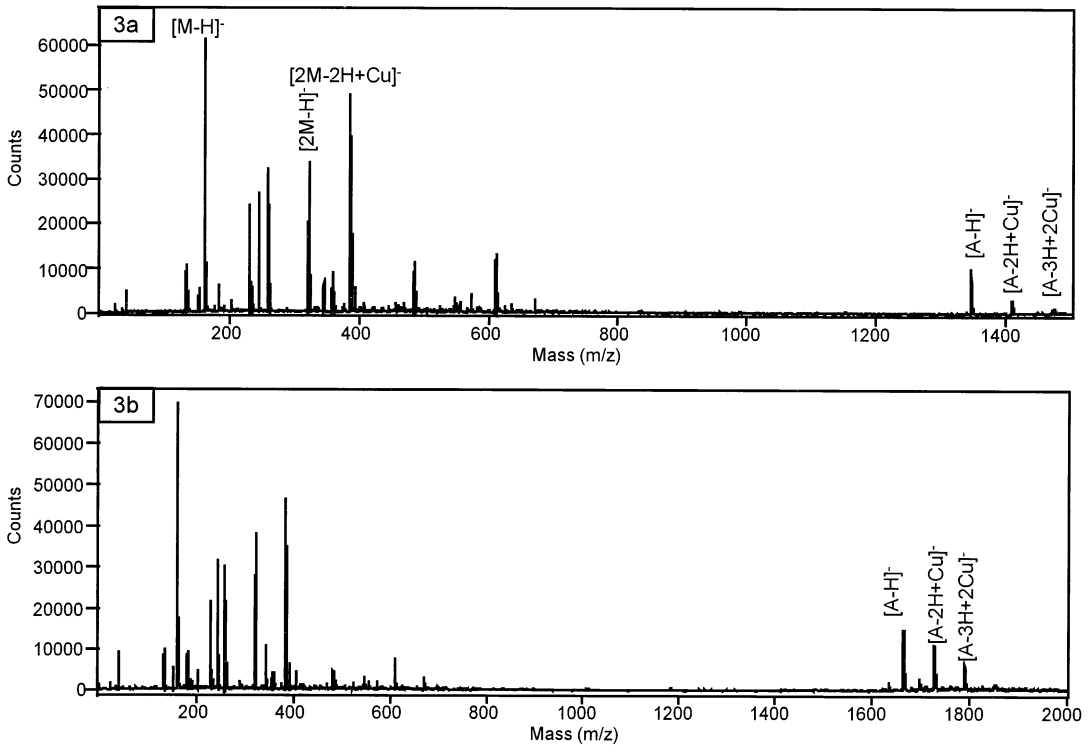

Fig. 3. Negative ion mass spectra of (a) substance P–NH_2 and (b) α-melanocyte STH desorbed from the copper sample stage with 7-hydroxycoumarin as the matrix.

producing gas-phase Cu^+ complexes of both the organic matrix and the biomolecule.

Because copper can exist as Cu^0, Cu^+, and Cu^{+2}, we used accurate mass measurements to establish the oxidation state of the copper complexed to the peptides. If the copper cation complexed to the peptide is Cu^{+2}, the peptide must lose a proton to maintain a formal +1 charge [reaction (1)]. On the other hand, if copper is complexed as a neutral, the addition of a proton would be necessary for ionization [reaction (2)]. The experimentally measured m/z value for $[M + Cu]^+$ is consistent with the assignment of the ions as $[A + Cu(I)]^+$ [reaction (3)] (see Table 1)

$$A + Cu^{+2} \rightarrow [A + Cu^{+2} - H]^+ \quad (1)$$

$$AH^+ + Cu^0 \rightarrow [AH^+ + Cu]^+ \quad (2)$$

$$A + Cu^+ \rightarrow [A+Cu]^+ \quad (3)$$

Although many models have been developed to describe the MALDI energy deposition and desorption event [40–44], it is generally assumed that surface–sample interactions do not play a significant role in these processes. The observation of copper adduct ions to the matrix and analyte when desorbed from the copper sample stage indicates a strong affinity of Cu^+ for peptide molecules. The studies performed to date do not permit us to determine whether Cu^+ is attached as a gas-phase process or by some surface derived reaction. At this point the origin of the Cu^+ complexed to the matrix and analyte appears to be from an oxide layer, Cu_2O, on the sample stage surface. For example, after washing a sample of matrix and analyte off the copper sample stage with methanol, the location where the sample was applied is much brighter than the adjoining area of the sample stage. This suggests that the sample

Table 2
The mass measurement accuracies and relative abundances of the negative peptide ions desorbed from the copper sample stage with 7-hydroxycoumarin as the matrix

Peptide	Experimental mass (da)	Calculated mass (da)	Error (ppm)	Relative abundance (%)
RKEVY				
$[A–H]^-$	692.381	692.373	12.0	44.1
$[A–2H + Cu(I)]^-$	754.304	754.295	11.8	37.9
$[A–3H + 2Cu(I)]^-$	816.223	816.217	8.1	18.0
KRQHPG				
$[A–H]^-$	720.401	720.391	14.5	29.4
$[A–2H + Cu(I)]^-$	782.325	782.312	15.8	49.6
$[A–3H + 2Cu(I)]^-$	844.251	844.234	20.4	17.0
$[A–4H + 3Cu(I)]^-$	906.176	906.156	21.6	4.0
Substance P				
$[A–H]^-$	1345.711	1345.720	−7.3	68.7
$[A–2H + Cu(I)]^-$	1407.633	1407.642	−6.3	20.6
$[A–3H + 2Cu(I)]^-$	1469.561	1469.564	−2.2	10.7
***α*-Melanocyte STH**				
$[A–H]^-$	1662.776	1662.785	−5.4	53.1
$[A–2H + Cu(I)]^-$	1724.705	1724.707	−1.3	33.4
$[A–3H + 2Cu(I)]^-$	1786.626	1786.629	−1.2	10.7
$[A–4H + 3Cu(I)]^-$	1848.565	1848.550	7.8	2.8
B-Chain of insulin				
$[A–H]^-$	3492.678	3492.636	12.1	87.2
$[A–2H + Cu(I)]^-$	3554.612	3554.557	15.3	12.8

(e.g. solvent, matrix, and analyte) is chemically etching the plate by removing the oxide layer. Studies by Cocke et al. [45] and Barr [46] show that the copper oxide film formed at room temperature is Cu_2O, whereas oxide films formed at high temperature (473 K) are CuO. In an attempt to determine whether the oxidation state of the copper oxide on the sample stage is important to the formation of $[M + Cu]^+$ or $[A + Cu]^+$ ions, we heated the sample stage at 473 K for 4 h in air and then allowed the stage to cool to room temperature before depositing the matrix and analyte mixture. The only analyte adduct ions observed from this sample corresponded to $[A + Cu(I)]^+$. This was not surprising because previous studies have shown that desorption of organic compounds in the presence of Cu(II) produces Cu(I) species [14,45]. Although we have not done extensive experiments, we did examine desorption from a silver sample stage and did not observe $[A + Ag]^+$ ions nor do we observe $[A + Fe]^+$ or other adduct ions from stainless steel surfaces irradiated at 337 nm.

Because $[A + H]^+$ and $[A + Cu]^+$ ions are formed from the copper stage, it is evident that there is competition between protonation and cationization regardless of the matrix used. The number of Cu^+ ions attached to the peptides roughly correlates to the number of basic residues contained in the peptide. For example, RKEVY contains two basic amino acids and the most abundant ions observed are $[A + Cu(I)]^+$ and $[A–H + 2Cu(I)]^+$ ions. A small amount, 4.1% relative abundance, $[A–2H + 3Cu]^+$ adduct ions are observed. For Ubiquitin (Fig. 2) there are 13 discernible copper adducts to this protein. Ubiquitin contains a total of 12 basic residues: 7 lysines, 4 arginines, and 1 histidine.

We also examined the Cu^+ ion binding to peptides containing only aliphatic side chains. The synthetic peptide, VGVAPG, was applied to the copper stage

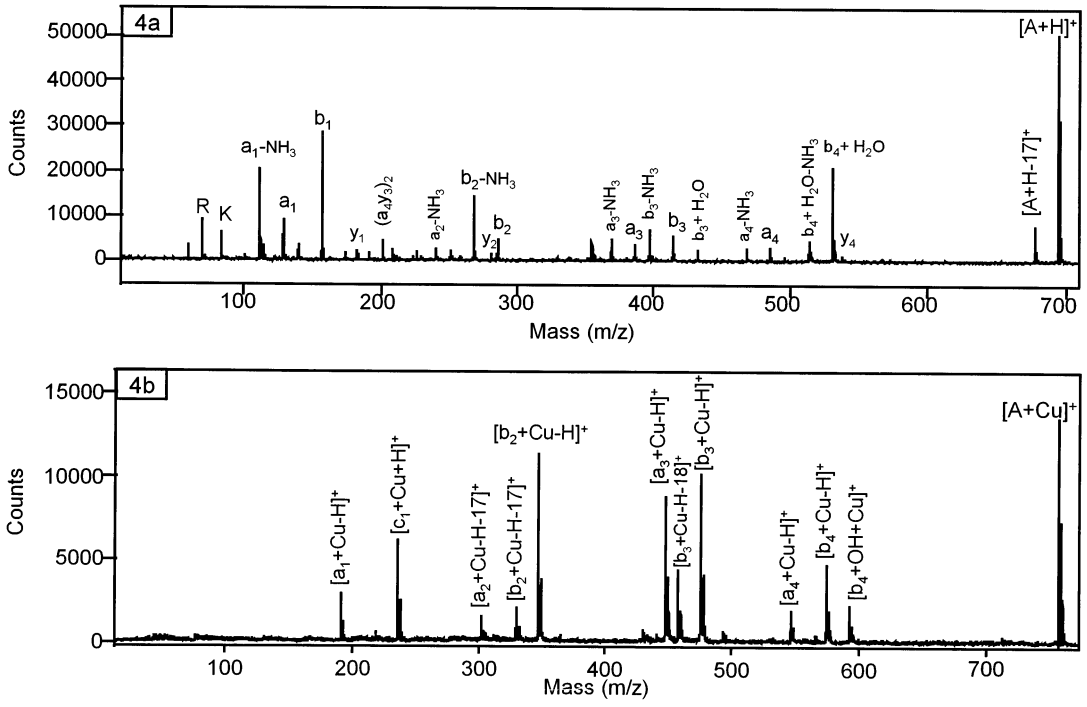

Fig. 4. MI spectrum of (a) $[A + H]^+$ ions and (b) $[A + Cu]^+$ ions of RKEVY with α-cyano-4-hydroxycinnamic acid as the matrix.

and no $[A + H]^+$ ions were observed and the signal-to-noise ratio for the $[A + Cu]^+$ ions was 2:1 at a high laser fluence. Due to a limited number of $[A + Cu]^+$ ions formed and the low signal-to-noise ratio, no fragment ions were observed from this peptide. In solution, Cu^+ is known to interact with sulfur on cysteine and methionine residues [47]. However, for the peptides methionine enkephalin (YGGFM) and KCTCCA no $[A + Cu]^+$ ions are observed in the MALDI mass spectrum. These observations suggest that basic residues have the greatest affinity for Cu^+ and without such residues, Cu^+ is not complexed to the peptide when desorbed from the copper sample stage. The difference in binding sites from solution to gas phase suggests that the gas-phase structure of the $[A + Cu]^+$ peptide ions is distinctly different from that of the peptide–Cu^+ complex in solution. The strong preference for Cu^+ binding to basic residues in the gas phase can be rationalized on the basis of the charge state, e.g. basic residues are protonated in solution, however, in the gas phase the side chains do not carry a charge, with the possible exception of a C-terminal arginine [48]. In addition, in solution the basic residues are hydrophilic and solvent stabilized.

Attachment of Cu(I) to peptides is also observed for negative ions, and for these ions, the species correspond to $[A–(x + 1)H + xCu]^-$. Figure 3 and Table 2 contain negative ion data collected from the same copper sample stage. Interestingly, multiple negatively charged ions, $[A–(x + 1)H + xCu(I)]^-$, are also formed. Table 2 illustrates that the number of negatively charged ions with Cu^+ adducts also correlates with the number of basic residues. According to Cerda and Wesdemiotis [10] and Hoyau and Ohanessian [36], arginine and lysine have the largest Cu^+ ion affinity. Therefore, regardless of whether the peptide is anionic or cationic, the number of copper adducts to the peptide should be consistent.

The metastable ion mass spectra of several peptides were recorded using postsource decay in an attempt to determine the Cu^+ ion binding site. The MI spectra for substance P and RKEVY are contained in

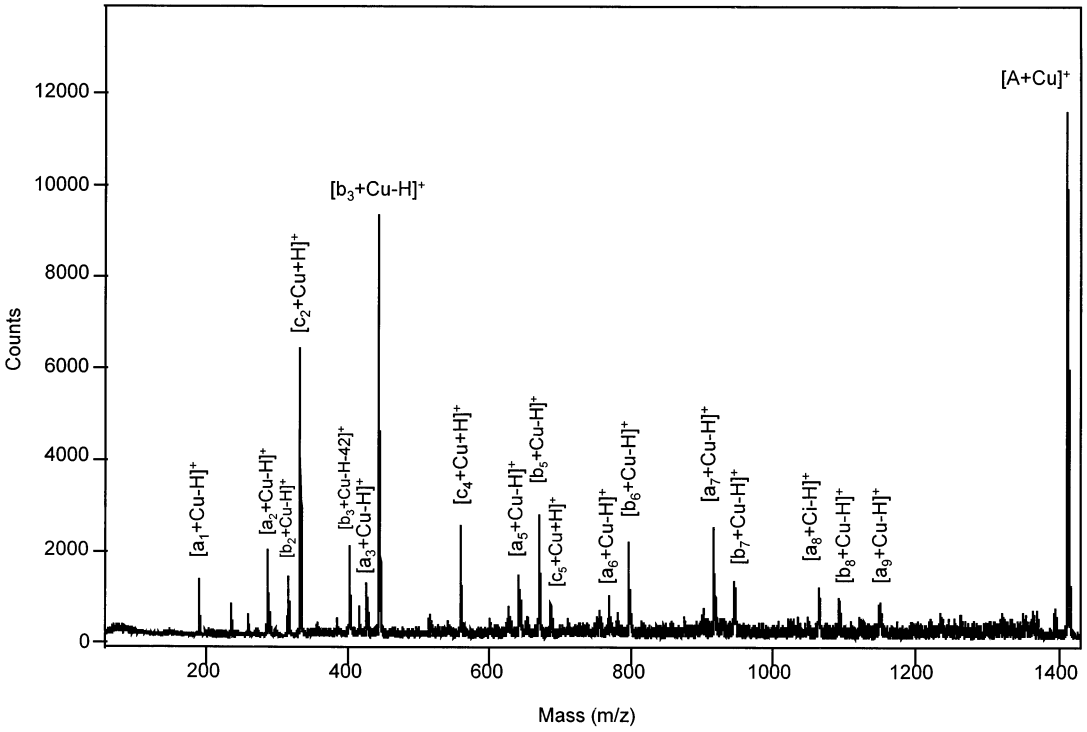

Fig. 5. MI spectrum of $[A + Cu]^+$ ions of substance P–NH_2.

Figs. 4 and 5, respectively, and have several similarities. First, ions at m/z 63/65 are not observed in either spectrum indicating that the Cu^+ ion is tightly bound to the peptide and does not detach under metastable ion dissociation conditions. Second, all metastable ions in both spectra contain the N-terminal arginine and the only immonium ion present is that of arginine complexed to copper. An anomaly to the metastable decay of protonated peptides is the formation of *c*-type fragment ions; however, these ions are observed in the MI spectra of both peptides complexed to Cu^+ and are associated with residues containing an NH_2 functional group on the side chain. The *c*-type ions are formed by cleavage of the HN–CH(R) bond where R corresponds to the side chain of lysine in both peptides and glutamine in the case of substance P. Since this *c*-type bond cleavage is only observed when there are NH_2 functionalities in close proximity to the N-terminal arginine, it is apparent that the basic residues in both substance P and RKEVY play a major role in the chemistry and structure of the peptide–Cu^+ complex.

MALDI/TOF mass spectra and MI spectra demonstrate the propensity for Cu^+ to bind to basic residues. Using this knowledge we undertook electronic structure calculations to probe the energetics of the [basic residue + $Cu]^+$ binding energies [49,50]. Both theory and experiment suggest that guanidine should have the greatest copper affinity. For example, molecular orbital calculations at the MP2 level of theory indicate that the Cu^+ ion is most tightly bound to the imine nitrogen of methylguanidine as shown in Fig. 7. The calculated binding energy of this interaction is 82 kcal/mol. The strong Cu^+ ion binding energy is consistent with the absence of Cu^+ in the MI mass spectrum in Figs. 4(b) and 5. Preliminary results show the Cu^+ ion affinity, in decreasing order, to be Arg > His > Pro > Asn > Lys, which is in conflict with results reported by Cerda and Wesdemiotis [10], whose kinetic method affinity scale is Arg > Lys >

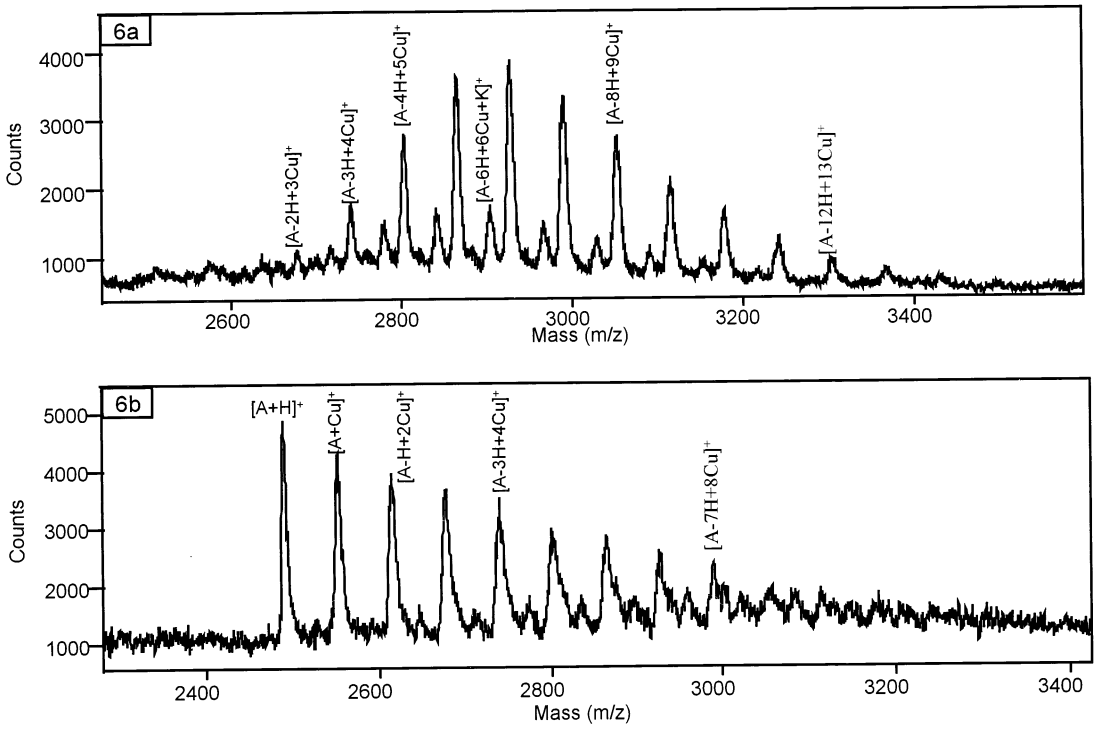

Fig. 6. Mass spectra of the (a) oligonucleotide 5′-pCTCTAGAG-3′ in ferulic acid and (b) 3-hydroxypicolinic acid desorbed from the copper sample stage.

His. Deng and Kebarle utilized ligand exchange equilibria experiments to determine the gas-phase Cu^+ ion binding energies to small organic molecules [51]. They found imidazole to have a much greater binding energy than the lysine model of $(CH_2)_4NH_2$, which supports our preliminary results. More extensive calculations are under way to evaluate this discrepancy; however, the anomaly may be due to lysine's flexibility allowing it to chelate the Cu^+ ion. That is, lysine, asparagine, and glutamine have nitrogen ligands preferred by Cu^+ and enough flexibility in the side chain to allow chelation with the N-terminal amine of that amino acid which increases the binding energy of the amino acid-Cu^+ ligand.

We also used the copper sample stage to form copper adduct ions to oligonucleotides. Typically, oligonucleotide analysis is performed in the negative ion mode. Because the negatively charged phosphate groups have to accept protons to neutralize the molecule and an additional proton to ionize the molecule, deprotonated oligonucleotides are more readily formed than are protonated oligonucleotides in the mass spectrometer. However, as evidenced by Fig. 6, positive ion analysis of oligonucleotides is easily achieved by Cu^+ adduction with the oligonucleotide when desorbed from the copper sample stage. The mass spectrum of 5′-pCTCTAGAG-3′ obtained with ferulic acid or 3-hydroxypicolinic acid illustrates this point. Note also, there are significant differences in the distribution of the copper adducts to this oligonucleotide with the different matrices. Also note that in Fig. 6(a) there are distinguishable ion series that correspond to $[A–xH + xCu + K]^+$ and to a lesser extent in Fig. 6(b). Oligonucleotides effectively bind Na^+ and K^+; however, in the gas phase it is apparent that the copper complexation dominates demonstrating a higher Cu^+ affinity for the oligonucleotide.

This technique for incorporating copper into a

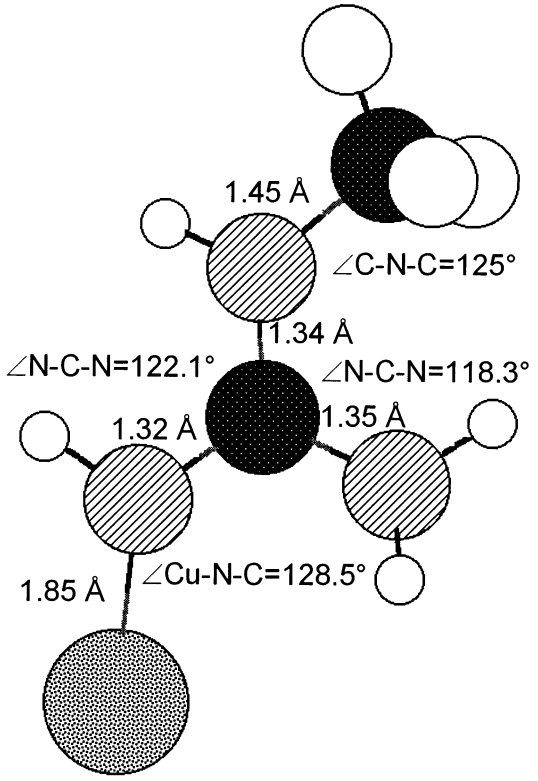

Fig. 7. The model system, methylguanidine, was used to approximate the binding energy of copper to arginine. Structures depicted were optimized using second-order Møller–Plesset perturbation theory (MP2) with inclusion of all electrons. Dunning's triple-ζ basis set was used for all nonmetal atoms with polarization functions included on C and N (C *d*: $\zeta = 0.72$, N *d*: $\zeta = 0.8$). Copper was treated with a basis set incorporating an effective core potential (ECP) developed by Ermler and Christiansen, which was of quadrupole-ζ quality in *d* space and triple-ζ quality in *s* and *p* space. Copper was found to have the greatest binding energy with imine nitrogen of 82 kcal/mol using the above theoretical models.

sample raises interesting questions regarding the fundamental desorption and ionization processes in MALDI. How does the organic sample interact with the surface or oxide layer? What is the mechanism by which the Cu^+ complexes to the analyte, as no free Cu^+ ions are observed in a mass spectrum or a MI mass spectrum? There are several possibilities: (1) $[A + Cu]^+$ and $[A–(x\text{-}1)H + xCu]^+$ could be preformed ions in the condensed media, (2) the copper is transferred to the peptide from the matrix, or (3) the peptide undergoes a gas-phase ion–molecule reaction with the Cu^+ ion. We are currently investigating these questions by probing the dynamics of multiple wavelengths for ion desorption.

5. Conclusions

In this article we demonstrate the utility of direct deposition of the matrix and analyte and desorption from a copper sample stage as a method for creating Cu^+ adducts to the matrix and analyte in MALDI. The adduction of Cu^+ to oligonucleotides aids in the formation of positive ions, which is generally difficult under normal desorption techniques.

In addition to oligonucleotides, the copper sample stage initiates Cu^+ complexation to peptides and proteins. The number of Cu^+ adducts is directly proportional to the number of basic amino acid residues found in the peptide or protein. Metastable decay of the $[A + Cu]^+$ ions of two peptides with N-terminal arginines fragment solely into N-terminal fragment ions also suggesting that the arginine anchors Cu^+. Moreover, a peptide complexed to Cu^+ with two basic residues in close proximity produces a fragment ion peculiar to metastable decay, a *c*-type fragment ion. The formation of this ion will be further examined by H/D exchange methods.

Future studies involve the mechanistic determination of the fragmentation reactions of both $[A + Cu]^+$ and $[A–2H + Cu]^-$ peptide ions. The fragmentation of $[A–2H + Cu]^-$ ions should offer complementary evidence to that of $[A + Cu]^+$ ions with respect to the binding site of Cu^+ and the gas-phase structure of the peptide–Cu^+ complex. Metastable ion decay studies of $[A–H + 2Cu]^+$ ions should reveal the location of both copper ions to determine if the basic sites are the area of complexation. This article has not investigated the effects of sulfur-containing residues because arginine and lysine dominate the gas-phase chemistry. However, in biological systems, the sulfur-containing residues, methionine and cysteine, complex to and stabilize Cu^+. The gas-phase interactions of Cu^+ with peptides containing a basic residue and a methionine or cysteine will also be investigated.

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