On-line Electrogeneration of Copper–Peptide Complexes in Microspray Mass Spectrometry

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The interaction of copper ions with peptides was investigated by electrospray mass spectrometry. Two electrospray micro-emitters were compared, the first one with a platinum electrode using a copper(II) electrolyte solution containing a peptide sample, and the second one with a sacrificial copper anode in a water/methanol solution containing only a peptide (i.e., angiotensin III, bradykinin, or Leu-enkephalin). The former yielded mainly Cu^{2+} complexes either with histidine residues or with the peptide backbone (Cu^{+} complexes can be also formed due to gas-phase reactions), whereas the latter can generate a mixture of both Cu^{+} and Cu^{2+} aqueous complexes that yield different complexation patterns. This study shows that electrospray emitters with soluble copper anodes enable the study of Cu(I)–peptide complexes in solution. (J Am Soc Mass Spectrom 2008, 19, 560–568) © 2008 American Society for Mass Spectrometry

Transition-metal ions and especially copper are essential elements in all living systems [1, 2], but can also activate pathological disorders [3–6]. Cuprous ions (Cu^{+}) are considered soft metal ions whereas cupric ions (Cu^{2+}) are intermediate. In vivo, the anchor sites for Cu^{+} include the thiol moieties of cysteines and those for Cu^{2+} include the imidazole residues of histidines [1, 7, 8]. In the gas phase, the binding sites of Cu^{2+} are quite identical [9] to those observed in solution whereas arginine becomes the preferred anchorage for Cu^{+} [10, 11]. Nevertheless, all these interactions are condition-dependent and other groups on a peptide can also be involved in the coordination of copper [12, 13]. Metal binding sites can be deduced by several techniques such as X-ray or nuclear magnetic resonance. Of course, mass spectrometry (MS) has been used for a long time to study inorganic complexes, especially since the introduction of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which are especially adapted to study bioinorganic complexes [14].

Copper complexes have been studied by different MS techniques. The traditional method is to mix a copper salt solution with the sample or by direct production of cations from metallic copper. For example, Cu^{+} ions have been generated in the gas phase by direct ionization of the metal in fast atom bombardment and in MALDI [15–17]. In aqueous solutions, Cu^{+} is unstable, and specific sample preparations are required (anaerobic conditions, adequate solvent). When copper salts are used, Cu^{+} ions are produced either by specific preparation of a copper(I) salt [18, 19] or by gas-phase reactions using a copper(II) salt. In ESI-MS studies, Cu^{+} ions are produced by reduction of Cu^{2+} during the ionization process [20–22] even in positive ionization mode. Lavanant et al. have observed the reduction of Cu^{2+} complexes by electron capture as well as by oxidation of the surrounding molecules such as methanol [22]. Gianelli et al. have reported the effect of the solvent on Cu^{2+} reduction and correlated the production of Cu^{+} with the ionization energy of alcohols [23]. Cu^{2+} reduction can be favored by increasing the nozzle-skimmer voltages or the source voltage. Schröder et al. have also observed this phenomenon by increasing the cone voltage [24].

An alternative method to produce metal ions is the direct oxidation of the metallic electrode tip [25–27]. Indeed, in positive ionization mode, the anode used to provide the spray current is the locus of an oxidation process that can generate reactive species that can in turn undergo further reactions, for example for the on-line tagging of peptides [28–33]. We have demonstrated that a sacrificial metal electrode coupled to microchip-ESI-MS can be used for the study of the metal complexes [34]. Moreover, sacrificial copper electrode leads to the formation either of Cu^{2+} or of Cu^{+} aqueous complexes depending on the ligands present in solution, as illustrated by using 8-hydroxyquinoline and bathocuproine [35].

In the present work, we investigate copper–peptide complexes formed in solution using a copper electrode. Peptides containing or not containing specific anchor sites for Cu^{2+} or Cu^{+} were sprayed to study the complexation sites. In addition, the influence of these cations on peptides fragmentation was studied.

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Experimental

Chemicals

Human angiotensin III (angIII, RVYIHPF, M = 931.1 g/mol), angiotensin I/II(1-7) (angI/II, DRVYIHP, M = 899.0 g/mol), (Sar^1) angiotensin I/II(1-7) amide (angI/II-NH_2, SarRVYIHP-NH_2, M = 854.0 g/mol), Leu-enkephalin (enke, YGGFL, M = 555.6 g/mol), were bought from Bachem (Bubendorf, Switzerland) and bradykinin (RPPGFSPFR, M = 1060.2 g/mol) from Sigma (St Louis, MO). Cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$) was from Acros Organics (Geel, Belgium) and methanol from Riedel-de-Haën (Seelze, Germany). Deionized water (18.2 MΩ·cm) was prepared using a Milli-Q system from Millipore (Bedford, MA). The peptides were used as received and diluted at a final concentration of 50 μM in 50/50 (vol/vol) MeOH/H_2O. All the solutions were prepared fresh daily.

MS Setup and Microspray Interface

Metal ion on-line complexation was carried out using a microspray interface described previously [34, 35]. In brief, it consists of a single microchannel (45 μm × 120 μm × 1 cm) polyimide microchip developed by DiagnoSwiss SA (Monthey, Switzerland) [36, 37]. A reservoir of polycarbonate (ø = 6.5 mm, h = 5 mm) was glued at the inlet of the microchannel. The sample was loaded (V = 100 μL) in the reservoir in which a metallic electrode was immersed. This electrode was made of platinum wire (blank and copper(II) salt experiments) or of copper plate (S = 30 mm^2). Both electrodes were sanded and rinsed with methanol before each experiment.

An LCQ DUO ion trap mass spectrometer (Thermo, San Jose, CA) was used in positive ion mode. The heated capillary was kept at 200 °C (except for the experiments with bradykinin). The commercial ESI interface was removed and the microchip was mounted on a plate fixed on the probe slide adapter of the mass spectrometer. After the MS power supply onset (U = 3.5 kV) the chip was moved closer to the entrance of the MS. The current was set between 30 and 80 nA by adjusting the distance between the microspray outlet and the entrance of the MS, and monitored by a nano-ammeter. The ion optics parameters were kept constant for each peptide studied. The flow rate was estimated at 300 nL/min according to Morier et al. [38]. The MS fragments were assigned based on the calculation of a web-based software, MS-Products from UCSF (http://prospector.ucsf.edu/prospector/4.0.8/html/msprod.htm).

Isotopic Distribution Calculation

The isotopes of copper are ^{63}Cu and ^{65}Cu with a natural abundance of 69.17% and 30.83%, respectively, and a mass of 62.93 and 64.93 u. Thus, a copper complex shows a typical isotopic distribution that can be altered by the different oxidation states of copper. Considering a mono-charged peptide, $^{63}Cu^+$ or $^{63}Cu^{2+}$ binding releases one or two protons, respectively, yielding a theoretical mass difference (with the native peak) of 61.9 or 60.9 Da.

With the aim to distinguish the proportion of the different oxidation states of copper in the peptide complexes, theoretical and experimental isotopic distributions were compared. Theoretical isotopes were obtained by the software CS ChemDraw Pro (CambridgeSoft Corporation, Cambridge, MA). According to the isotope of $[M + Cu^I]^+$ and $[M + Cu^{II} - H]^+$ or others, a weighted average of the relative abundance of each isotope was calculated.

$$I_{i,\mathrm{cal}} = \alpha \cdot I_{i,\mathrm{Cu(II)}} + (1 - \alpha) \cdot I_{i,\mathrm{Cu(I)}} \quad (1)$$

where $I_{i,cal}$ represents the calculated relative abundance of the isotope i, $I_{i,Cu(II)}$ the theoretical relative abundance of the isotope i where only Cu^{2+} is linked, $I_{i,Cu(I)}$ the theoretical relative abundance of the isotope i where only Cu^+ is linked, and α is a variable comprised between 0 and 1. The optimum ratio α was obtained with minimizing the difference (r) between experiments and calculations.

$$r = \sum_{i=1}^{n} (I_{\mathrm{i,cal}} - I_{i,\mathrm{exp}})^2 \quad (2)$$

where $I_{i,exp}$ stands for the relative abundance obtained by mass spectrometry. Thus, α gives the ratio Cu^{2+}/Cu^+ coordinated to a peptide. The experimental error on the relative abundance of isotopes induced by the data processing is lower than 2%. This error was calculated for ions of known isotopic composition, such as $[M + H]^+$ for angiotensin III. α-Values were averaged on several experiments for the same experimental conditions and the standard deviations were precised on the given values.

Results and Discussion

Copper–Angiotensin III Complexes

Copper(II) and copper(I) ions that can be produced in solution by a sacrificial copper electrode present a good affinity for histidine and arginine, respectively. Therefore, a histidine- and arginine-containing peptide, namely angiotensin III (RVYIHPF), has been chosen to study the relative complexation properties of the two oxidation states. Experiments were first carried out classically with a Pt electrode for reference (data not shown). The mono and doubly charged peptides were observed at m/z = 931.5 Th and at m/z = 466.3 Th, respectively, and no fragment ions could be detected. When the copper electrode is used described above, copper ions are electrogenerated by anodic dissolution of the electrode and Cu^{n+}–angIII complexes are formed as clearly shown in Figure 1a where two copper complexes can be observed. The mono-copper complex

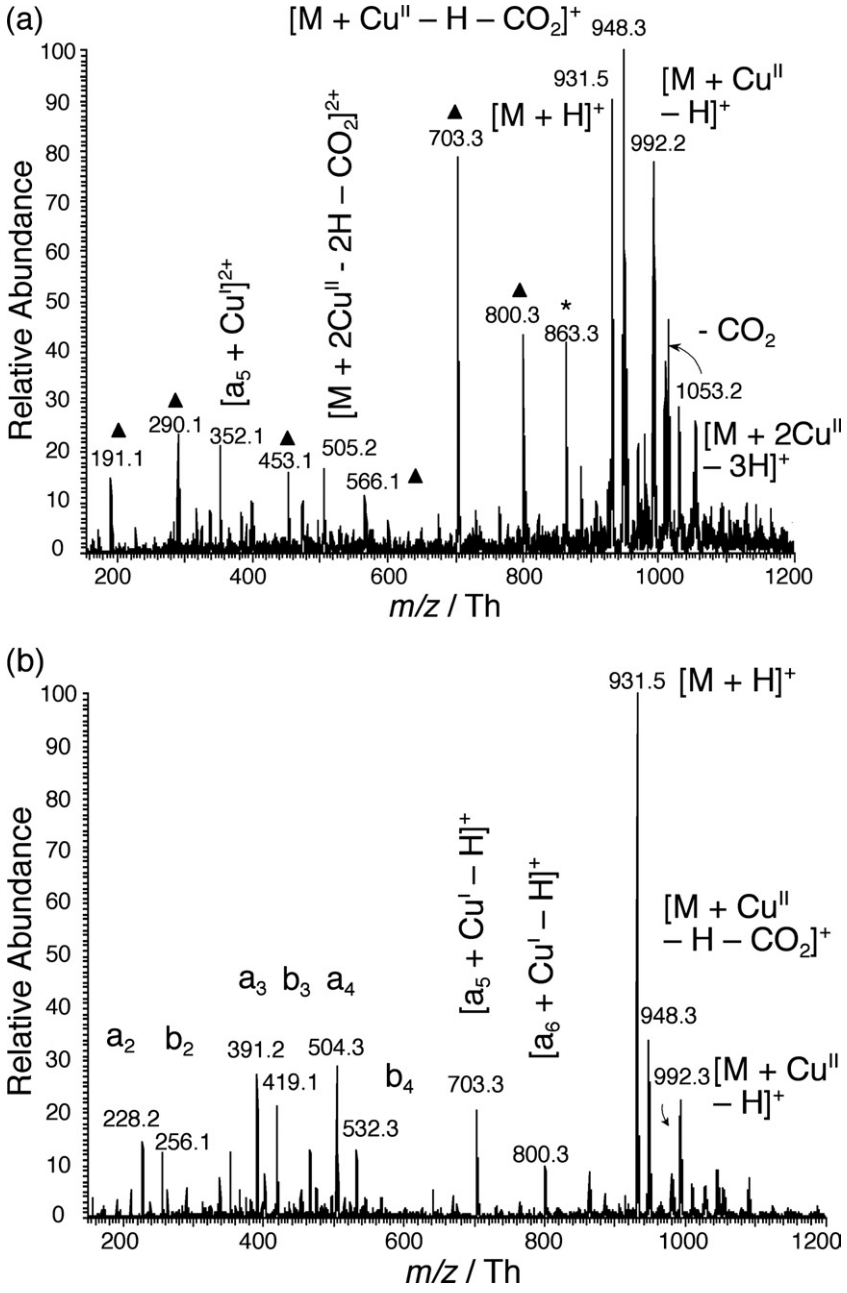


Figure 1. Mass spectra of angiotensin III (RVYIHPF, 50 μM) in 50/50 (vol/vol) MeOH/H_2O obtained with (**a**) a Cu electrode, t = 59 min, (same pattern was observed after 25 min) and (**b**) a Pt electrode in presence of $CuCl_2$ at 400 μM; (filled triangle) a_n-ions bound to Cu^+; (asterisk) x_6 bound to Cu^+. Cu^{2+} ions generated from either copper electrode or copper(II) salt induce the fragmentation of angiotensin III. Cu electrode clearly gives the access to Cu^+ complexes.

(copper:peptide, 1:1) at *m/z* = 992.2 Th, previously reported [34, 39], appeared straightaway whilst the bimetallic complex (2:1) at *m/z* = 1053.2 Th appeared after few minutes of electrolysis. Focusing on the isotopic distribution of the mono-copper complex, four peaks can mainly be observed where the relative abundances show in fact an overlap of two oxidation states. To determine the ratio between Cu^{2+} and Cu^+, the theoretical isotopic distribution has been calculated (see Table 1). In comparison with the experiment, these data clearly show that the mono-copper complexes are constituted by 76% ± 3% of Cu^{2+} under these experimental conditions. This can be due either to the binding of copper ions on different sites, (RVYIH(Cu^{2+})PF or R(Cu^+)VYIHPF), or to a mixed population of Cu^{2+}/Cu^+ on a given site, (e.g., RVYIH($Cu^{2+/+}$)PF), induced by an in situ reduction of Cu^{2+}–histidine complexes in the gas phase [20, 22–24, 35]. As the copper electrode is able to produce either Cu^{2+} or Cu^+ aqueous ions, Cu^{2+}–histidine and/or Cu^+–arginine complexes can in

Table 1. Relative abundance of the isotopes of Cu^{n+}–angiotensin III complexes

	I/%			
m/z/Th	Cu^{n+}–angIII experimental	Cu^{2+}–angIII theoretical[a]	Cu^{+}–angIII theoretical[a]	Cu^{n+}–angIII calculated[b]
992.2	100 ± 0	100	0	100 ± 4
993.2	89 ± 5	55	100	87 ± 4
994.3	76 ± 6	61	55	78 ± 4
995.3	45 ± 3	28	61	47 ± 4
996.2	20 ± 3	7	28	16 ± 4
997.2	7 ± 1	1	8	4 ± 4

[a]Obtained from the software CS ChemDraw Pro.
[b]Calculated according to eq 1 with $\alpha = 0.76 \pm 0.03$.

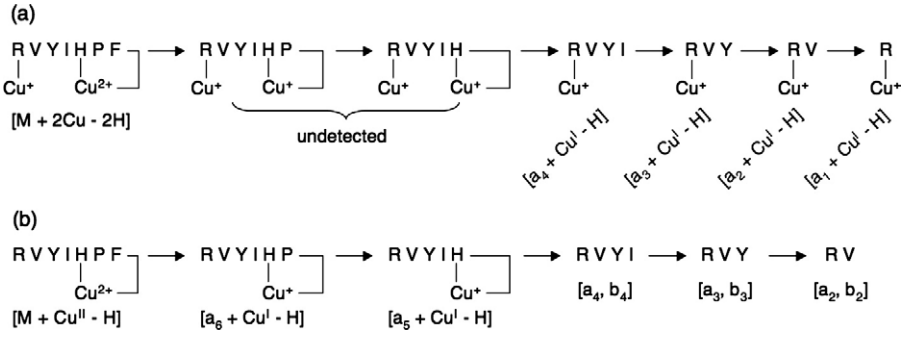
principle be concomitantly formed. The peak pattern observed for the bimetallic complex at $m/z = 1053.2$ Th also shows the presence of both Cu^{2+} and Cu^{+} species (data not shown).

In contrast, the experiments performed with a platinum electrode (with the same MS parameters) in the presence of $CuCl_2$ added to the peptide solution revealed only a mono-copper complex at $m/z = 992.3$ Th (Figure 1b) with a lower conversion rate. Moreover, the copper(II) salt concentration required (400 μM) had to be in excess compared with that of the peptide (50 μM) to achieve an equivalent result to that obtained with the copper electrode (5 μM Cu^{2+} electrogenerated in 30 min according to the Faraday's law). These results suggest a difference of reactivity between copper ions added as a salt or electrogenerated. In the first case, the counterions, namely chloride, can lead to the formation of different chloride complexes, thereby decreasing the free ion concentration whereas the electrogenerated copper ions can only be complexed by water or methanol. Then, these cations are more reactive towards angiotensin III, yielding a higher conversion rate. In addition, reactions at the surface of the electrode could also contribute to this phenomenon.

Copper–Angiotensin III Complexes Fragmentation

The binding of copper ions to angIII was found to induce its fragmentation (Figure 1). The degradation induced by electrogenerated copper ions presents all the a-type fragments linked to one copper ion (Figure 1a, Scheme **1**a). All these fragments contain an arginine residue, which is able to coordinate Cu^{+}. In comparison, $CuCl_2$ also induced a fragmentation, but only histidine-containing fragments complexed with Cu^{+} are observed (Figure 1b, Scheme **1**b). The fragments $a_{n,n<5}$ are not complexed and no copper ions were found anchored to arginine. These Cu^{+}–histidine-containing fragments must result from Cu^{2+} ions initially bound to histidine but reduced in the gas phase during the fragmentation. The fragmentation patterns (not induced by MS/MS experiments) show that the mono-copper complex discussed above is a mixture of two complexes, one with Cu^{2+} bound to histidine and one with Cu^{+} bound to arginine.

When Cu^{2+} binds to the histidine of angIII, the C-terminus is involved in the formation of a tetracoordinated complex including the oxygen of the carboxylate [7]. Figure 1a shows, for the native peak tagged, a loss of 43.9 Da corresponding to the loss of the carboxylate group of the C-terminus in which the oxidation state of Cu^{2+} is maintained, as observed by several studies [39, 40]. Indeed, focusing on the isotopic distributions of the mono-copper complex peaks and of the decarboxylated one, the calculations gave 76% ± 3% (see Table 1) and 96% ± 3% (data not shown) of Cu^{2+}, respectively. This suggests that the loss of CO_2 is induced by the presence of Cu^{2+} and not its reduction. However, the other fragments are complexed with Cu^{+} (the isotopic distribution of the a_n-ions bound to copper shows 100% of Cu^{+}). These complexed a_n-ions can be explained as follows. After the loss of CO_2, an electron-transfer reducing Cu^{2+} to Cu^{+} oxidizes the peptide and the observed complexes are coordinated with Cu^{+}. When the fragmentation reaches the histidine, copper unbinds from the peptide and stays on the departing histidine (experiments performed with angiotensin II (DRVYIHPF) showed a (a_6y_3) fragment linked to Cu^{+}, i.e., the immonium ion of histidine, corroborating this particular loss with Cu^{+} ions). In addition, the fragments $a_{n,n<5}$ (Figure 1a) are bound to Cu^{+}. Therefore, it seems that the complexed ions a_5 and a_6 come from a monometallic complex and that the $a_{n,n<5}$ fragments come from a bimetallic peptide after release of a Cu^{+}-



Scheme 1. Fragmentation of angiotensin III induced by copper ions. Experiments ran with (**a**) a Cu electrode and (**b**) a Pt electrode in presence of $CuCl_2$.

histidine complex (Scheme **1**a). This fragmentation is also coherent with the experiment run with the platinum electrode in presence of $CuCl_2$, where the histidine-free fragments are not linked to copper (Figure 1b, Scheme **1**b). In these peaks, only the histidine-containing fragments are complexed (with Cu^+) and all the fragments are present. All these experiments suggest that the initiation of the fragmentation is induced by Cu^{2+}.

To understand the influence of the C-terminus carboxylate on the fragmentation process, angiotensin I/II(1-7) and (Sar[1])angiotensin I/II(1-7) amide were sprayed with a copper electrode (Figure 2). A quick view of these spectra clearly exhibits the difference. The first peptide, DRVYIHP, enables the binding of two Cu^{2+} ions by the presence of two anchor sites, i.e., aspartic acid and histidine (Figure 2a). Consequently, these sites undergo the formation of a bimetallic complex at m/z = 1021.1 Th (the monometallic complex being at m/z = 960.2 Th). As with angIII, the decarboxylation occurs and then Cu^{2+} can oxidize the peptide. All $[a_{n,n>5} + Cu^{I} - H]^+$ fragments and immonium ions of arginine bound to copper ($[(a_2y_6) + Cu^{I} - H]^+$) are

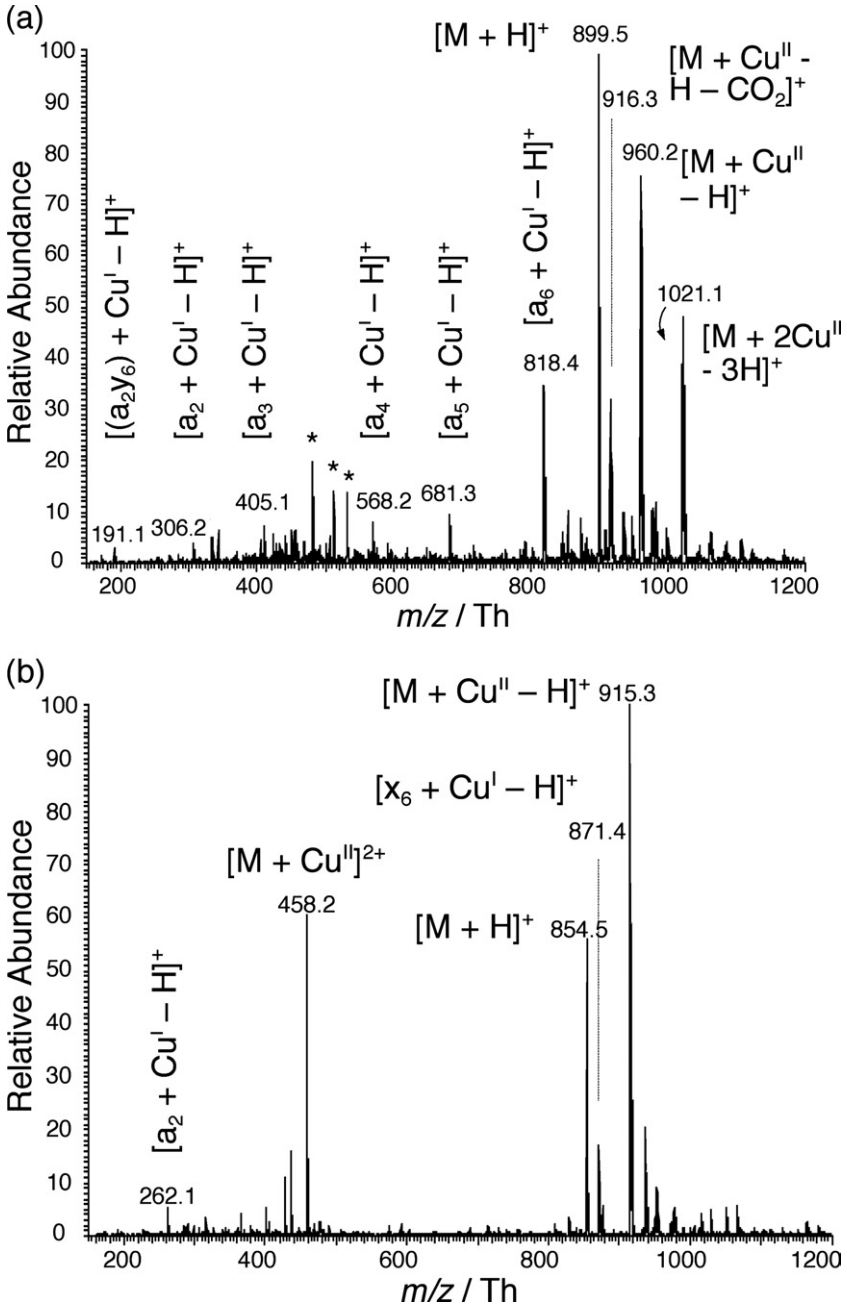


Figure 2. Mass spectra of (**a**) angiotensin I/II (DRVYIHP, 50 μM), t = 1h23, and (**b**) (Sar[1])angiotensin I/II amide (SarRVYIHP-NH_2, 50 μM), t = 1h21, in 50/50 (vol/vol) MeOH/H_2O performed with a Cu electrode; (asterisk) doubly charged species. The amidation of the C-terminus stops the fragmentation induced by copper ions.

also observed. In contrast, the peptide with the modified C-terminus (SarRVYIHP-NH_2) does not show a clear fragmentation (Figure 2b). There is only one adduct of Cu^{2+} at m/z = 915.3 Th, which loses 43.9 Da corresponding to the loss of the fragment a_1, the immonium moiety from *N*-methylglycine (Sar group).

These mass spectra confirm the influence of the C-terminus on the fragmentation pathway. The replacement of aspartic acid by *N*-methylglycine prevents the addition of a copper ion at the N-terminus but does not change the binding to the arginine. When the carboxylate group is unmodified, the histidine-containing fragment $[a_6 + Cu^I - H]^+$ is intense and many others fragments, including $[(a_2y_6) + Cu^I - H]^+$ corresponding to a copper ion linked to arginine, are observed. These results are consistent with the interpretation made for angiotensin III. When the amidated peptide is studied, only a mono-copper complex and a fragment $[a_2 + Cu^I - H]^+$ are present. No histidine-containing fragments are observed. Copper ions bind to arginine when the carboxylate group is not free and probably with the participation of nitrogen from the amide bound. This adduct being a Cu^{2+} complex, a binding between the guanidine and the imidazole residues is not excluded. The loss of 43.9 Da corresponds to the fragment a_1 and the peak at m/z = 871.4 Th is $[x_6 + Cu^I - H]^+$. The amidation of the C-terminus stops the fragmentation due to the formation of a Cu^{2+}–histidine complex, as reported by Hu and Loo [39].

Copper–Bradykinin Complexes

Histidine-free peptides were then used to test the production of Cu^+–peptide complexes from a sacrificial copper electrode. Bradykinin (RPPGFSPFR) contains two arginines able to bind theoretically two Cu^+ ions. Again, this peptide was sprayed with a copper electrode and with a platinum electrode in presence of $CuCl_2$. The most intense peak was the mono charged peptide at m/z = 1060.6 Th. With both methods, the addition of one Cu^+ ion was observed at m/z = 1122.4 Th even if the proportion of Cu^+ was less important when using a copper(II) salt (roughly 65% versus 90% of Cu^+ with the copper electrode). According to the peptide structure, the formation of Cu^+ complexes should be enhanced and the spray condition could facilitate the Cu^{2+} reduction in the gas phase. The comparison between the use of a copper electrode and a copper(II) salt was not straightforward. Decreasing the heated capillary temperature has been found in this case to enhance the formation of doubly charged peptides and also the formation of Cu^{2+} complexes, probably helped by a lower desolvation rate. Thus, the temperature of the heated capillary was fixed at 100 °C instead of 200 °C. The relative abundance of the doubly charged bradykinin (m/z = 530.9 Th) and that of mono charged at m/z = 1060.6 Th were 100% and 20%, respectively. First, bradykinin was sprayed with a copper electrode and the oxidation produced mainly a doubly charged Cu^+–bradykinin complex at m/z = 561.7 Th. However, after 15 min the proportion changed and a Cu^{2+} complex was observed to reach ~60% and 80% of Cu^{2+} after 30 and 60 min of electrospray, respectively. According to the soft ionization, the mass spectrum of bradykinin obtained with a platinum electrode in presence of $CuCl_2$ shows the formation of a Cu^{2+}–bradykinin complex at m/z = 561.1 Th. Indeed, under these conditions, this copper adduct was only formed by Cu^{2+}. Bradykinin has two anchor sites for Cu^+, via arginine [10, 11], but also can complex Cu^{2+} via the amide group of the peptide backbone or different part of the amino acids side chain [12, 13].

Copper–bradykinin complexes did not fragment like the copper-angiotensin III complexes. Collision-induced dissociation (CID) was thus required. Table 2 summarizes the fragment ions coming from tandem mass spectrometry (at 33% of collision energy) of $[M + Cu^{II}]^{2+}$ and $[M + H + Cu^I]^{2+}$. The CID spectrum patterns and several peaks are different. For the Cu^{2+} complexes, the most intense peaks are the immonium ions of arginine bound to Cu^+ (either from the N-terminus or the C-terminus after a CO_2 loss) and several fragments bound to Cu^{2+}. The presence of Cu^{2+} induces the loss of CO_2 (44 Da), of formaldehyde coming from the serine side chain (CH_2O, 30 Da) [41, 42], of water and the loss of a part of guanidine moiety from arginine (HN=C=NH, 42 Da) [41, 43]. Several a- and b-ions (a_8, a_6, a_5, b_4) are linked to Cu^+ (including the immonium ion of arginine), suggesting the N-terminus arginine is the anchor site after Cu^{2+} reduction. Intense x- and y-ions (x_8, x_7, y_7) are mainly linked to Cu^{2+}. In addition, internal fragments corresponding to PPGF and FSP or SPF are also complexed with Cu^{2+}. As a result, copper(II) seems fixed in the middle of the structure and copper(I), probably coming from Cu^{2+} reduction, is coordinated to one of the arginine. As for the CID spectrum of $[M + H + Cu^I]^{2+}$, the fragments are coordinated with Cu^+. The loss of the serine side chain, water, and part of the guanidine represents the most abundant species and the decarboxylation is not observed (phenomenon also observed with angIII where CO_2 loss was related to Cu^{2+}). Several a- and b-ions support the formation of Cu^+ complexes at the N-terminus and the immonium ion of arginine (less abundant compared with the fragmentation of $[M + Cu^{II}]^{2+}$) corroborates the binding between copper(I) and arginine. Taking into account the absence of CO_2 loss and the relative weak abundance of $[Im R + Cu^I]^+$ (27.2%) compared with the fragmentation of the Cu^{2+}-complex (100%), it seems that Cu^+ (produced at the copper electrode) is linked to the N-terminus arginine; and that $[Im R + Cu^I]^+$ produced from $CuCl_2$ stems from the C-terminus as well as the N-terminus arginine. These CID spectrum patterns emphasize the difference in Cu^{2+} and Cu^+ binding site on bradykinin. In the absence of histidine, the oxidation of the copper electrode produced copper(I) ions able to bind at the arginine. On the other hand, copper(II) ions coming

Table 2. Fragments in CID spectra of the doubly charged copper(II)– and copper(I)–bradykinin complexes

$[M + Cu^{II}]^{2+}$ (561.1 Th)[a]			$[M + H + Cu^{I}]^{2+}$ (561.7 Th)[b]		
	m/z/Th	*I*/%		*m/z*/Th	*I*/%
$[x_8 + Cu^{II} - 2H]^+$	991.3	40	$[x_8 + Cu^{II} - 2H]^{+f}$	991.1	10
$[y_8 + Cu^{I} - H]^+$ or $[b_8 + H_2O + Cu^{I} - H]^+$	966.2	15	$[b_8 - NH_3 + Cu^{I} - H]^+$	931.1	6
$[b_8 - NH_3 + Cu^{I} - H]^+$	931.3	21	$[a_8 + Cu^{I} - H]^+$	920.2	8
$[a_8 + Cu^{I} - H]^+$	920.3	33	y_8 or $b_8 + H_2O$	904.4	8
y_8 or $b_8 + H_2O$	904.3	31	$[x_7 + Cu^{II} - H_2O - 2H]^{+f}$	876.4	7
$[x_7 + Cu^{II} - H_2O - 2H]^+$	876.3	56	a_8	858.5	5
a_8	858.4	26	$[y_7 + Cu^{II} - 2H_2O - 2H]^{+f}$	832.3	5
$[y_7 + Cu^{II} - 2H_2O - 2H]^+$	832.3	18	$[b_6 - H_2O + Cu^{I} - H]^+$	686.3	5
$[a_6 + Cu^{I} - H]^+$	676.3	12	b_6	642.1	6
a_6	614.2	27	a_6	614.3	7
$[a_5 + Cu^{I} - H]^+$	589.1	53	$[a_5 + Cu^{I} - H]^+$	589.2	20
$[M + Cu^{II} - CH_2O]^{2+c}$	546.1	23	$[M + H + Cu^{I} - H_2O]^{2+}$	552.7	34
$[M + Cu^{II} - CO_2]^{2+}$	539.1	47	$[M + H + Cu^{I} - CH_2O]^{2+c}$	546.7	100
$[M + Cu^{II} - H_2O - HN{=}C{=}NH]^{2+d}$	531.1	34	$[M + H + Cu^{I} - H_2O - CH_2O]^{2+c}$	537.7	21
a_5	527.1	21	$[M + H + Cu^{I} - H_2O - HN{=}C{=}NH]^{2+d}$	531.7	11
$[M + Cu^{II} - CO_2 - CH_2O]^{2+c}$	524.1	23	$[M + H + Cu^{I} - 2H_2O - HN{=}C{=}NH]^{2+d}$	522.7	10
$[M + Cu^{II} - 2H_2O - HN{=}C{=}NH]^{2+d}$	522.1	15	$[M + H + Cu^{I} - H_2O - CH_2O - HN{=}C{=}NH]^{2+cd}$	516.7	41
$[M + Cu^{II} - H_2O - CH_2O - HN{=}C{=}NH]^{2+cd}$	516.1	24	$[y_3 + Cu^{I} - H]^+$	481.1	13
$[b_4 + Cu^{I} - H]^+$	470.0	14	$[b_4 + Cu^{I} - H]^+$	470.0	11
$[(a_5x_8) + Cu^{II} - 2H]^+$	460.0	15	$[a_4 + Cu^{I} - H]^+$	442.1	6
x_3	445.0	13	b_4	408.1	5
$[(a_7x_5) + Cu^{II} - 2H - H_2O]^+$ or $[(a_8x_4) + Cu^{II} - 2H - H_2O]^+$	375.1	9	b_2	253.9	8
$[Im\ R + Cu^{I}]^{+e}$	191.1	100	$[Im\ R + Cu^{I}]^{+e}$	191.0	27
$[Im\ R + Cu^{I} - NH_3]^{+e}$	174.0	13			

[a]Produced by the addition of $CuCl_2$.
[b]Produced by the oxidation of a Cu electrode.
[c]Loss of Ser side chain.
[d]Loss of part of guanidine moiety of Arg.
[e]Immonium ion of Arg from *C*- or N-terminus.
[f]Presence of Cu^{II} complexes probably due to the isolation window (±1 Th).

from the salt are fixed in the middle of the structure but are also able to produce Cu^+-arginine complexes after reduction of Cu^{2+}-bradykinin complexes during CID experiment. These fragments suggest a rearrangement in the copper coordination after the reduction of Cu^{2+}.

Copper–Enkephalin Complexes

Leu-enkephalin (YGGFL) was finally used as a histidine and arginine free sequence (Table 3). In the absence of coordination sites, the metallic ions will anchor to the amide groups of the peptide backbone via nitrogen [12]. The mass spectra obtained with a Pt electrode show the presence of the mono charged peptides at *m/z* = 556.2 Th, the fragments b_2, b_3, a_4, and b_4, and also the formation of noncovalent dimers [44, 45] and trimers at *m/z* = 1111.2 Th and 1665.9 Th. The use of a copper electrode instead of a platinum wire produced two copper adducts at *m/z* = 618.1 Th and *m/z* = 679.9 Th. The 1:1 complex was formed after a few seconds, whereas the 2:1 complex appeared after a few minutes. The mono-copper complex corresponds to a mixture of $[M + Cu^I]^+$ (*m/z* = 618.1 Th) and $[M + Cu^{II} - H]^+$ (*m/z* = 617.1 Th) in equivalent quantity according to the isotopic distribution. The dimer is mainly complexed with one Cu^{2+} (*m/z* = 1172.1 Th) and two Cu^+ ions (*m/z* = 1235.1 Th). As observed above, the copper ions induce some fragmentations. The losses of CO_2 and then the radical propyl and the tyrosine side chain were observed at *m/z* = 573.1 Th, *m/z* = 530.1 Th and *m/z* = 510.0 Th, respectively. These two last fragments are formed of Cu^+ and come from the reduction of Cu^{2+}, as explained by Tabet and coworkers [40, 46]. Indeed, the fragmentation of $[M + Cu^I]^+$ cannot give these fragments. Finally, MS/MS experiments (data not shown) performed on the peaks *m/z* = 618.1 Th and *m/z* = 530.0 Th confirmed the binding site of Cu^+ in the middle of the sequence, i.e., on Gly. Obviously, the coordination is achieved by the peptidic bond.

When a copper(II) salt is used a platinum electrode, the results are almost identical and the difference stems mainly from the ratio between copper ions and Leu-enkephalin. The first adduct (*m/z* = 616.9 Th) is mainly formed by Cu^{2+} (roughly 80%) and the bimetallic complex is $[M + 2Cu^I - H]^+$ at *m/z* = 680.0 Th. As for the fragments, the same pattern as before has been observed. However, few differences in the ratio must be pointed out. The isotopic distribution around *m/z* = 510.0 Th ($[M + Cu^I - H - \cdot C_7H_7O]^+$) revealed the presence of a Cu^{2+} complex at *m/z* = 511.0 Th ($[M + Cu^{II} - H - C_7H_6O]^+$, not observed with the copper electrode). This fragment corresponds to the loss of 105.9 Da. As described by Bossée et al. [47], this molecule is obtained by the loss of the tyrosine moiety without Cu^{2+} reduction. Finally, the binding site of Cu^{2+} remains the same as before (according to CID experiments) even if the fragmentation of $[a_4 + Cu^I - H]^+$ shows a copper adduct on YG structure. Furthermore, MS/MS experiments carried out on the bimetallic complex at *m/z* = 680.0 Th highlighted the binding of two Cu^+ ions on the GF part of Leu-enkephalin.

The experiment performed with the sacrificial copper electrode produced more Cu^+ ions than the one carried out with a copper(II) salt. Approximately 40% of Cu^+ were produced by the anodic dissolution of the copper electrode. The fragments (those induced by the metastable ions formed by addition of copper ions, see difference in Table 3) are most abundant when using $CuCl_2$. These fragments mainly stem from the reduction of Cu^{2+} complexes in Cu^+ and are explainable by the higher presence of mono-copper(II) complex. Indeed, the fragments $[M + Cu^I - H - CO_2 - \cdot C_3H_7]^+$ and $[M + Cu^I - H - \cdot C_7H_7O]^+$ can only come from Cu^{2+} reduction. These experiments are equivalent although the copper electrode produces more Cu^+ ions. Moreover, the

Table 3. Peaks observed when spraying Leu-enkephalin (50 μM) with different electrodes or in presence of $CuCl_2$ (50 μM)

		I/%		
	m/z/Th	Pt electrode	Cu electrode[a]	Pt electrode, $CuCl_2$
$[M + 2Cu^I - H]^+$	680	—	8	11
$[M + Cu^I]^{+b}$	618	—	49	62
$[M + Cu^{II} - H]^{+b}$	617	—	39	100
$[M + K]^+$	594	42	99	11
$[M + Na]^+$	578	78	100	30
$[M + Cu^{II} - H - CO_2]^+$	573	—	25	62
$[M + H]^+$	556	100	52	90
$[M + Cu^I - H - CO_2 - \cdot C_3H_7]^+$	530	—	24	54
$[M + Cu^I - H - \cdot C_7H_7O]^+$	510	—	9	14
$[a_4 + Cu^I - H]^+$	459	—	11	26
b_4	425	7	6	10
a_4	397	6	6	12
b_3	278	4	—	14
b_2	221	2	2	6

[a]Obtained after 30 min of experiment.
[b]*I* is influenced by the isotopic distributions (see text for details).

Cu^+ and Cu^{2+} anchor sites are the same in this sequence in absence of particular copper binding sites.

Conclusions

As reported previously for small molecules, sacrificial copper electrode leads to the formation of either copper(I) or copper(II) aqueous complexes depending on the ligands present in solution. The present work highlights the behavior of a soluble copper anode during the analysis of cysteine-free peptides in positive ionization mode. Indeed, copper cations can oxidize the thiol moiety of cysteine to form disulfide bridges. Further experiments will be performed in such direction and with more complex samples.

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