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Formation and Fragmentation of α-Amino Acids Complexed by Cu^+

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Gas phase complexes of α-amino acids with Cu^+ have been engendered in a plasma desorption ion source by bombarding a mixture of α-amino acids and cupric salts. The resulting adducts MCu^+ and the fragments that include copper were studied using a time-of-flight analyser. The main fragments result from a loss of 46 u, this fragmentation being very similar to the one observed for protonated molecules. One noticeable exception, however, is arginine and lysine which give very little loss of 46 u as protonated molecules but do give a significant amount of it when cationized by Cu^+. The study of valine-d_8 has shown that the migration accompanying the fragmentations principally involves the hydrogens linked to heteroatoms. The deuteration of exchangeable hydrogens, done on several amino acids, confirmed these results. This fact, plus evidence of an interaction between Cu^+ and the side chain of non-aliphatic α-amino acids, has led us to suggest several mechanisms. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Gas phase complexes of bare transition metal cations with simple organic molecules have been the object of considerable interest in the last decade.[1] So far, however, little work has been done on their interaction with biological molecules. Concerning simple organic molecules, many studies have been carried out on saturated or unsaturated hydrocarbons and simple organic compounds such as alcohols,[2] alkylchlorides,[3,4] amines,[5–9] ketones and derivatives,[10–17] etc. Little doubt is left now of the fact that bare transition metal cations have a peculiar and interesting reactivity in the gas phase, as they are able to activate C—H and C—C bonds.

Among all transition metals Cu^+ stands out because of its complete shell (d^{10}) electronic configuration. Although as reactive as the others, it is thought to react via a different mechanism ('dissociative attachment') than the classical insertion/β-hydrogen shift/competive ligand loss sequence.[1] Both these factors, conjugated with the fact that copper plays an essential role in many biological processes[18] such as oxidation, dioxygen transport and electron transfer, make it an interesting object of study.

One of the most probable reasons why there are few fundamental studies on metal interaction with biological molecules is that these compounds, being thermolabile, necessitate the use of desorption/ionization sources. The essential requirement is thus to be able to engender organometallic ions, i.e. to find a way to put biological molecules in the presence of transition metal cations. Several methods have been used which all give interesting and various results: laser bombardment of a deposit made on a target of the pure metal[19,20] and particle beam bombardment of a mixture of the compound with transition metal salts. The latter was done using various primary particles for the bombardment: keV-energetic ions (secondary ion mass spectrometry),[21] MeV-energetic ions (plasma desorption mass spectrometry),[22] keV-energetic atoms (fast atom bombardment mass spectrometry)[23,24] and photons in the presence of an energy-absorbing matrix (matrix-assisted laser desorption ionization mass spectrometry).[25] Recently, Lei and Amster[26] have used an FT-ICR (Fourier transform ion cyclotron resonance) cell to complex, directly in the gas phase, thermalized transition metal cations produced from the laser bombardment of a pure metal target with laser-desorbed amino acid neutral molecules. A few electrospray studies[27–33] have been carried out as well.

In the present work we have used a plasma desorption (PD) source. Ten α-amino acids were studied in mixture with copper salts. This results in the formation of adducts with Cu^+ that have a very general fragmentation pattern. These findings show the specificity of copper reactivity.

EXPERIMENTAL

Instrumentation

The PD mass spectra were recorded on the time-of-flight DEPIL-X instrument constructed at IPN (Institut

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Table 1. Relative intensities (%) of characteristic ions with various proportions of copper sulfate pentahydrate for spectra recorded in direct mode (600 s)

		Characteristic organic ions						Organometallic adducts			
		MH^+	I					MCu^+	$MCu^+ - 46$	Other	Pollution
Gly	*m/z*	*76*	*30*					*138/140*	*92/94*	*90/92*	*152*[a]
	Alone	100	53								
	1:1	—	77					59	100	58	230
	1:10	21	100					94	83	72	47
Tyr	*m/z*	*182*	*136*	*135*	*107*			*244/246*	*198/200*	*169/171*	
	Alone	100	81	15	64						
	1:1	—	50	33	83			—	100	83	170
	1:10	—	44	22	58			25	100	31	27
Glu	*m/z*	*148*	*102*	*84*	*56*			*210/212*	*164/166*		
	Alone	100	53	51	64						
	1:1	—	—	35	81			42	100		120
	1:10	74	44	78	100			90	63		90
Arg	*m/z*	*175*	—	*87*	*70*	*60*	*43*	*237/239*	*191/193*	*174/176*	
	Alone	100		25	70	35	84				
	1:1	17			100	40	43	10	93		440
	1:10	39		15	100	42	91	39	61	14	60

[a] In the right-hand column is recorded the relative height of the peak at *m/z* 151.8, which is one of the isotopic peaks of the polluting cluster ion $(CuCN)Cu^+$; its relative intensity exceeds 100% because in some cases it is the highest peak in the spectrum, but as it carries no information, we have chosen not to use it as the base peak.

de Physique Nucléaire), Orsay, France. It is fitted with a ^{252}Cf source that emits fission fragments whose energy is around 100 MeV. It has an electrostatic mirror and is designed with axial symmetry. The activity of the source is 2 μCi, the acceleration distance is 3 mm and the distance between the sample and the end of the tube is 894 mm. A voltage of 10 kV was applied on the sample. Three microchannel plate detectors are used to record the time of flight: one, close to the ^{252}Cf source, records the ion departure times and the other two detect the ion arrivals; the first is at the end of the flight tube and the second is annular and detects the ions reflected by the electrostatic mirror.

A 'direct spectrum' is obtained without the mirror. It is usually acquired over a 600 s period, which corresponds to an average of 2×10^6 correlated fission fragment impacts. The electrostatic mirror can be operated in two modes: to provide velocity focusing and achieve higher resolution or as a means for metastable ion analysis.[34] A 'high-resolution spectrum', obtained using the mirror in a two-stage mode, is usually acquired over a longer period.

Peak intensities are proportional to the number of ions reaching the detector; these ions are counted in channels related to their times of flight. The PD mass spectra are given in all tables as a function of the base peak. Some peaks such as *m/z* 45–41 and 30–27 appear in all spectra; they are therefore not included in the tables unless they are of unusual importance. These ions, which are almost always present in PD spectra, are thought to originate from direct impact of the fission fragments. This impact results in small organic fragments that are not very characteristic except in rare cases such as that of glycine, for which the peak of the immonium at *m/z* 30 is much more intense. Peaks whose intensity is less than 10% of the base peak are neglected. For species with copper, whose isotopic distribution is 69% for *m/z* 62.9 and 31% for *m/z* 64.9, intensities were obtained by summing the intensities of the two isotopic peaks.

Metastable ion studies

The analysis method for metastable ion studies (P^+ decomposing in the field-free region to A^+ and N) has been extensively described by Della-Negra and Le Beyec.[34] Briefly, it consists of first recording a 'neutral TOF spectrum'; the neutrals are detected by their impact on the detector at the end of the flight tube, the electrostatic mirror being activated. The impact of a neutral signifies that a metastable ion has fragmented in the field-free tube. Since the neutral time of flight is roughly the same as that of the parent corresponding stable ion, the metastable ions are easily identified. The fragment ions are then determined by recording their corresponding arrival on the annular detector and by calculating their mass using their typical time of flight.

The percentage of 'in-flight decomposition' is obtained by dividing a peak intensity of the 'neutral TOF spectrum' by the corresponding peak intensity in the 'direct TOF spectrum' (the mirror is not activated) recorded in the same time period (neutrals and ions are counted). The decompositions in flight depend strongly on the pressure (2×10^{-6} Torr). One must keep in mind, however, that the reactivity of metastable ions does not depend on their mode of formation but solely on their internal energy. The latter may be approached by the ion lifetimes, which in time-of-flight analysers are dependent on the spectrometer geometry. In our spectrometer a metastable ion of 200 u has a lifetime superior to 6×10^{-8} s and inferior to 9.4×10^{-6} s.

Remember also that while metastable transitions may give information on the parent ion of fragment ions, the same fragment ion may also be generated by other com-

petitive, faster fragmentations with no metastable transition component and possibly from other parent ions.

Sample preparation

The α-amino acids (Gly, Val, Phe, Tyr, Trp, His, Asp, Asn, Glu, Gln, Arg, Lys) were purchased from Aldrich Chemie and used as received without any further purification. As for the copper salts, $CuCl_2$(dihydrate) comes from Schuchardt München and all the others ($CuSO_4 \cdot 5H_2O$, $CuBr_2$, CuAc) from Aldrich Chemie. Valine-d_8 was purchased from the Cambridge Isotope Laboratories. For each α-amino acid, two solutions were prepared: one consisting of the pure α-amino acid at a concentration of 10^{-2} M (sometimes 5×10^{-3} M), which served for the reference spectra, and the other consisting of a mixture of α-amino acid (concentration of 10^{-2} M) and copper salt (for concentrations, see below). The solvent used was a 1:1 mixture of 2-propanol and ultrapure water. Thirty microlitres of these solutions were then deposited on a Mylar film by the electrospray method described by McNeal *et al.*[35] (this represents an amount of about 60 μg cm^{-2}).

For the experiments on deuteration of exchangeable hydrogens the sample was prepared as before and some deuterium oxide was introduced in the airlock where the sample is stored before its introduction in the tube of flight. This method of saturating the atmosphere of the airlock with heavy water, although it does not permit complete deuteration, was found to be the best method. This non-total exchange needed to be carefully evaluated to allow a correct interpretation of the experiments. Therefore all the data were treated by evaluating the 'noise' in the non-deuterated compound and subtracting it from the deuterated experiment. Similarly the relative proportions used for the ^{13}C and ^{65}Cu were extracted from the non-deuterated spectrum rather than calculated, to obtain the various rates of exchange with as much accuracy as possible. The main source of error here was the evaluation of the noise and the evaluation of peak areas. It was estimated by comparing different measurements and data treatments that either underestimated or overestimated the background noise. All the figures in Table 5 are therefore rounded to the nearest 10 and the possible error on calculations is ±10%.

Several proportions of the copper salts were examined as well as the influence of the anion in the salt.

Two different molar proportions—1:1 and 1:10 in copper salt versus α-amino acid—were tested on glycine, glutamic acid, arginine and tyrosine (Table 1). In Fig. 1 the highest-intensity peaks of the spectrum of the 1:1 molar proportion sample are due mainly to copper and to cluster ions of the type $(CuA)Cu^+$ (A is an anion such as OH^-, CN^-, Cl^-). These ions are also mentioned by Grade and Cooks in their SIMS study.[21] $(AgBr)Ag^+$ ions have also been observed in FAB[36] using AgBr as reagent salt. They must undoubtedly be due to the desorption/ionization source. With a 1:10 molar ratio the relative intensity of these peaks is much lower, while the organometallic adducts still appear quite clearly. All the ions originating from the α-amino acid glycine are the same in both cases.

Various cupric salts were tested with glycine using a proportion of 10 mol of α-amino acid to 1 mol of salt

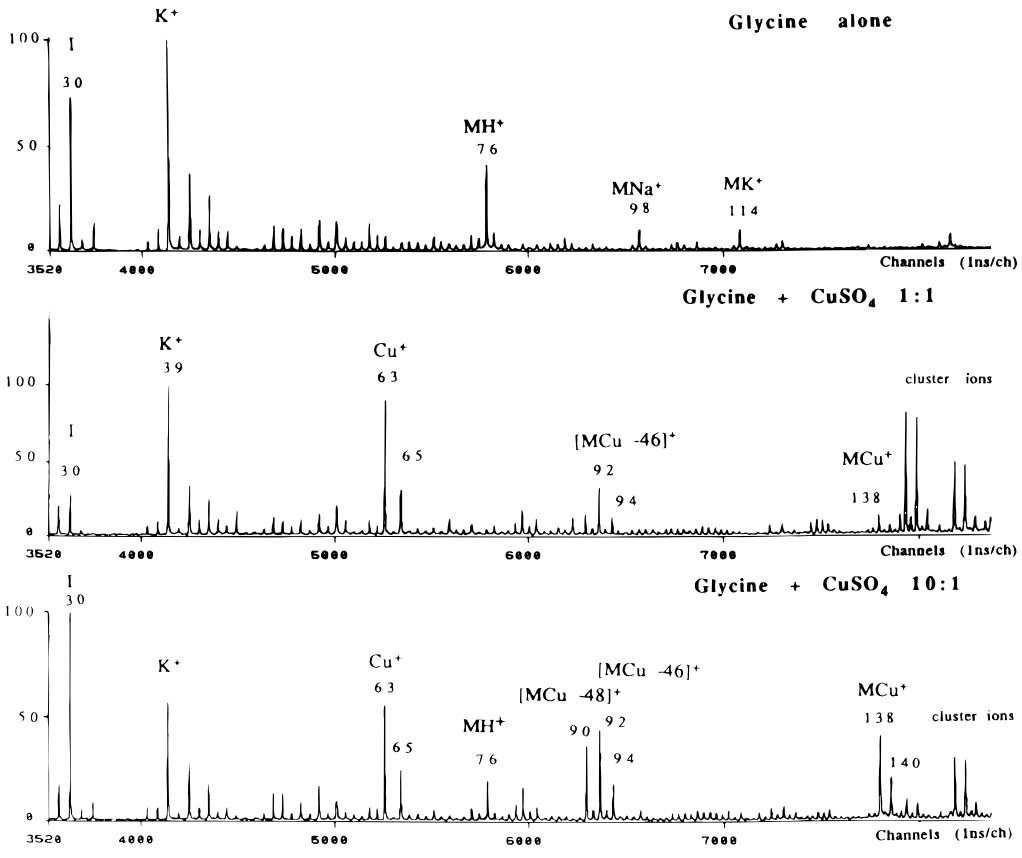


Figure 1. Direct spectra of glycine with different proportions of copper sulfate.

Table 2. Relative intensities (%) of main peaks of spectra of glycine in mixtures (10:1 molar ratio) with various copper salts

	MH^+	I	MCu^+	$MCu^+ - 46$	$MCu^+ - 48$	Cu^+	Cluster ion[a]
m/z	*76*	*30*	*138/140*	*92/94*	*90/92*	*63/65*	*Peak 152*
$CuCl_2$	36	100	77	53	46	6	27
$CuBr_2$	54	100	65	48	45	53	20
$CuSO_4$	—	100	94	93	75	150[a]	76
CuAc	53	100	2	9	7	33	10

[a] Here Cu^+ is actually the highest peak in the mass spectrum, but as it carries no information, we have chosen not to use it as the base peak.

(Table 2). We have chosen cupric salts because they are reasonably soluble in aqueous solutions. The appearance of the spectrum virtually does not change with the salt (Fig. 2). On the other hand, the relative intensities of the peaks due to a protonation and those due to an interaction with copper vary. Apparently the best salts regarding organometallic reactivity are $CuCl_2$ and $CuSO_4$. In the following experiments we have mostly used copper sulfate.

As a conclusion to these preliminary studies, we can say that this simple method of adding cupric salts to our sample is surprisingly efficient for the formation of organometallic adducts.

It is remarkable, however, that we exclusively observed copper in its cuprous oxidation state [Cu(I)], whereas we have only used cupric salts [Cu(II)]. For instance, in the cluster ions $(CuA)Cu^+$ the oxidation state of the metal is Cu(I). In our spectra the copper/α-amino acid complexes we eventually observed were either MCu^+ or $[M - H)Cu_2]^+$. Such adducts, in which the metal has obviously been reduced, have been observed before when metal salts are added to organic compounds in desorption/ionization sources.[21,37] A similar reduction has also been observed in studies on inorganic complexes.[38] Where does this reduction happen? Let us examine the phenomena that occur in solution, in the plasma and in the gas phase.

It is well known that peptides form various complexes with Cu^{2+} in solution.[39] Moreover, three different copper–glycine complexes are said to be formed in various proportions depending on the pH of the solution: $[(H_3N^+CH_2CO_2^-)Cu^{2+}]$, $[(H_2NCH_2CO_2^-)Cu^{2+}]$ and $[(H_2NCH_2CO_2^-)_2Cu^{2+}]$.[40] On the other hand, Cu^+_{aq} is quite unstable and tends to disproportionate. In addition, if a reduction took place in the presence of isopropanol, traces of acetone would then be detected,

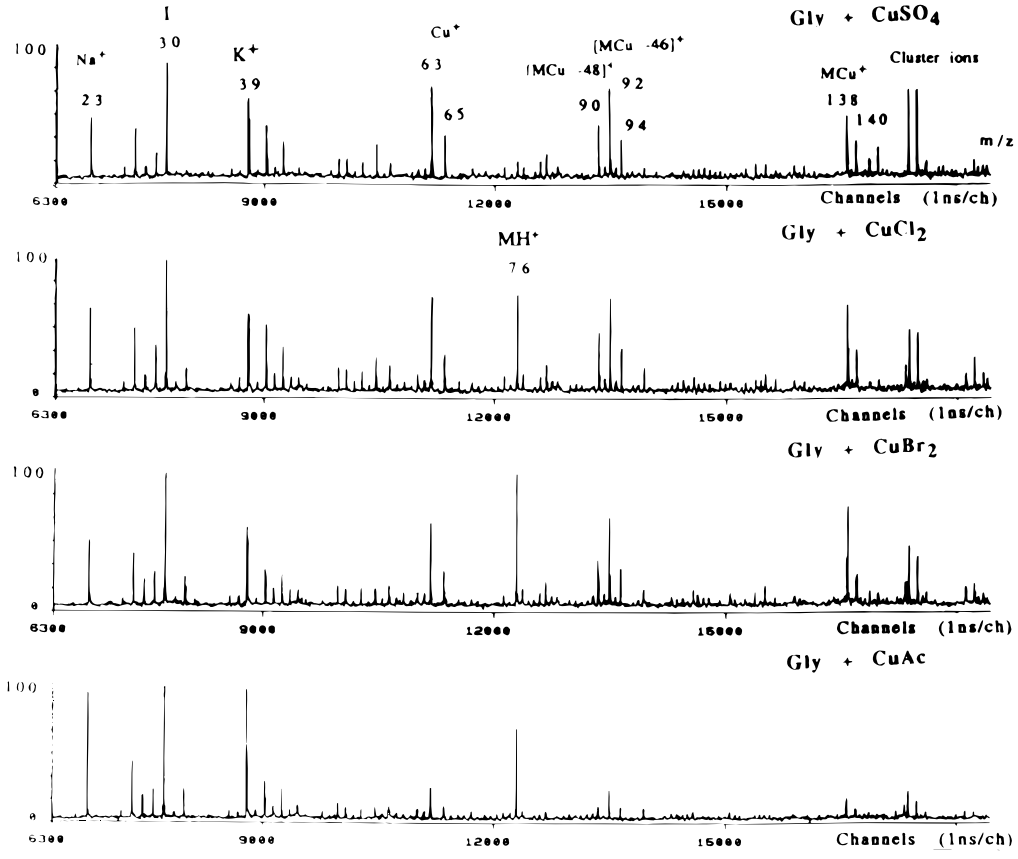


Figure 2. 'High-resolution' spectra of glycine in mixtures (10:1 molar ratio) with different cupric salts.

whereas in fact the classical tests with 2,4-dinitrophenylhydrazine were all negative. Furthermore, changing the solvent from water/isopropanol to water/acetone, although less convenient for the deposit, did not change the spectra. It is thus unlikely that the reduction should take place in solution.

Classical models of desorption[41] usually distinguish two regions in which chemical reactions can occur: the 'selvedge' and the free vacuum. The selvedge, in which neutral or ionic species are present for a very short time, is the region where fast ion/molecule or electron ionization is thought to occur. With such a model in mind it seems logical that the reduction observed should rather take place in this region, all the more so as in our case the 'selvedge' corresponds to a superficial part of the plasma engendered by the impact of a fission fragment and it has been proved[42] that there are considerable amounts of free electrons with energies ranging from a few eV to several tens of eV. The reduction could therefore take place by the simple capture of an electron by one of the complexes that were formed in solution and deposited as such on our target.

$$[(H_3N^+CH_2CO_2^-)Cu^{2+}] + e^- \rightarrow [(H_3N^+CH_2CO_2^-)Cu^+]$$

$$[(H_2NCH_2CO_2^-)Cu^{2+}] + e^- \rightarrow [(H_2NCH_2CO_2^-)Cu^+]$$

$$[(H_2NCH_2CO_2^-)_2Cu^{2+}] + e^- \rightarrow [(H_2NCH_2CO_2^-)_2Cu^+]$$

Protonated molecules are also thought to be formed in this region from fast bimolecular reactions.[43,44] A similar model could also be used to explain the formation of $[(M - H)Cu_2]^+$ and MCu^+.

$$[(H_2NCH_2CO_2^-)Cu^+] + [(H_2NCH_2CO_2^-)Cu^+] \rightarrow [(H_2NCH_2CO_2)Cu_2]^+ + [H_2NCH_2CO_2]^-$$

$$[(H_2NCH_2CO_2^-)Cu^+] + [H_3N^+CH_2CO_2^-] \rightarrow [(H_2NCH_2CO_2H)Cu]^+ + [H_2NCH_2CO_2]^-$$

Among the resulting compounds of the α-amino acid are zwitterions. Theoretical calculations[45] have shown that in the gas phase the complex with the neutral form of the α-amino acid is much more stable than the complex with the zwitterion. A rearrangement is therefore possible.

$$[(H_3N^+CH_2CO_2^-)Cu^{2+}] + e^- \rightarrow [(H_3N^+CH_2CO_2^-)Cu^+] \rightarrow [(H_2NCH_2CO_2H)Cu^+]$$

RESULTS AND DISCUSSION

Our long-term objective in this work is to study the influence of copper on the fragmentation of peptides to see if it is possible to increase the amount of structural information available in a mass spectrum.

As a starting point, in addition to two neutral α-amino acids (Gly, Val), three different families of α-amino acids were studied: the aromatic α-amino acids (Phe, Tyr, Trp, His), the ones with a carboxylic acid or amide function on their side chain (Asp, Asn, Glu, Gln) and the basic α-amino acids (Lys, Arg). All these compounds have been shown by Cerda and Wesdemiotis[46] to have very high affinities for Cu(I) and in fact all form significant amounts of the adduct MCu^+. Experimental results (Tables 1 and 3) have shown us that the addition of copper brings out new ions while suppressing none of the information already available in the spectra without copper. In fact, two types of structurally informative ions are observed: regular organic ions and ions with copper. Consequently, some of the organic ions may very well be formed from the fragmentation of the copper adduct MCu^+. This hypothesis is supported by the fact that the quantity of MH^+ tends to diminish when cupric salts are added, while the main organic fragments remain in large quantity. It is, however, very difficult to evaluate the real amount of organic fragment ions (in particular of immonium ion I) actually coming from MH^+ or MCu^+ respectively. The peak of the protonated molecule completely disappears in conditions where the overall information in the spectrum is overflowed by the 'noise' created by the cluster ions, while in 'milder' conditions (1:10 molar ratio) its relative intensity is still quite important. Furthermore, metastable ion studies of MCu^+ exclusively lead to fragments with copper. The reactions leading to organic fragment ions, probably too fast, could not be observed. We have therefore chosen to discuss here only the structure and formation of the fragment ions that include copper.

Among these the most important one is the ion at a mass-to-charge ratio corresponding to that of $[MCu - 46]^+$. It is present in all the α-amino acids tested, without any exception (Table 3). This ion has been observed before[22] and, as discussed by Wen *et al.*,[24] may correspond either to the loss of formic acid, HCOOH, or to the consecutive loss of H_2 and CO_2 or H_2O and CO. In our metastable ion studies this ion is formed directly from the adduct MCu^+. No intermediary ion such as $[MCu - H_2O]^+$ or $[MCu - CO]^+$ was observed. This loss of 46 u is quite parallel to the one observed for the protonated α-amino acids. In fact, protonated α-amino acids also typically lose 46 u to form the characteristic immonium ion I ($H_2N - HC^+ - R$).[47,48] This particularly stable ion I is used in protein sequencing to point out the presence of α-amino acids in the sequence and is therefore particularly valuable for structural analyses. The ion $[MCu - 46]^+$ will thus often be referred to as $[I - H + Cu]$.

For arginine and lysine, however, the appearance of ions at mass $[MCu - 46]^+$ is new. For these two basic α-amino acids the spectrum of the protonated molecule is quite complicated: there is no (or very little) immonium ion and the fragmentations result from multiple rearrangements and give several ions of a lower mass range (m/z 70, 60 and 43 for arginine and m/z 56 and 30 for lysine).[47,48] The formation of an ion $[I - H + Cu]$ is thus doubly remarkable. First, it shows that the addition of Cu^+ engenders a fragmentation pattern even more general than that engendered by the addition of a proton. Second, it points out a way to bypass the strong basicities of these α-amino acids which prevents the formation of the corresponding immonium ions.

Table 3. Direct spectra [relative intensities (%)] of 12 α-amino acids studied, alone and with a 1:10 molar proportion of α-amino acid versus copper sulfate pentahydrate. Only the most characteristic peaks are shown

		Characteristic organic ions						Organometallic adducts		
		MH^+	I					MCu^+	$MCu^+ - 46$	Other
Gly	*m/z*	*76*	*30*					*138/140*	*92/94*	*90/92*
	Alone	100	53							
	1:10	21	100					94	83	72
Val	*m/z*	*118*	*72*					*180/182*	*134/136*	
	Alone	57	100							
	1:10	49	100					11	12	
Phe	*m/z*	*166*	*120*	*91*				*228/230*	*182/184*	
	Alone	55	100	32						
	1:10	20	81	57				52	100	
Tyr	*m/z*	*182*	*136*	*107*				*244/246*	*198/200*	*169/171*
	Alone	100	81	64						
	1:10	—	44	58				25	100	31
Trp	*m/z*	*205*	*159*	*130*				*267/269*	*221/223*	*192/193*
	Alone	28	26	100						
	1:10	19	19	100				21	24	10
His	*m/z*	*156*	*110*	*81*	*82*	*83*		*218/220*	*172/174*	
	Alone	100	77	14	35	19				
	1:10	100	86	17	42	22		38	44	
Asp	*m/z*	*134*	*88*	*74*	*70*			*196/198*	*150/152*	
	Alone	100	56	13	60					
	1:10	78	57	64	63			100	60	
Asn	*m/z*	*133*	*87*	*74*	*70*			*195/197*	*149/151*	
	Alone	100	55	55	49					
	1:10	47	37	43	31			100	59	
Glu	*m/z*	*148*	*102*	*84*	*56*			*210/212*	*164/166*	
	Alone	100	53	51	64					
	1:10	74	44	78	100			90	63	
Gln	*m/z*	*147*	*101*	*84*	*56*			*209/211*	*163/165*	
	Alone	100	27	51	39					
	1:10	50	18	51	43			100	88	
Lys	*m/z*	*147*	*101*	*84*	*56*			*209/211*	*163/165*	*161/163*
	Alone	51	—	100	97					
	1:10	19		67	88			73	100	32
Arg	*m/z*	*175*	*129*	*87*	*70*	*60*	*43*	*237/239*	*191/193*	*174/176*
	Alone	100	—	25	70	35	84			
	1:10	39		15	100	42	91	39	61	14

In order to have more information on the mechanism of this fragmentation, we studied the fragmentations of several deuterated compounds. First we chose valine-d_8, which is an α-amino acid whose every aliphatic hydrogen has been exchanged with a deuterium. This choice was made principally for the continuation of previous studies.[22] We had investigated before the fragmentation of the adduct of valine-d_8 with cobalt, for which the mechanism suggested was the classical insertion/competitive β-hydrogen shift/ligand loss sequence. If this mechanism holds for copper, then two different immonium ions at masses $[MCu - 46]^+$ and $MCu - 47]^+$ should appear (Fig. 3). Results are summarized in Table 4. The rate of exchange being about 98%, the peak of the copper adduct is preceded by another peak at the mass $MCu^+ - 1$ which represents the contribution of valine-d_7, which is about 16% (two times eight) of the total population, i.e. 20% in relative intensity. The same phenomenon exists for every ion with eight deuteriums. The peak corresponding to the loss of 46 u is thus also accompanied by another at the mass MCu − 47 which could be attributed either to the loss of 47 u from valine-d_8 or to the loss of 46 u from the fraction of valine-d_7. Its relative intensity, however, barely approaches 23%, i.e. about the same as the natural amount of valine-d_7 (Fig. 4). This shows that the loss of 46 u is dominant: practically no hydrogen from the aliphatic side chain is involved in the fragmentation. This result was further confirmed by metastable ion studies.

The consequence of this finding is that if one refers to the mechanism in Fig. 3, then path B, in which the β-hydrogen migration involves a hydrogen from the aliphatic chain, probably does not take place. The fact that only the product with structure A was observed suggests either that this β-hydrogen migration strongly

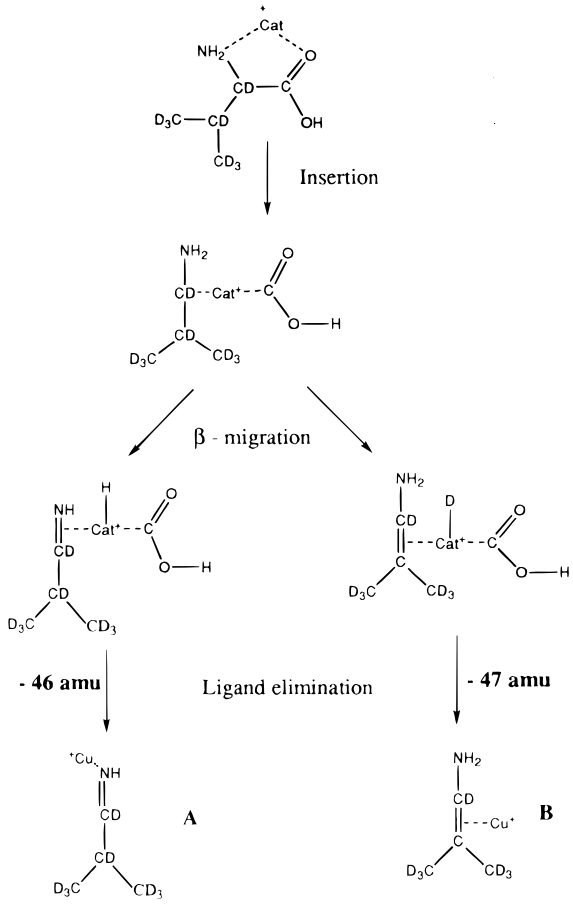


Figure 3. Classical insertion/competitive β-hydrogen shift/ligand loss sequence applied to valine-d_8 cationized with Cu^+, with insertion of metal cation into covalent C—CO bond.

favors exchangeable hydrogens or that the formation of [I − H + Cu] results from a totally different process that would only involve exchangeable hydrogens.

In order to further investigate the role of exchangeable hydrogens, we selected one α-amino acid in each group (valine, glutamine, histidine and arginine) and put it in an atmosphere of deuterium oxide in order to exchange acidic hydrogens and see how the fragments would shift. Owing to the fast process of back exchange, complete deuteration was not possible and therefore all interpretation must be done on distributions of differently deuterated compounds. For example, glutamine appeared with a mass shift of from 1 to 7 u, in agreement with five hydrogens exchanged and the two isotopes of copper (Fig. 5). With a completely deuterated product we would expect to see an [I − D + Cu] ion with the structure shown in Fig. 5, in which the three hydrogens on the nitrogens have been exchanged with deuteriums. The fact that a large part of the ions were not completely deuterated and the presence of the ^{65}Cu isotope make the distribution appear wider and distorted. Our first observation, however, was that for glutamine (Fig. 5) and the other three α-amino acids chosen, the distribution of the [I − H + Cu] ion was narrower than the one of MCu^+. Since two exchangeable hydrogens have been eliminated, the fragment ions were expected with a maximum shift of 1 u for valine, 2 u for histidine, 3 u for glutamine and 5 u for arginine; each distribution should be two units narrower, which is what was observed (Table 5). We then proceeded to check this by calculating the theoretical relative intensities, treating every exchangeable hydrogen statistically. The observed intensities for the [I − H + Cu] isotopic

Table 4. Relative intensities (%) of characteristic peaks of direct spectra of valine-d_8

	MH^+ valine-d_8	MH^+ valine-d_7	I-d_8	I-d_7	MCu^+ valine-d_8	MCu^+ valine-d_7	$MCu^+ - 46$	$MCu^+ - 47$
m/z	*126*	*125*	*80*	*79*	*188*	*187*	*142*	*141*
α-amino acid alone	45	10 (18%)[a]	100	26				
α-amino acid with 10% $CuSO_4$	41	10 (23%)[a]	100	28	18	4 (22%)[a]	18	4 (23%)[a]

[a] Relative intensity compared with the corresponding valine-d_8 ion.

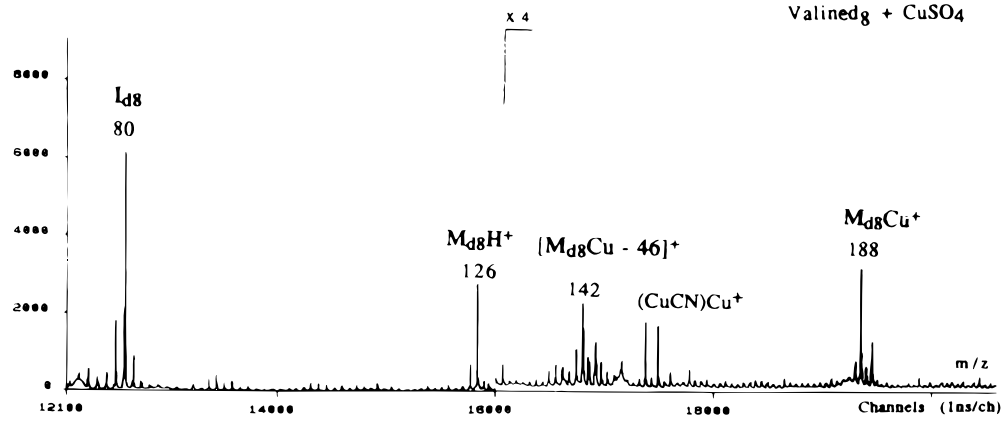


Figure 4. High-resolution spectrum of valine-d_8 with 10% copper sulfate (in molar ratio)

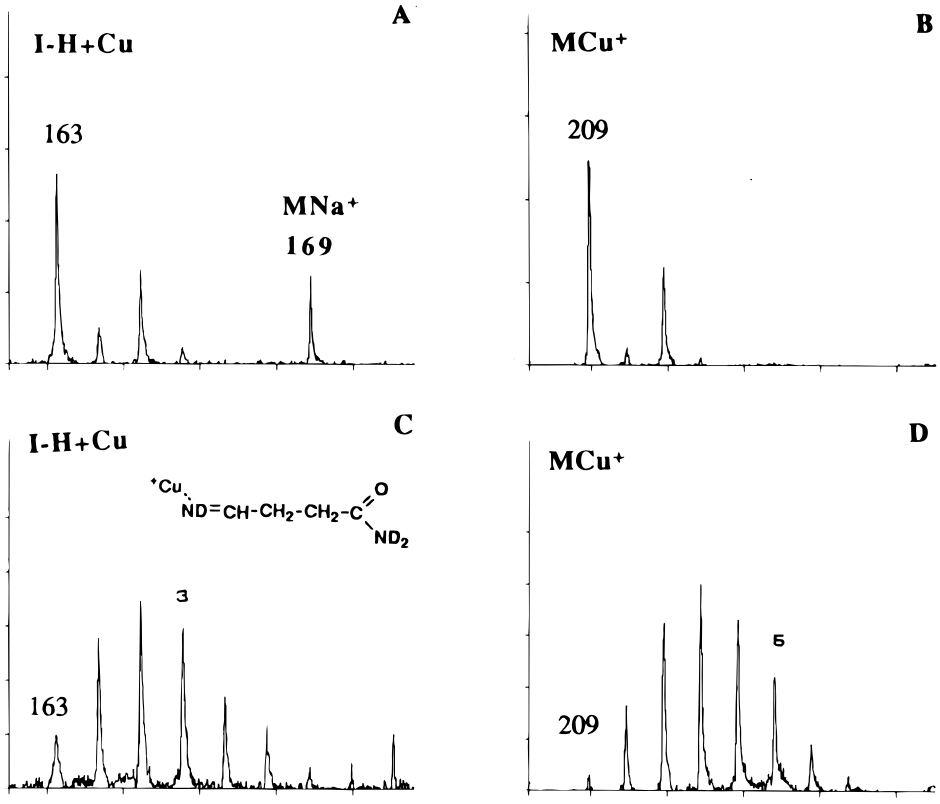


Figure 5. Spectra of glutamine in a 5:1 molar mixture with $CuSO_4$: A, [I − H + Cu] ion of non-deuterated glutamine; B, MCu^+ ion of non-deuterated glutamine; C, [I − H + Cu] ion of deuterated glutamine; D, MCu^+ ion of deuterated glutamine.

cluster are, within the error margin, in agreement with our calculated values. For valine the result found using valine-d_8 is confirmed. If any ion with structure B of Fig. 3 was present, it could only be a very minor product. It is therefore confirmed that the fragmentation process largely involves exchangeable hydrogens.

In order to get more information and possibly distinguish between the three different fragmentations that

Table 5. Relative intensities (%) of [I − H + Cu] and MCu^+ isotopic clusters for deuteration experiments on valine, histidine, glutamine and arginine

		MCu^+								[I − H + Cu]						
		0	1	2	3	4	5	6	7	0	1	2	3	4	5	6
Valine ($n = 3$)	*m/z*	*180*	*181*	*182*	*183*	*184*	*185*			*134*	*135*	*136*	*137*			
Relative intensity		100	30	70	40	20	10			100	50	40	20			
Intensity of ^{63}Cu isotope		100	30	20	20	—	—									
Calculated intensity[b]										100	30	50	20			
Histidine ($n = 4$)	*m/z*	*218*	*219*	*220*	*221*	*222*	*223*			*172*	*173*	*174*	*175*	*176*		
Relativity intensity[c]		20	70	100	60	40	20			60	100	70	50	30		
Intensity of ^{63}Cu isotope[a]		20	70	80	10	—	—									
Calculated intensity[b]										60	100	70	50	10		
Glutamine ($n = 5$)	*m/z*	*209*	*210*	*211*	*212*	*213*	*214*	*215*		*163*	*164*	*165*	*166*	*167*	*168*	
Relative intensity		10	30	80	100	80	60	20		20	80	100	80	40	20	
Intensity of ^{63}Cu isotope[a]		10	30	70	70	30	10	—								
Calculated intensity[b]										30	80	100	70	40	10	
Arginine ($n = 7$)	*m/z*	*237*	*238*	*239*	*240*	*241*	*242*	*243*	*244*	*191*	*192*	*193*	*194*	*195*	*196*	*197*
Relative intensity[c]		—	30	70	90	100	70	40	15	—	70	80	100	70	40	20
Intensity of ^{63}Cu isotope[a]			30	60	60	50	10									
Calculated intensity[b]										10	60 100	90	70	30	—	

[a] These are the proportions of differently deuterated precursor ions with which we have calculated the intensities of [I − H + Cu].
[b] Intensities were calculated treating each exchanged deuterium statistically: the loss of H_2CO, HDCO and D_2CO occurs in proportion to the number of deuteriums compared with the total number of exchangeable hydrogens (n) without considering any preferences as to the position of deuteration. The isotopic distribution was then reconstructed using the proportions for ^{13}C and ^{65}Cu that were measured from the spectrum of the non-deuterated sample.
[c] The relative proportions measured for $A + 1$ (20% of the monoisotopic peak) make the distribution look one peak larger.

Table 6. Rate (expressed as a percentage) of 'in-flight' decomposition of MH^+ and MCu^+ into I and [I − H + Cu] respectively for amino acids for which loss of 46 u is only metastable decomposition

	Gly	Phe	Tyr	His	Asp	Asn	Glu	Gln
$MH^+ \rightarrow I$	10%	31%	16%	40%	12%	15%	19%	21%
$MCu^+ \rightarrow [I - H + Cu]$	12%	28%	16%	40%	18%	20%	15%	14%

can account for the loss of 46 u, we have compared the metastable fragmentations of MH^+ and MCu^+. For protonated α-amino acids, several arguments[49–51] point toward a mechanism in which the loss of 46 u should result from a consecutive loss of H_2O and CO. The observation, in low abundance, of $[MCu - H_2O]^+$ in the gas phase study of Lei and Amster[26] suggested a similar process for cationized α-amino acids. In our metastable ion analysis the ions I and [I − H + Cu] have been observed to result from the in-flight fragmentation of MH^+ and MCu^+ respectively. It is even most of the time the only metastable fragmentation observed for these parent ions. The rate of 'in-flight' decomposition (Table 6), which represents the proportion of parent ions which decompose through this process, was found to be about the same for MH^+ and MCu^+. These ions being close in mass-to-charge ratio, the calculated lifetime of their metastable components, which is given by the time during which they 'fly' in the field-free region, is fairly similar: MH^+ must exist for longer than 5×10^{-8} s and less than 7.85×10^{-6} s; the corresponding limits for MCu^+ are 6×10^{-8} and 9.36×10^{-6} s. The fact that the same proportions of these metastable ions fragment indicates that the kinetic rates of both fragmentation reactions should be similar. This similarity between protonated compounds and organometallic adducts thus confirms previous observations, strengthening the hypothesis that the loss of 46 u should result from the consecutive loss of water and carbon monoxide.

We have represented in Fig. 6 several possible mechanisms for the formation of [I − H + Cu]. The initial structure of the cationized molecule, proposed in Fig. 3 and 6, is the metal cation in interaction with the nitrogen atom and the oxygen atom of the carbonyl group. In the case of glycine, among six different structures, this structure was *ab initio* calculated to be the most stable one.[45] Then the rearrangement of the acidic hydrogen may occur after the insertion of the metal in the CH—COOH bond (a), as usually suggested, or in other covalent bonds such as CO—OH (b) or N—H (c). It may also very well happen through an electrostatic complex (d). After transfer of an acidic hydrogen the molecular ion may rearrange into an intermediate form, which is the metal cation in interaction with three ligands (a molecule of water, a carbon monoxide and an imine). This intermediate has the advantage of being an interesting precursor for the losses of water and carbon monoxide leading to the formation of the [I − H + Cu] ion. Theoretical calculations on these different hypotheses have been undertaken.

The participation of exchangeable or acidic hydrogens, as well as the similarity between protonated compounds MH^+ and adducts with copper MCu^+, was

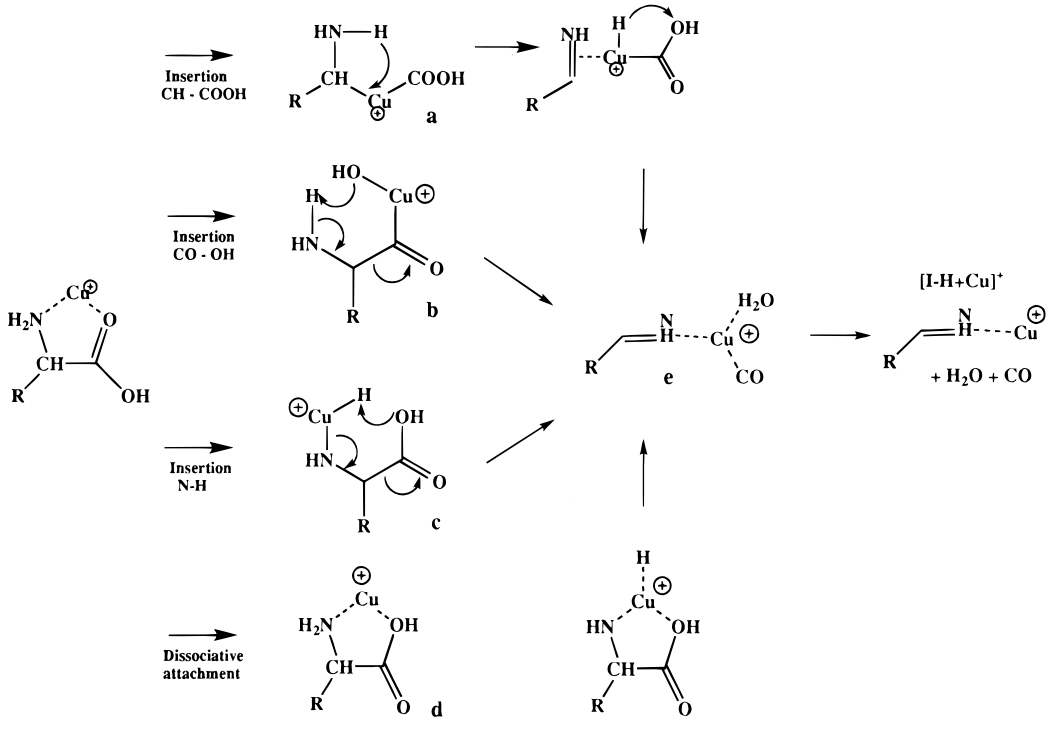


Figure 6. Mechanisms proposed for loss of 46 u from MCu^+.

further confirmed by another interesting family of fragment ions: the $[R - H + Cu]^+$ formed from the aromatic α-amino acids except phenylalanine (Table 7). These ions result from a loss of 75 u from the adduct MCu^+ and correspond to the aromatic side chain R minus a proton and complexed by Cu^+. Under energetic particle bombardment the aromatic α-amino acids alone are known[47] to give these very stable benzylic ions which also appear in electron impact ionization.[52] They are thought to result from fast processes (no metastable transition is observed) involving protonation on the aromatic ring followed by a migration of the protonating hydrogen leading to the loss of $H_2N{-}CH_2{-}COOH$. The addition of copper seemed again to engender a reactivity similar to that issuing from protonation. It is particularly true for histidine, for which the side chain appears at *m/z* 81 (R^+), *m/z* 82 ($RH^{\cdot +}$), *m/z* 83 (RH_2^+) and is found with copper at masses 143, 144, 145, 146, 147. This $[R - H + Cu]^+$ ion is however, completely absent in the spectrum of phenylalanine. This implies that its presence is not linked to a complexation of Cu^+ on the aromatic ring but rather to the presence of a heteroatom on the side chain. This hypothesis has led us to the possible structures for that $[R - H + Cu]^+$ ion illustrated in Fig. 7, in which an exchangeable proton has migrated to the α-amino acid chain. Here also, no metastable transition could be associated with their formation.

In addition to showing the dominant participation of exchangeable hydrogens, the presence of these fragment ions $[R - H + Cu]^+$ points out the fact that the side chains must undoubtedly play a role. This is not surprising in the least. When one examines the relative affinities for Cu(I) of the various α-amino acids,[46] it seems clear that copper should complex the side chain. Furthermore, Hoyau and Ohanessian have recently demonstrated[45] with serine and threonine that Cu^+ complexes three different sites at the same time, one of which is the heteroatom on the side chain.

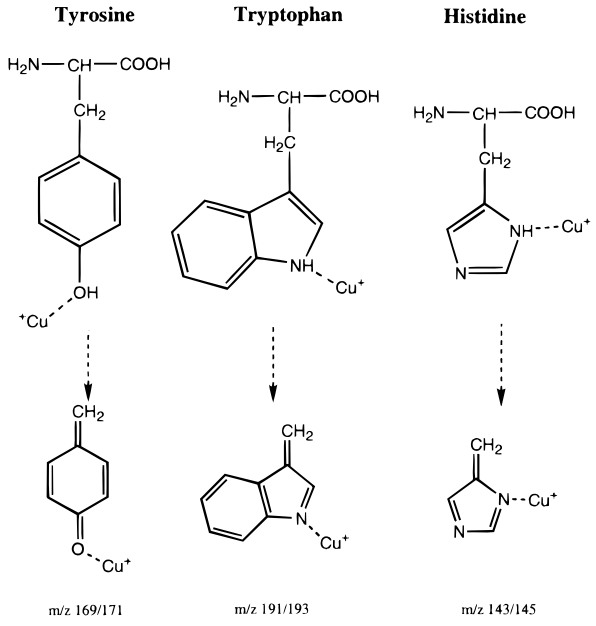


Figure 7. Structures of $[R - H + Cu]^+$ ions.

For arginine and lysine, participation of the side chain in the loss of 46 u implies that the complexation by Cu^+ affects several different sites of the molecule. This phenomenon reminds us of the 'remote functionalization'[53] which is common with organometallic adduct ions with long and flexible aliphatic chains. It can also be interpreted in terms of co-ordination: copper simultaneously co-ordinates several sites of the molecule, which leads to different parts to approach so that a proton can migrate from the side chain to the carboxylic acid function (Fig. 8).

This perspective opens for α-amino acids such as glutamic acid, glutamine, aspartic acid and asparagine other possible mechanisms for the loss of 46 u than the one suggested in Fig. 6. These compounds have reasonably long and flexible chains and most probably offer

Table 7. Relative intensities (%) of most characteristic peaks in spectra of four aromatic α-amino acids obtained in direct mode. The reference spectra of the α-amino acids alone are prepared with the usual concentration (30 µl deposit of 10^{-2} M solution); the mixtures with copper are prepared with copper chloride dihydrate in 1:1 proportion

		MH^+	I	R			MCu^+	[I − H + Cu]	[R − H + Cu]		
Phe	*m/z*	*166*	*120*	*91*			*228*	*182*	*152*		
							230	*184*	*154*		
	Alone	55	100	32							
	With $CuCl_2$ 1:1	8	100	27			19	75	—		
Tyr	*m/z*	*182*	*136*	*107*			*244*	*198*	*169*		
							246	*200*	*171*		
	Alone	100	81	64							
	With $CuCl_2$ 1:1	—	100	54			—	100	30		
Trp	*m/z*	*205*	*159*	*130*			*266*	*221*	*192*		
							264	*223*	*194*		
	Alone	28	26	100							
	With $CuCl_2$ 1:1	—	22	100			4	18	14		
His	*m/z*	*156*	*110*	*81*	*82*	*83*	*218*	*172*	*143*	*145*	*147*
							220	*174*	*145*	*147*	*149*
	Alone	100	77	14	35	19					
	With $CuCl_2$ 1:1	56	100	12	22	18	19	69	10		

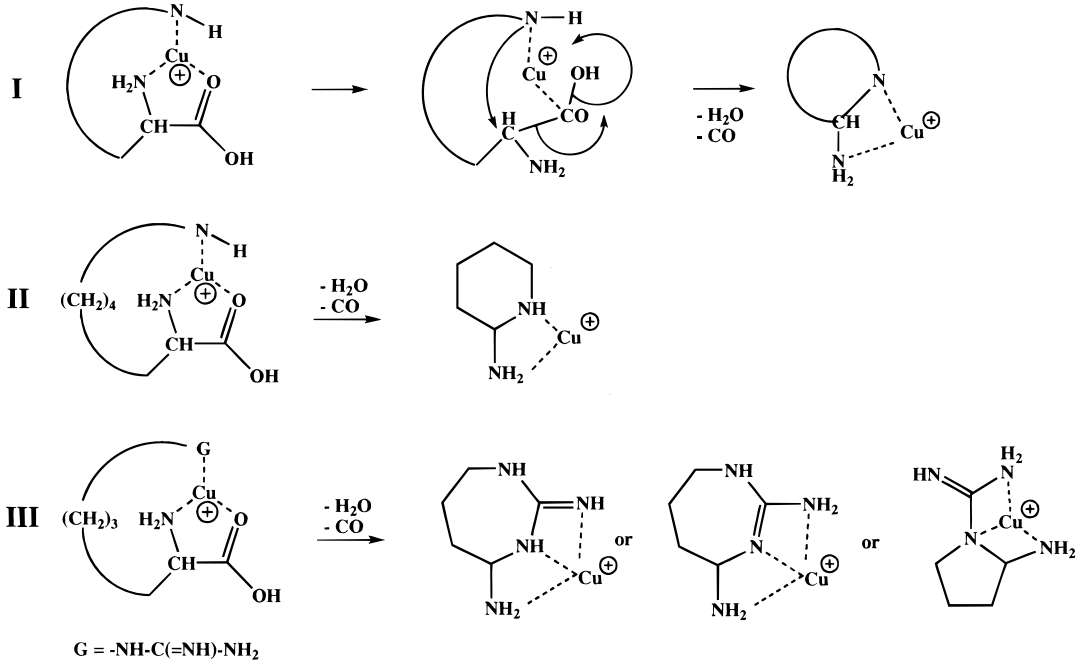


Figure 8. Formation mechanism of $[MCu-46]^+$ involving 'remote functionalization' or multiple co-ordination of Cu^+: I, general mechanism; II, case of lysine; III, case of arginine.

co-ordination sites on their side chains with hydrogens susceptible to migration. This would leave at least two different structures existing for their $[I - H + Cu]$ ion (Fig. 9).

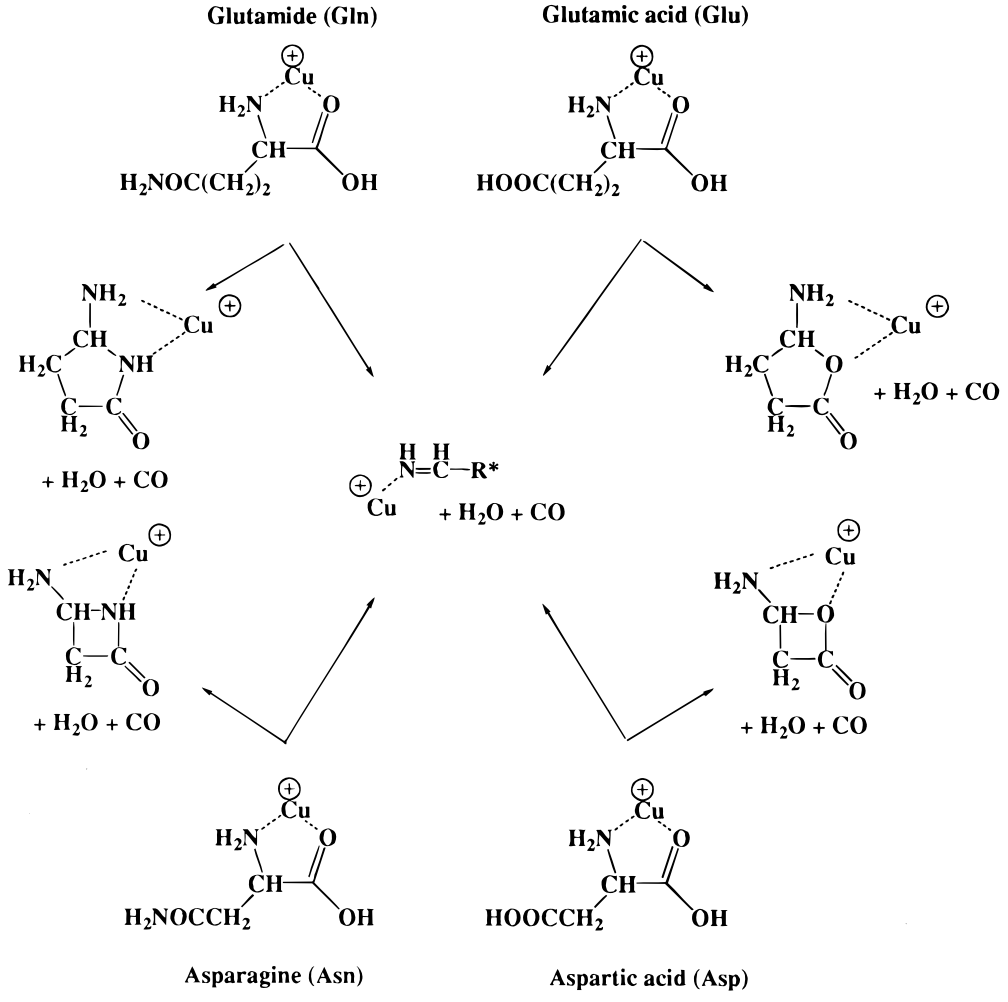
In the deuteration experiments on histidine, glutamine and arginine the calculated intensities treat every exchangeable hydrogen statistically. This treatment therefore takes into account the fact that the migrating hydrogens could originate from the side chain, resulting in the structures shown in Figs 8 and 9. For these three α-amino acids the measured relative intensities were found to be close to the calculated ones. The differences of around 10% or 20% are therefore not very significant. In each case, however, the higher isotopes are a bit larger than predicted.

The underlying hypothesis implied by a calculation that treats exchangeable hydrogens statistically is that the exchange rate should be the same for all the different acidic hydrogens in the molecule, which is obviously not true. The hydrogen on the carboxylic acid function probably exchanges the fastest, which means that it will be the first to exchange back with the residual water in our tube of flight. This hypothesis would explain the slightly larger intensities of the higher isotopes: the probability of losing $HDCO_2$ is higher than that of losing D_2CO_2. Could a similar argument be applied to differentiate between the involvement of the side chain and that of the α-amino acid skeleton? For this we would need to know the probability of exchange for each acidic hydrogen. Even with smaller fragments present, this was found to be very difficult to evaluate. Furthermore, apart from the difference in exchange rate, some intramolecular exchanges also probably play a role, making any attempts at estimating probabilities surely unreliable. With these figures, therefore, we can conclude that the hydrogens involved in the loss of 46 u are mainly exchangeable hydrogens, even if their provenance is not clear. In the case of arginine and lysine, however, the appearance of $[I - H + Cu]$ when the corresponding I is absent or of very low abundance in the spectra of the protonated molecules showed a specific reactivity for MCu^+. A co-ordination of several different sites that would result in an involvement of the side chain, unlikely when the cationizing agent is a proton, appeared to us a possible explanation of the difference in reactivity between MH^+ and MCu^+.

Several other ions with copper appear in the spectra of various α-amino acids. The ion $[MCu - 48]^+$ appears in significant quantities in the spectra of glycine and lysine and is also present, but much less intensively, for valine, arginine, histidine, glutamine and glutamic acid. This additional loss of H_2 from $[I - H + Cu]$ could not be observed in the metastable mode. Its appearance is, however, not very surprising: copper is known to have a high affinity for hydrogen (it was observed to react by hydride abstraction and dehydrogenation)[7,54] and, according to our findings, all these $[I - H + Cu]$ ions have some exchangeable hydrogens left for further reactions. An ion corresponding to the loss of 47 u from MCu^+ was observed in the spectra of tyrosine and tryptophan, which are both aromatic α-amino acids, but could not, here either, be observed in our metastable ion studies. Both these ions must therefore be attributed to fast fragmentation processes. Finally, arginine showed the ion *m/z* 174 (176) which results from the loss of ammonia from $[I - H + Cu]$; this fragmentation was observed in the metastable mode.

CONCLUSIONS

In view of our objective, which was to increase the amount of structural information in mass spectral data of α-amino acids and peptides, these preliminary results



* R = $-(CH_2)_2-CONH_2$ for Gln, $-(CH_2)_2-COOH$ for Glu, $-CH_2-CONH_2$ for Asn and $-CH_2-COOH$ for Asp.

Figure 9. Structures of $[MCu-46]^+$ ions of Glu, Gln, Asp and Asn.

are quite promising.

This plasma desorption method for engendering complexes with Cu^+ in the gas phase is quite effective, although it undoubtedly involves both reactivities in solution and in the gas phase. In fact, it is quite likely that some complexes are formed in solution while mixing the α-amino acids with the cupric salts. These complexes, deposited in the solid state, are then brought to react in the plasma engendered by the particle bombardment in the ion source. The resulting adducts MCu^+ show an extremely interesting reactivity that appears to parallel that of MH^+—with which mass spectrometrists are quite familiar—while bringing new reactions precisely where protonation failed: the fragmentation of basic α-amino acids. The $[I - H + Cu]$ ions in the spectra of lysine and arginine bring new insights on how to bypass the basicity of these two residues. Studies on simple peptides with these two residues are now beginning at the laboratory.

The mechanism involved in these fragmentations remains largely unknown, although certain facts stand out. First, the study of valine-d_8 has shown that exchangeable hydrogens appear to play a dominant role in the migrations accompanying the loss of 46 u. This result was further confirmed by experiments on α-amino acids whose acidic hydrogens were exchanged with deuteriums. Second, the fragmentation of the aromatic α-amino acids (among other things) reveal that the side chain must participate, probably in proportion to Cu^+ affinities. Finally, the participation of the side chain in α-amino acids such as arginine and lysine means that the loss of 46 u comes from a process similar to the 'remote functionalization' concept which has been described before as a specific reactivity for organometallic adducts in the gas phase.

Although many things remain to be studied concerning the precise action of transition metal cations on α-amino acids and peptides, all these facts open interesting perspectives on how organometallic reactivity in the gas phase may be useful for the obtention of structural information on biological compounds via mass spectrometry.

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