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Copper-binding Abilities of the Tripeptide Diglycylhistidine Studied by Laser-induced Liquid Beam Ionization/Desorption Mass Spectrometry in Aqueous Solution

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Laser-induced liquid beam ionization/desorption (LILBID) mass spectrometry is capable of desorbing ions directly from a liquid beam into the gas phase by means of an IR laser pulse tuned to an absorption band of the solvent used. Up to now, it has been restricted to the use of alcoholic solutions owing to the limitations of the desorbing IR laser system. This paper presents the first results using an Nd:YAG–optical parametric oscillator laser system that enables us to study aqueous solutions. Using this laser system, studies on the Cu(II)-binding abilities of the tripeptide diglycylhistidine are presented. Complex formation with various concentrations of Cu(II) ions was observed in water at basic pH. The use of buffer solutions did not affect the signal intensity of the peptide-related peaks. In acidic solutions, the complex partially dissociates. The free tripeptide and the released Cu(II) can be observed in the mass spectrum. The results obtained with this method were compared with measurements undertaken with electrospray ionization mass spectrometry. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Human serum albumin has a specific copper(II)-binding site and is considered to be the transport form of copper(II) ions in blood. After comparing the metal binding sites of albumin from various organisms, the tripeptide L-glycyl-L-glycyl-L-histidine (GGH) was chosen to mimic the native Cu(II)-binding site of albumin by Lau *et al.*[1] The reaction studied in their work is depicted in Fig. 1. The structure of the complex was elucidated by x-ray analysis[2] and analytical potentiometry. The binding features of this model system were then compared with those of human albumin by analytical potentiometry. GGH binds Cu(II) ions in the pH range 6.5–11 with a dissociation constant of 1.2×10^{-16} M compared with the dissociation constant of human albumin of 6.6×10^{-17} M. The lower binding strength is mainly influenced by the COOH-terminal free carboxyl group of the peptide.[3] The copper-chelating properties can be useful for the treatment of Wilson's disease, a genetic defect that leads to increased copper(II) deposition in the body. This otherwise lethal disease is treated by removing the copper with chelating reagents and excretion via the kidnies. A chelating agent could be GGH, as shown in animal test studies.[1] More recently, the GGH–Cu(II) complex has been used to synthesize sequence-specific DNA-cleaving proteins[4] and anthraquinone derivatives with antitumor properties.[5] A GHL–Cu complex with a similar structure has been found more recently.[6]

The study of the binding of new metal-chelating entities using classical analytical methods is a time-consuming task that could be accelerated by the use of mass spectrometry. This is especially important for medical applications, with the aim of improving the design of new drugs. In this work, we studied the Cu(II)-binding properties of the tripeptide GGH with laser-induced liquid beam ionization/desorption mass spectrometry (LILBID-MS). With this technique, it is possible to desorb ions dissociated in solution intact into the gas phase with an IR laser pulse and to analyze them in a mass spectrometer. Protein and DNA dimerization and the metal binding of ionophores in alcoholic solutions have been studied successfully with this method.[7–10] The latest advance of this method, reported here, is the introduction of a new laser system that allows one to study analytes embedded in an aqueous environment.

This work had two main scopes. First, we wanted to study the feasibility of desorbing ions directly from a pure aqueous liquid beam with an IR laser pulse. The extensive formation of H-bridges in water should make it more difficult to remove the solvation sphere from charged molecules and thus desorb them into the gas

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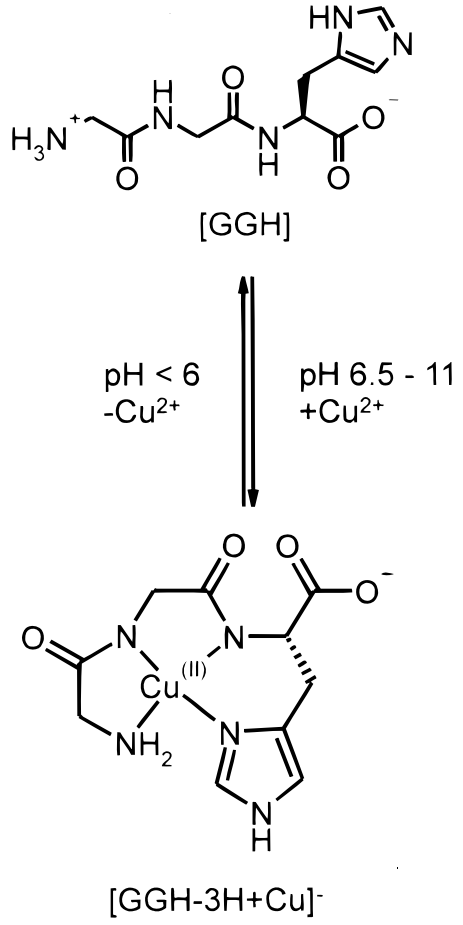

Figure 1. Scheme of the chelating reaction of Cu(II) ions with the tripeptide GGH. The binding takes place at pH 6.5–11.

phase. The second aim was to show that LILBID-MS can give some insight into the solution properties of analyte ions, so that this method can be used to study non-covalent interactions in a native environment. The results obtained were compared to those given by electrospray ionization (ESI) MS, which is an established technique for the investigation of non-covalent complexes.[11,12]

The GGH–Cu(II) system was chosen for this study, as its complexation behavior is well known and because this small peptide allowed us to resolve the isotopic patterns with the limited resolution of our mass spectrometer.

EXPERIMENTAL

In the following, the set-up of the laser system and the mass spectrometer used for LILBID-MS is discussed briefly.

Laser system

Compared with our previously conducted experiments, the main new feature in the experimental set-up is a laboratory-built, $LiNbO_3$ IR optical parametric oscillator/amplifier (OPO/OPA) system as the IR radiation source. The OPO delivers mid-IR radiation which is continuously tunable between 2.5 and 4 μm for the idler component and between 1.55 and 1.85 μm for the corresponding signal component.

The oscillator is built around a 5 cm long $LiNbO_3$ crystal cut at an angle of 47° to its optical axis. It is pumped by the 1064 nm fundamental wavelength of an injection seeded Q-switched Nd:YAG laser (Continuum Powerlite 9000). The cavity of the OPO has a length of 12 cm and consists of two plane CaF_2 mirrors which are coated for high reflectivity at signal wavelengths between 1.55 and 1.85 μm. The pump laser is coupled in and out of the resonator by two mirrors which are highly reflective under an angle of 60° for 1064 nm and which are transparent for the signal and idler radiation. Behind the OPO the IR output around 3 μm is then coupled into a second $LiNbO_3$ crystal, which acts as an amplifying stage. The same in- and outcoupling mirrors are used to combine the IR radiation from the oscillator with a second pump beam from the Nd:YAG laser and to separate the pump beam from the mid-IR radiation again after amplification. For the adjustment of the respective wavelengths, both $LiNbO_3$ crystals are mounted on motorized rotation stages. The wavelength of the OPO was tuned to the absorption band of bulk water by photoacoustic measurements.

The output energies of the OPO/OPA system can range up to 40 mJ per pulse for the idler radiation when the OPO and OPA stages are pumped with an energy of 120–160 mJ each. The repetition rate of the laser system was 10 Hz. In the experiments shown, the output energy was 24 mJ. The whole OPO set-up is built into a housing which can be flooded with nitrogen gas to avoid absorption from water vapor present in the laboratory air.

Mass spectrometer

The mass spectrometer used for these measurements is a laboratory-built reflectron time-of-flight (Re-TOF) instrument; a schematic diagram of the ion source chamber is depicted in Fig. 2. The analyte solution is introduced into the vacuum chamber of the ion source through a nozzle with a platinum–iridium aperture of 10 μm diameter. In order to form a liquid beam, a backing pressure of 30 bar (3000 kPa) is provided by a high-performance liquid chromatographic (HPLC) pump (L-6000A; Merck, Darmstadt, Germany) operating in the constant-pressure mode. This corresponds to a flow rate of about 1 ml min^{-1}. After 10 cm, the beam passes through a skimmer and is then frozen out on a cold trap filled with liquid nitrogen in order to maintain a pressure of 10^{-6} mbar (0.1 Pa). The initial temperature of the solution is kept at 15 °C (288 K) with a thermostat. A more detailed description of the liquid beam in vacuum can be found in papers by Faubel and co-workers.[13,14]

The focused laser beam is intersected with the liquid beam 2–5 mm behind the aperture, between a repeller and accelerator grid. The ions formed in this region are accelerated in two stages from about 1200 V to ground potential. An electrostatic lens provides some focusing of the ions towards the reflectron. After passing through

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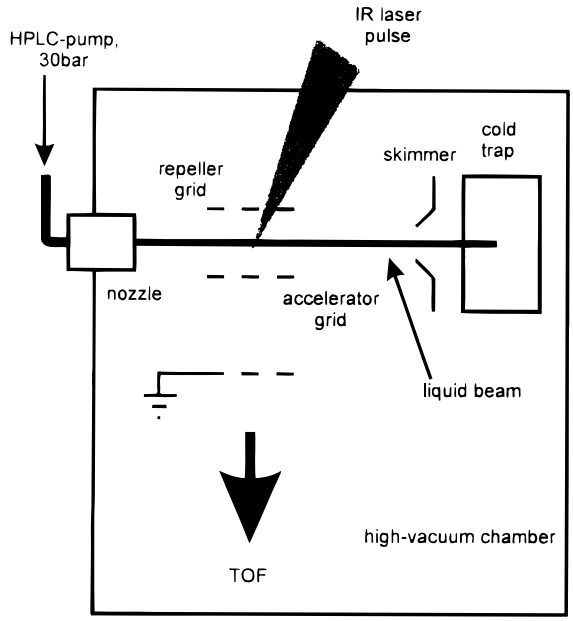

Figure 2. Schematic diagram of the LILBID ion source. The liquid beam is introduced into the vacuum chamber by a nozzle with a 10 μm aperture and a backing pressure of 30 bar. The focused IR laser pulse is intersected with the liquid beam to desorb the solvated ions, which are accelerated into a time-of-flight mass spectrometer.

a 1 m long field-free drift region, the ions enter a two-stage reflectron; the entrance potential is defined by a 91% transmission grid and the potential of the back plate is about 1450 V. After the ion mirror, the ions pass through a second 1 m long drift region. The angle between the field-free parts of the ion trajectory is 12°. At the end of the flight path the ions meet two electrostatic lenses that focus the ion beam and reject metastable fragments formed during the flight time. In order to enhance the detection probability in the high-mass region, the ions are post-accelerated to 20 kV into a ion–electron converter that is coupled with a scintillator and a photomultiplier. The detector signal is registered and stored by a Model 9410 storage oscilloscope (LeCroy, Chestnut Ridge, NY, USA) and read out with a PC for further processing. Typically, 200 spectra are averaged to improve the signal-to-noise ratio. All spectra were calibrated internally with the signals of the cluster series.

Electrospray mass spectrometry

A Fisons VG Platform II ESI-MS system fitted with a single-quadrupole mass analyzer was used. The scans were recorded in the mass range m/z 80–3000. The capillary was held at ± 2.8 kV and an extraction cone voltage of 33 V was applied. The capillary was heated to 313 K. The spray was maintained by means of an HPLC pump with a methanol–water (1:1) as solvent at a flow rate of 40 μl min^{-1}. The sample was injected using a sample loop. Calibration was carried out using NaI and CsI.

Chemicals and reagents

All measurements have been carried out in aqueous solutions. The water used was singly distilled. All reagents were of the highest purity obtainable and were used as purchased. All solvents were filtered with a 0.5 μm filter (Schleicher & Schüll, Dassel, Germany). GGH was supplied by Bachem (Heidelberg, Germany) and $CuCl_2$ and ammonium acetate by Fluka (Buchs, Switzerland).

After GGH had been dissolved in water or ammonium acetate buffer to a concentration of 10^{-3} M, the appropriate amount of $CuCl_2$ was added to the solution. To obtain pH 7.4 the solution then was titrated with 1 M NaOH. The solution was incubated for ~ 1 h at room temperature. For the ESI-MS experiments the buffered solutions at basic pH were treated with 20% (v/v) formic acid in order to perform pH dependence measurements.

RESULTS AND DISCUSSION

Mass spectra that were obtained by laser desorption from liquid beams formed by pure aqueous solutions are presented. These are the first measurements undertaken to elucidate the feasibility of desorbing ions from a water beam with this method.

A typical negative ion LILBID mass spectrum of an aqueous solution is shown in Fig. 3. The aqueous solution analyzed contained 10^{-3} M GGH at pH 7.4. In the low-mass region the spectrum is dominated by cluster series of $AcO^-(H_2O)_n$ and $Cl^-(H_2O)_n$ with n up to 30. These ions are impurities introduced by the water and the reagents used. These cluster series show up in the mass region <500 Da, needed for the present work, and it therefore was necessary to use a high concentration of 10^{-3} M for the tripeptide GGH in order for it to be easily distinguishable from the ionic underground. In the anion mode the tripeptide GGH gives rise to a signal at m/z 268 that corresponds to the singly deprotonated species $[GGH - H]^-$. A peak corresponding to the doubly deprotonated species cannot be detected. At higher masses, GGH with up to nine water molecules can also be observed, thus retaining part of its solvation sphere throughout the desorption process. No peak related to $[GGH + H]^+$ could be observed in the positive ion mode (data not shown). This finding is surprising at the pH value given, if one assumes that ions are directly desorbed from solution.[17] The pK values for the titratable protons are 2.72–2.84, 6.74–6.99 and 8.04–8.23 for the carboxyl, the imidazole and the amino group, respectively.[1,15,16] Hence at this pH, the peptide exists mainly in a zwitterionic form with the amino terminus protonated, the carboxylic group deprotonated and the imidazole being neutral. Still, about 40–50% of the peptides carry a positive or negative net charge in solution. Under these conditions, the peptide GGH should be observable in both the positive and negative ion modes. Further research has to be done to clarify this point.

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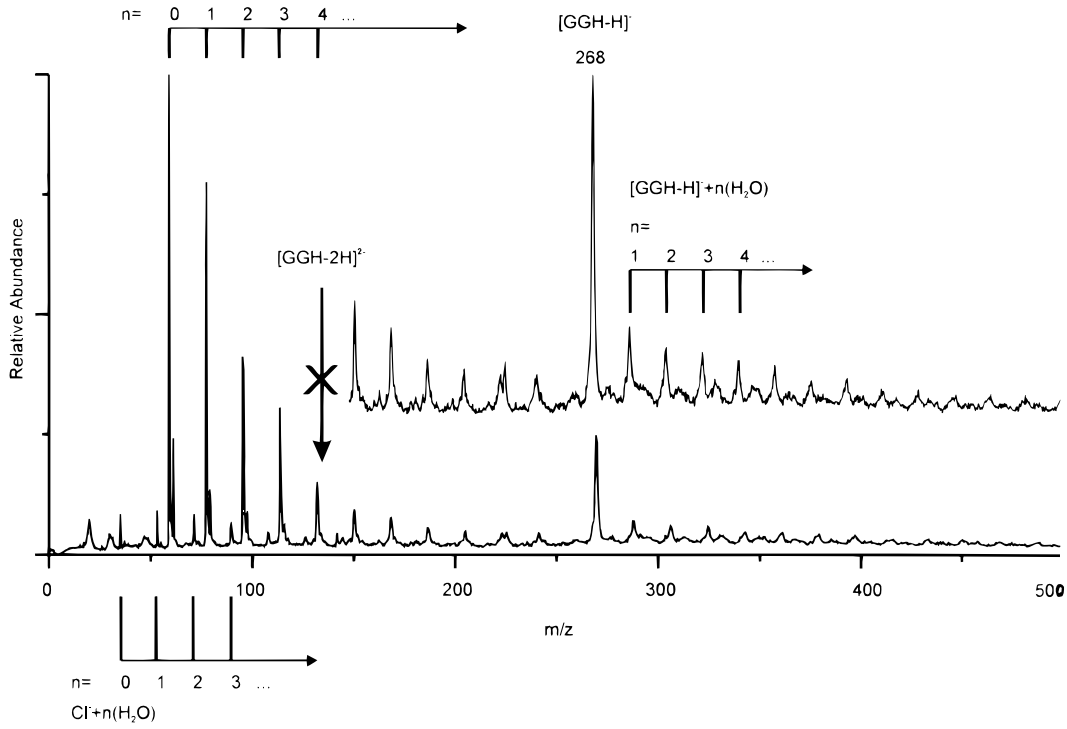

Figure 3. A typical negative ion LILBID mass spectrum of an aqueous solution. The solution contains 10^{-3} M of the tripeptide GGH in pure water at pH 7.4. A notable feature of this spectrum is the formation of water clusters with charged particles, leading to a noisy background signal.

First, we studied the stoichiometry of the complexation of copper(II) ions by GGH in pure aqueous solution at pH 7.4. It is known that at pH 6.5–11 the tripeptide binds copper(II) ions with $k_D = 1.2 \times 10^{-16}$ M.[1] Hence at the peptide concentrations used, GGH should complex virtually all copper(II) ions in solution. We therefore varied the GGH-to-copper(II) ratio and studied the effects on the LILBID mass spectra. At the basic pH used, it was not possible to use an excess of copper(II) ions in solution, as these ions will precipitate immediately as $Cu(OH)_2$. Figure 4 shows the mass spectra of GGH at different concentrations of copper(II).

After adding 0.5 equiv. of $CuCl_2$ to the 10^{-3} M solution of GGH, a new signal corresponding to the GGH–copper complex (*m/z* 329/331) is observed in addition to the signal of the free tripeptide [Fig. 4(A)]. The mass of this peak corresponds to the $[GGH - 3H + Cu]^-$ species. At this pH the imidazole is mainly deprotonated, so that in addition to the protonated amino terminus two more protons are removed from the peptide upon metal binding. This is in accordance with the proposed structure of the complex entity, where two amide protons are removed during complexation. The low-mass region now is dominated by $Cl^-(H_2O)_n$ clusters. The identification of the complex is simplified by the natural isotopic distribution of copper that can be found in the complex peak but not in the signal of the free tripeptide. Again, clustering of water to the complex is observed.

Upon addition of 1 equiv. of $CuCl_2$ to the solution of GGH, the signal of the free tripeptide disappears, as all GGH is now complexing Cu(II) [Fig. 4(B)]. This shows that the peptide–metal complex is not dissociated during the desorption process. LILBID-MS reflects the solution properties of the chelating agent in an appropriate way and is shown to be a soft desorption method.

A striking feature of the spectrum in Fig. 4(B) is the very intense salt cluster signals. This is due to the addition of $CuCl_2$ and NaOH to the solution in order to maintain the pH of 7.4, as outlined in the Experimental

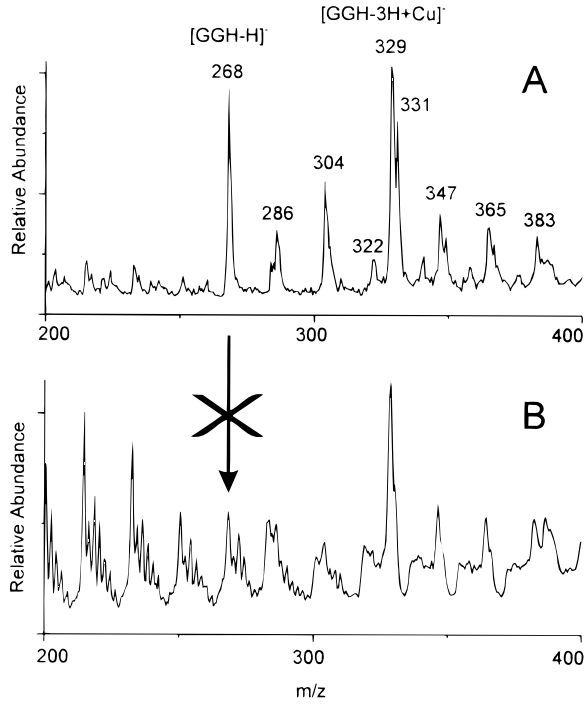

Figure 4. Negative ion LILBID mass spectra of 1×10^{-3} M GGH in pure aqueous solution at pH 7.4, containing (A) 5×10^{-4} M $CuCl_2$ and (B) 1×10^{-3} M $CuCl_2$.

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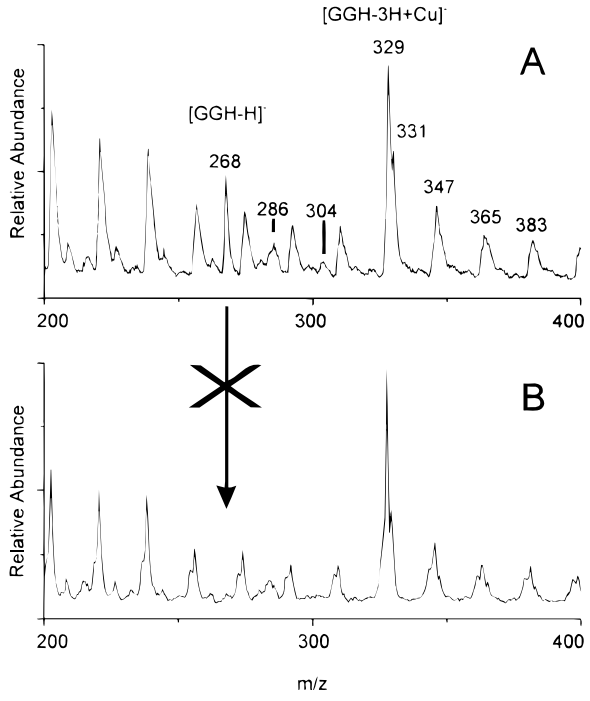

Figure 5. Negative ion LILBID mass spectra of 1×10^{-3} M GGH in an aqueous solution containing 10^{-2} M ammonium acetate buffer (pH 7.5) with (A) 5×10^{-4} M $CuCl_2$ and (B) 1×10^{-3} M $CuCl_2$.

section. In solution NaCl is formed and observed as $NaCl_2^-(H_2O)_n$ clusters, which show a very complex isotopic pattern. This broad isotope distribution together with the dominant $Cl^-(H_2O)_n$ cluster series complicates the interpretation of the mass spectra and reduces the sensitivity of this method. One possibility of circumventing this problem is to use an excess of buffer salts. Spectra of good quality could be obtained from solutions buffered by 10^{-2} M ammonium acetate at pH 7.5 (Fig. 5), showing the same concentration dependence of the complex formation as the spectra recorded before using pure water as a solvent. Because the dominant cluster series deriving from the buffer is essentially monoisotopic compared with the cluster series deriving from sodium chloride, the spectra are far easier to interpret. In particular, these measurements show that LILBID mass spectra in the case studied are tolerant to higher salt concentrations, since no quenching effect can be observed.

The buffered solutions were also analyzed using ESI-MS. In the solution containing only GGH at basic pH the free peptide can be observed in both negative and positive ion modes. Nevertheless, the cationic signal is weaker than its anionic counterpart (data not shown). The complexation behavior can also be monitored with ESI-MS. The solution containing equimolar portions of GGH and $CuCl_2$ shows a strong peak corresponding to the GGH–Cu complex in the negative ion mode, as proposed by the structure in Fig. 1. Interestingly, a weak GGH–Cu complex peak can also be found in the positive ion mode at *m/z* 331/333. No peak corresponding to the free peptide can be detected. The work with ESI-MS was carried out using a methanol–water mixture, whereas the LILBID mass spectra were recorded using pure aqueous solutions.

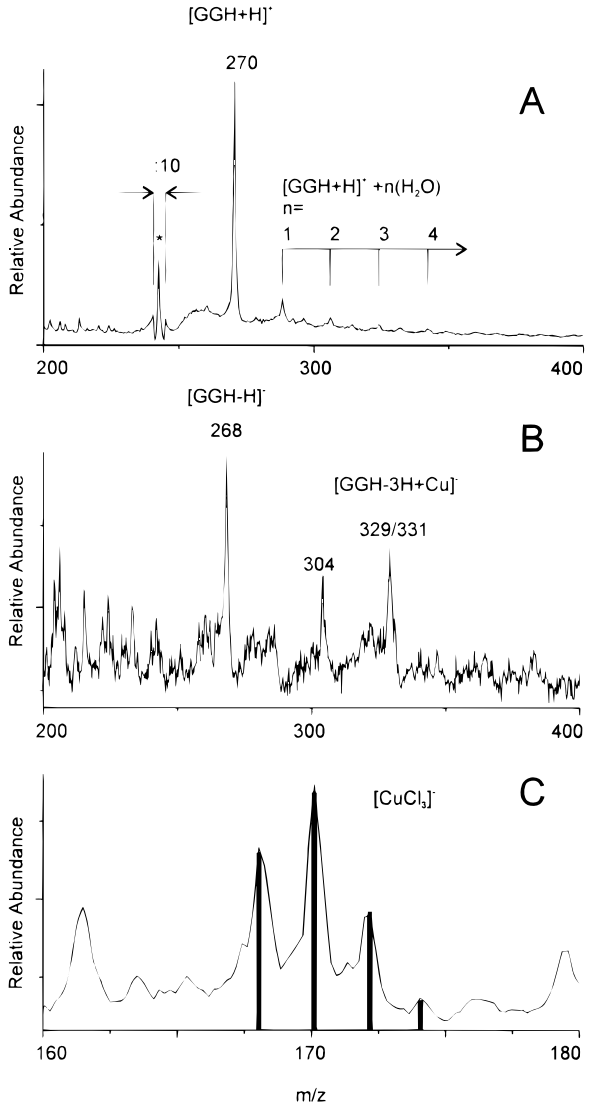

Figure 6. LILBID mass spectra of 1×10^{-3} M GGH and 1×10^{-3} M $CuCl_2$ in pure aqueous solution at pH 4.8. (A) The positive ion spectrum shows a strong signal corresponding to the free peptide and no signal corresponding to the complex entity. The asterisk indicates a peak of a calibration substance. The peak intensity was diminished by factor of 10. (B) The negative ion signals of the peptide–copper complex and of the free peptide exhibit a poor signal-to-noise ratio. (C) The isotopic pattern of the free copper in solution, compared with the theoretical isotopic distribution.

Next we investigated the pH dependence of the copper binding abilities of GGH. At a more acidic pH, the protonation equilibrium is on the side of the free peptide (Fig. 1). Hence the complexation of the copper(II) ions is decreased. Lau *et al.*[1] observed that the binding of Cu(II) begins at pH 4 and is completed before pH 6 is reached. If we can study the behavior of the GGH–copper complexation reaction in solution with LILBID-MS, a solvent-induced dissociation of the complex should have a pronounced effect on the mass spectrum. For this purpose, we dissolved the tripeptide with 1 equiv. of $CuCl_2$ in water without adjusting the pH. The solution obtained had a pH of 4.8, just at the beginning of the region in which binding is observed. At this acidic pH one expects to observe the protonated species $[GGH + H]^+$ in the mass spectrum, as the histidine residue is now protonated.

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The LILBID mass spectrum [Fig. 6(A)] shows the corresponding peak in the cation mode. No peak related to the GGH–Cu complex can be detected. Only a few water molecules are bound to the GGH at this pH. The complex peak $[GGH - 3H + Cu]^-$ should be observed in the negative ion mode. In addition to the GGH–Cu complex, signals of the free peptide [Fig. 6(B)] and $CuCl_3^-$ [Fig 6(C)] are detected, albeit with a poor signal-to-noise ratio. The LILBID mass spectrum shows that most of the complex is dissociated. A comparison of the $CuCl_3^-$ signal with the theoretical isotopic distribution clearly identifies this compound. Thus, the pH-induced dissociation of the complex into its components can be observed with this method. Interestingly, a peak of the mass 304 is observed that could correspond either to $Cu_2Cl_5^-$ (*m/z* 304.4), to $[GGH - H + 2H_2O]^-$ (*m/z* 304.3) or to the addition of a Cl^- ion to the neutral peptide. The first and last cases are unlikely, as no isotope pattern is observed, and the addition of water cannot be found in spectra without copper(II) ions present. Comparing the LILBID mass spectra at acidic and basic pH shows that the absolute signal intensities of the peptide-related peaks do not change within the error of measurement. In contrast, the relative signal intensities vary according to the protonation equilibrium in solution. This is due to the extensive ion recombination that takes place after the desorbing laser pulse is applied. The ions having lost their solvation sphere start to recombine because of coulombic attraction. In the LILBID process we only can detect a small fraction of ions that are able to escape this recombination. Hence this ion current should be approximately constant. A detailed discussion of the desorption mechanism will be given in a forthcoming publication.[17]

Measurements undertaken with ESI-MS in the positive ion mode also show that the complex is dissociated upon addition of formic acid to the buffered solution. The peak corresponding to the GGH–Cu complex disappears and at the same time the peak of the free peptide shows up in the spectra. Monitoring the total ion current at the mass of the free peptide clearly shows that the peak intensity increases as the pH is decreased. This also has been shown by other workers.[18,19]

CONCLUSION

LILBID-MS is a method for desorbing ions directly from the liquid phase into the gas phase by means of an IR laser pulse. In this work, we have shown that LILBID-MS is capable of analyzing pure aqueous solutions. A higher concentration of salts does not influence the intensity of the peptide-related peaks observed. Measurements undertaken with ESI-MS show the same complexation behavior as with LILBID-MS. Differences between the two methods can be shown for the detection of the peptide-related peaks in the positive and negative ion modes.

With LILBID-MS we could reproduce the (known) solution dependences of the GGH–copper(II) binding, such as pH-induced complex dissociation. The GGH–copper complex could be desorbed intact into the gas phase, partially with some of its solvation sphere still attached. This is an example showing that LILBID-MS is a 'soft' desorption method, allowing some insight into solution-phase properties. Further work is necessary to improve the sensitivity and resolution of the mass spectrometer and to explore the benefits and limitations of this promising method. Encouraged by these first results, it is proposed to expand the scope of the method to examine large biomacromolecules such as proteins or DNA and their interactions.

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