Hen Egg White Lysozyme–Metal Ion Interactions: Investigation by Electrospray Ionization Mass Spectrometry

Sabine Moreau,† Abalo C. Awadé,*,‡ Daniel Mollé,‡ Yvon Le Graet,‡ and Gérard Brulé†

Laboratoire de Technologie Alimentaire, Ecole Nationale Agronomique de Rennes, 65 Rue de St. Brieuc, 35042 Rennes Cedex, France, and Laboratoire de Recherches de Technologie Laitière, Institut National de la Recherche Agronomique, 65 Rue de St. Brieuc, 35042 Rennes Cedex, France

The present paper reports the first investigation of HEL interactions with metal ions using a powerful technique, namely electrospray ionization mass spectrometry (ESI-MS). The protonation states are the same with a HEL–metal ion mixture as with HEL taken alone. HEL binds from 1 to 8 ions of Cu^{2+}, where the ratios of Cu^{2+} varied from 1 to 86 ions of Cu^{2+} per molecule of HEL within the mixture. HEL was also able to bind from 1 to 6 ions of Zn^{2+} when the ratio of this metal ion varied in the same manner as copper. No effect on the enzyme activity was provoked by Cu^{2+} or Zn^{2+}.

Keywords: *Lysozyme; protein–metal ions; mass spectrometry*

INTRODUCTION

Lysozyme is a protein widely distributed in nature (Jollès *et al.*, 1963; Fernandez-Sousa *et al.*, 1977; Panfil-Kuncewicz and Kisza, 1988; Aramini *et al.*, 1992; Lyster, 1992). A peculiarity of this protein is its lytic activity on the cell walls of Gram-positive bacteria (Wang *et al.*, 1990). Two main kinds of lysozyme were identified: the c-type, which corresponds to the hen egg white lysozyme (HEL), and the g-type, which corresponds to the goose egg white lysozyme. The primary structure of lysozymes of several types, including HEL, were determined (Jollès *et al.*, 1963; Blake *et al.*, 1965; Jollès and Jollès, 1984). In this protein, basic amino acid residues (e.g. lysine or arginine) are present in high proportion and they give positive charges at neutral pH.

Metal binding to proteins such as lysozyme has been investigated to obtain information about the behavior of these proteins in biological media and the effect of metal ions on their structure (Gorini and Felix, 1953; Ramadan and Porath, 1985; Kuroki *et al.*, 1989). Interactions of lysozyme with different metal ions, such as Ca^{2+}, Fe^{3+}, Mn^{2+}, and Cu^{2+}, have thus been observed. These interactions were studied using techniques such as immobilized metal ion affinity chromatography (Ramadan and Porath, 1985; Belew *et al.*, 1987), circular dichroism (Desmet *et al.*, 1989), nuclear magnetic resonance (Tsuge *et al.*, 1991; Aramini *et al.*, 1992), fluorescence spectroscopy (Nitta and Watanabe, 1991), and X-ray crystallography (Tsuge *et al.*, 1992). More recently, a new technique, electrospray ionization mass spectrometry (ESI-MS), was used to evaluate the interactions of metal ions with peptides. Hutchens *et al.* (1992) demonstrated that during electrospray ionization the specific interactions between peptides and metal ions (Cu^{2+}, Zn^{2+}) are stable and, therefore, this method seems to be suitable for the investigation of such complexes. Siuzdak *et al.* (1993), using the same technique, studied Ca^{2+} association with a carbohydrate.

A molecular mass of 14 305 was determined for HEL using ESI-MS (Suckau *et al.*, 1992; Katta *et al.*, 1993; Matsubara *et al.*, 1993). In this study, we describe for the first time the use of ESI-MS to investigate HEL interactions with metal ions. This investigation explores the association of a protein with metal ions by ESI-MS. Interactions between HEL and Cu^{2+} or Zn^{2+} at different metal ion concentrations are investigated to determine the HEL binding capacity.

MATERIALS AND METHODS

HEL Purification and Activity Assay. HEL was purified according to the method of Awadé *et al.* (1994). Fresh egg white was diluted with 2 volumes of Tris-HCl (0.05 M, pH 9) containing 0.4 M NaCl and 10 mM β-mercaptoethanol. This solution was stirred gently and then applied to a gel filtration Superose 6 prep grade column (Pharmacia Biotechnology S.A., France) using a FPLC system from Pharmacia. Elution was performed with the same buffer but containing 0.2 M NaCl and no β-mercaptoethanol. The fraction containing the enzyme was dialyzed against water using a Centriprep 3 system from Amicon (Epernon, France).

Lysozyme activity was determined according to a turbidimetry method based on the method proposed by Weaver *et al.* (1977), using *Micrococcus lysodeikticus* cells from Sigma (L'Isle d'Abeau Chenes, France) as substrate. For this purpose, 2.5 mL of the *M. lysodeikticus* suspension in 0.066 M potassium phosphate buffer (pH 6.24) (absorbance at 450 nm between 0.6 and 0.7 nm) was placed into a glass cuvette with a light path of 1 cm; 0.1 mL of a HEL solution in the same buffer was added to the cells, and the decrease of the absorbance at 450 nm was recorded every 30 s for 3 min, at 25 °C. One enzyme unit will produce a decrease of the absorbance of 0.001 per minute.

Protein Assay. Protein concentration was determined using the Bio-Rad protein assay, based on the Bradford method (1976), with bovine serum albumin as the standard.

Preparation of HEL–Metal Ion Solutions for ESI-MS. Metal ion solutions were prepared from $CuCl_2$ and $ZnCl_2$ (Merck, Darmstadt, Germany) and analyzed with an atomic absorption spectrometer (flame AAS) (Varian AA 300, Les Ulis, France). The concentration of the copper solution was 3.8×10^{-2} M (pH 5.6) and of the zinc solution 1.8×10^{-2} M (pH 5.9). Aliquots of the copper solution, 1.56, 4.64, 7.73, 12.37, 15.47, 23.3, and 31 μL, were added to 1 mL of the HEL solution in water (pH 7.01) to obtain copper/HEL molar ratios of 1, 3, 5, 8, 10, 15, and 20, respectively. To obtain a copper/HEL ratio of 86, 72.9 μL of a 7×10^{-2} M solution of copper (pH 5.6) was added to 1 mL of the HEL solution. For zinc, aliquots of 3.3, 9.9, 16.5, 26.4, 33.1, 49.5, and 66.3 μL were added to 1 mL of the HEL solution to obtain zinc/HEL ratios of 1, 3, 5, 8, 10,

* Author to whom correspondence should be addressed [fax (33) 99 28 53 50].

† Laboratoire de Technologie Alimentaire.

‡ Laboratoire de Recherches de Technologie Laitière.

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15, and 20, respectively. For a zinc/HEL ratio of 86, 69.2 μL of a 7.38×10^{-2} M solution of zinc (pH 5.9) was added to 1 mL of the HEL solution. The final concentration of HEL was estimated as 59.4 μM. The pH values of the copper–HEL mixtures were 6.9, 6.8, 6.6, 6.6, 6.6, 6.6, 6.6, and 6.4 for copper/HEL ratios of 1, 3, 5, 8, 10, 15, 20, and 86, respectively. In the case of zinc–HEL mixtures, the pH values were 6.9, 6.8, 6.7, 6.6, 6.6, 6.5, 6.5, and 6.4 for zinc/HEL ratios of 1, 3, 5, 8, 10, 15, 20, and 86, respectively. These mixtures were incubated at 4 °C for 12 h and were then analyzed, without any supplementary treatment, by ESI-MS. A total of 326 pmol of HEL was consumed in acquiring one spectrum.

Electrospray Mass Spectrometry. All experiments were performed with a quadrupole mass spectrometer (API, I, Sciex, Toronto, Canada). Solutions were sprayed through a stainless steel capillary held between +5 and +5.2 kV, generating multiply charged ions. Positive ionization was used. The liquid nebulization was assisted with a coaxial air flow along the sprayer. The nebulizer pressure was usually adjusted within the range 0.3–0.4 MPa. The solution was delivered to the sprayer by a syringe infusion pump (through a fused silica capillary of 75 μm i.d.) at 5 μL/min. The interface between the sprayer and the mass analyzer consisted of a conical orifice of 100 μm diameter. To enhance ion signals, the potential on the orifice was 100 V. In the interface region, the aerosol droplets were evaporated and the formed clusters were broken up with a gas curtain maintained by a continuous flow of N_2. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adduct of polypropylene glycols. Unit resolution was maintained across the *m/z* region, according to the 55% valley definition. The same resolution setting was used for molecular mass measurements on proteins. Solutions were electrosprayed at 55 °C. The mass spectra of the proteins that are shown are averages from 10 scans. Scans were accumulated with Tune 2.3, and masses were calculated with Macspec 3.2.

RESULTS AND DISCUSSION

Molecular Mass Determinations on Native HEL. Native HEL was studied in aqueous solutions (pH 7.01) (MilliQ water, Millipore, St-Quentin-Yvelines, France) at a concentration of 59.4 μM. Only 326 pmol of HEL was consumed in the acquisition of one spectrum. The mass spectrum for HEL is shown in Figure 1. The average molecular mass was calculated from the five multiply charged molecular ions in Figure 1 (Table 1) as 14 303.4 ± 0.6, consistent with the value of 14 304.2 obtained from the formula for HEL. These results reveal that the molecule was homogeneous. The ion producing the most intense signal (at *m/z* 1590.2) in the mass spectrum (Figure 1) carried nine positive charges. HEL is a highly basic protein (p*I* 11.1) containing 19 basic groups. Under our conditions (pH 7.01), lysine, arginine, histidine, and any free amino termini may carry positive charges in the positive ion ESI-MS. These results are in line with those previously published by Mirza *et al.* (1993).

Interactions between HEL and Copper. Figure 1 shows the mass spectrum of HEL in comparison with mass spectra obtained after the addition of copper to HEL to achieve copper/HEL molar ratios of 3, 5, or 86. As shown in Figure 1, the most intense signals in these mass spectra come from ions with nine positive charges, irrespective of the presence or absence of copper. This observation indicates that the addition of even a large excess of copper (up to 86 mol/mol of HEL) did not affect HEL ionization. As expected, the intensities of the peaks decreased with the increasing concentration of the metal ions, because complexation must reduce the free enzyme concentration. The signals for HEL and 0, 3, 5, 8, 10, 15, 20, and 86 mol of copper added/mol of

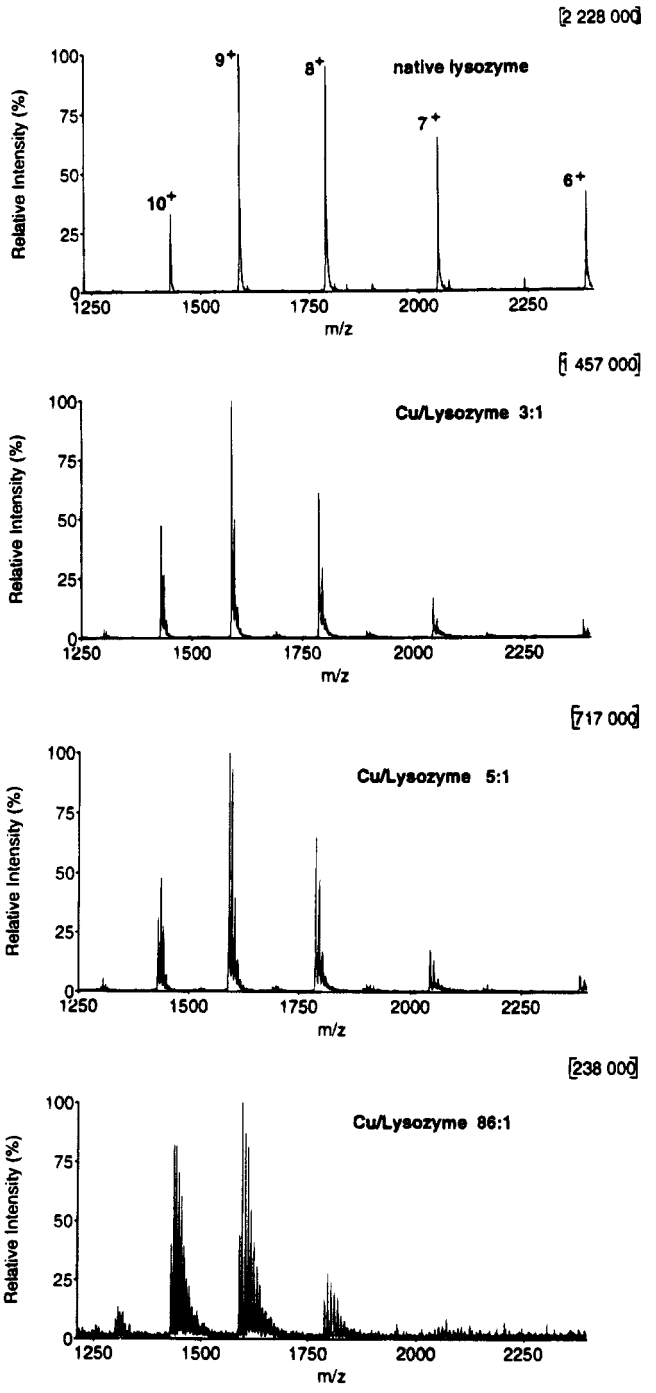

Figure 1. Electrospray ionization mass spectra at pH 7.01 of uncomplexed and complexed copper/HEL at molar ratios of 3, 5, and 86. Relative intensity (%) represents the relative intensity of each multiply charged ion. The protonation states are indicated above the peaks in the top panel. The numbers in brackets correspond to the intensities (arbitrary units) of the most abundant ion.

HEL are presented with a molecular mass scale on the horizontal axis (Figure 2). These spectra were obtained by multiplying the *m/z* values for the signal clusters arising from the ions with nine positive charges by nine. These spectra indicate clearly, that the number of complexes HEL–$(Cu)_n$ increases with increasing molar ratio of copper to HEL. At a molar ratio of 86, eight HEL complexes with $n = 1-8$ can be identified in the spectrum. The observed and calculated molecular masses for HEL and HEL–copper complexes are summarized in Table 2. The incremental difference of 61.6 between

Table 1. Determination of the HEL Molecular Mass by the HyperMass Method

actual peak at m/z	intensity (arbitrary units)	predicted m/z[a]	charge	molecular mass[a]
1431.4	735 000	1431.4	10	14 303.9
1590.2	2 228 000	1590.3	9	14 302.7
1789.0	2 131 000	1789.0	8	14 303.9
2044.4	2 131 000	2044.4	7	14 303.7
2384.8	929 000	2385.0	6	14 302.7

[a] Predicted m/z = (molecular mass obtained from the formula for HEL + nH)/n, with n corresponding to the protonation state. Average molecular mass: 14 303.4 ± 0.6.

two neighboring molecular masses suggests that binding of one atom of copper releases two protons.

The experiment with mixtures containing copper and HEL in 86:1 molar ratio revealed that one molecule of HEL can bind up to eight ions of copper. Belew *et al.* (1987) studied the interactions of HEL with immobilized Cu^{2+} and found that 1 mol of HEL binds approximately 2.5 mol of Cu^{2+}. They postulated the presence of two or three binding sites on the surface of HEL. Figure 3, which presents the percentage of HEL–$(Cu)_n$ complexes as a function of copper/HEL molar ratio, indicates the presence of two or three major complexes corresponding to the first two or three additional peaks (Figure 2). These complexes may result from the binding of copper to the two or three main binding sites of HEL. It is noteworthy that in the case of an excess of copper (up

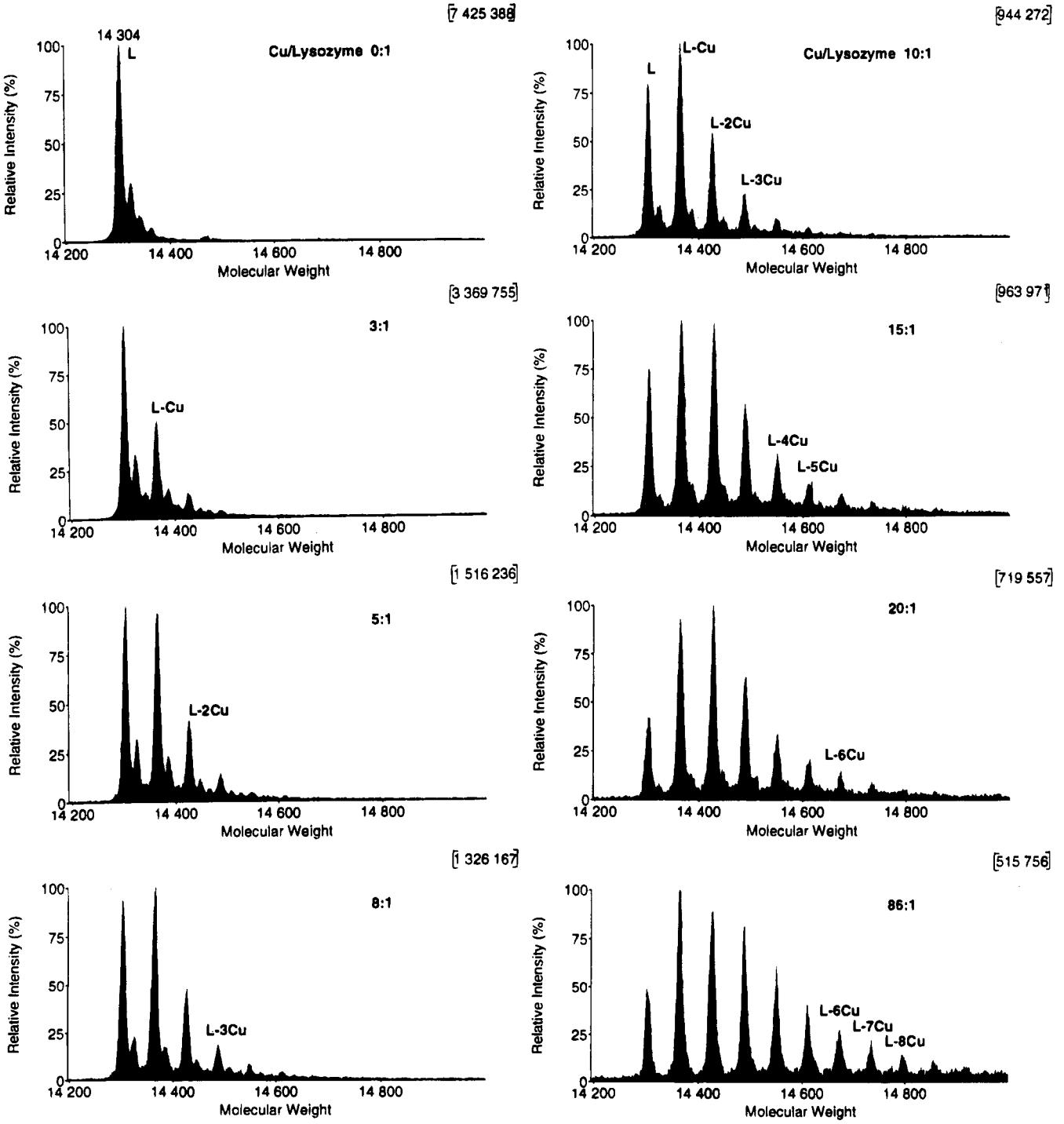

Figure 2. Mass spectra of HEL in the absence and in the presence of copper based on the ions with nine positive charges with molecular masses on the horizontal axis. The numbers in brackets correspond to the intensities (arbitrary units) of the most abundant complex.

Table 2. Mass Assignments for Molecular Ions of HEL and HEL–Copper Complexes Observed by Electrospray Ionization

no. of Cu atoms (n) bound to molecular ion	[M − 2nH + nCu] calcd	mass obsd	incremental difference in mass[a]
0	14 304.2	14 303.5	
			62.0
1	14 365.7	14 365.5	
			61.7
2	14 427.2	14 427.2	
			61.4
3	14 488.7	14 488.6	
			62.4
4	14 550.0	14 551.0	
			60.7
5	14 611.7	14 611.7	
			61.9
6	14 673.2	14 673.6	
			61.8
7	14 734.7	14 735.4	
			61.0
8	14 796.2	14 796.4	

[a] The incremental difference in mass is the difference between two consecutive calculated masses.

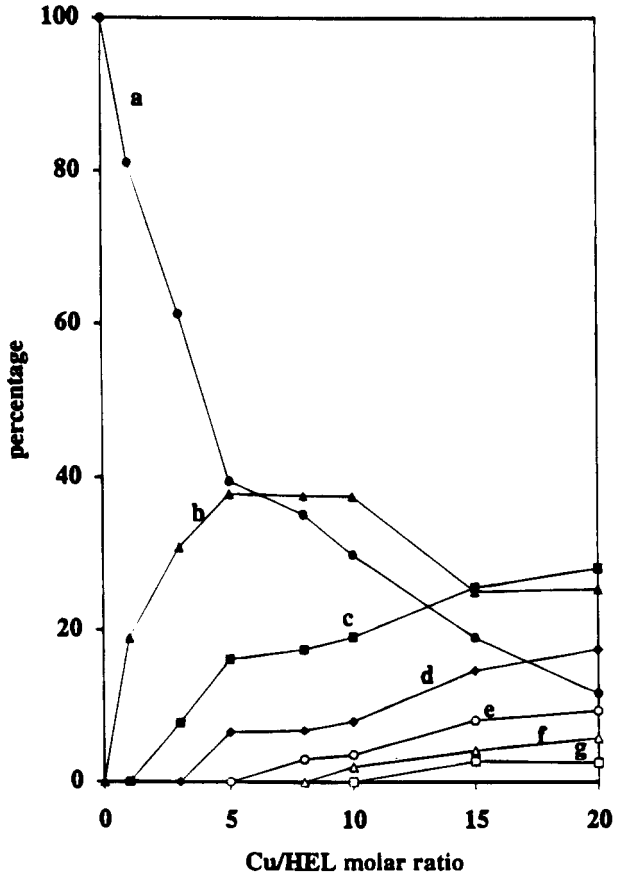

Figure 3. Percentage of HEL–$(Cu)_n$ complexes as a function of copper/HEL molar ratio. This figure was obtained from the results shown in Figure 2: (a) uncomplexed HEL; (b) HEL–$(Cu)_1$; (c) HEL–$(Cu)_2$; (d) HEL–$(Cu)_3$; (e) HEL–$(Cu)_4$.

to 86 mol of copper/mol of HEL), HEL was never totally bound in a complex, although it decreased as copper was added (Figure 3). Approximately 19.4% of the initial HEL remains uncomplexed in the presence of 86 mol of copper/mol of HEL. Probably an equilibrium is established between uncomplexed HEL and complexed forms. At lower concentrations of copper (3 mol of copper/mol of HEL), even though the two complexes HEL–$(Cu)_1$ and HEL–$(Cu)_2$ were detected together, HEL–$(Cu)_1$

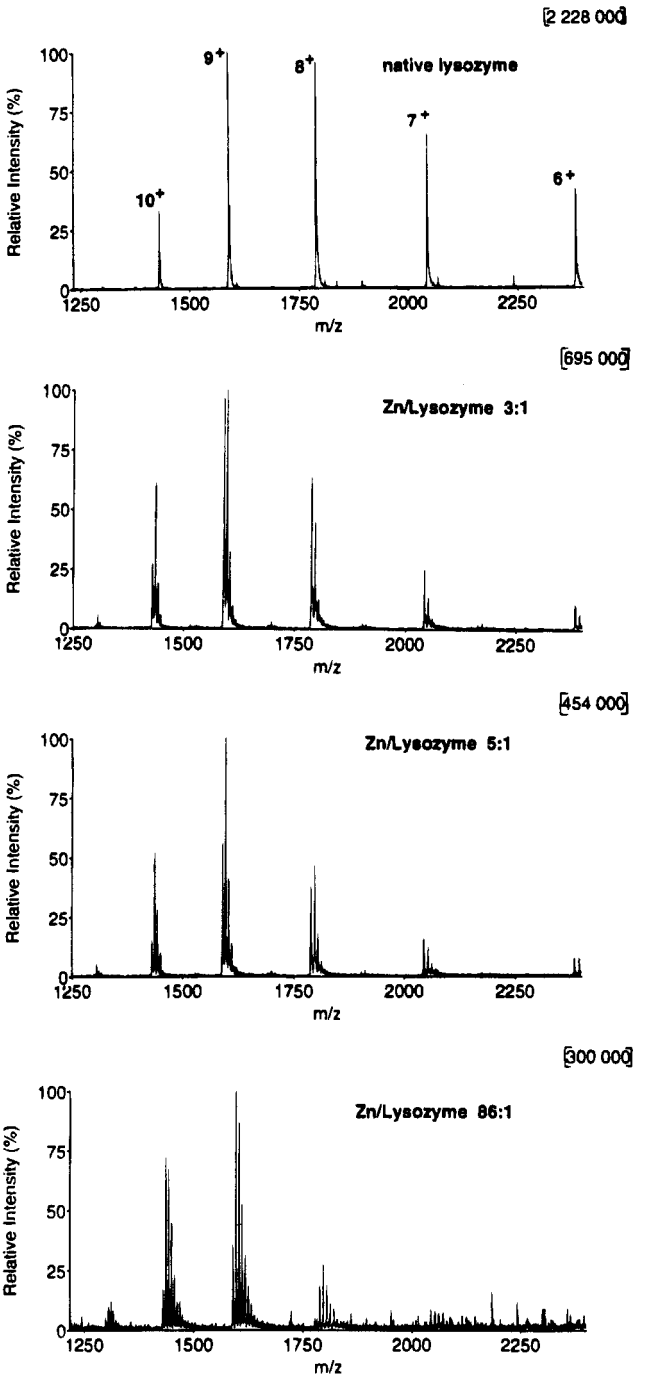

Figure 4. Electrospray ionization mass spectra at pH 7.01 of uncomplexed and complexed HEL at zinc/HEL molar ratios of 3, 5, and 86. Relative intensity (%) represents the relative intensity of each multiply charged ion. The protonation states are indicated above the peaks in the top panel. The numbers in brackets correspond to the intensities (arbitrary units) of the most abundant ion.

formation is greater. The relative percentage of the peak corresponding to HEL–$(Cu)_1$ was 30.6%, whereas that of the peak corresponding to HEL–$(Cu)_2$ was 8.3% (Figure 3). This led us to suppose that the site which binds Cu^{2+} to HEL–$(Cu)_1$ may exhibit a higher association constant than the second site which led to HEL–$(Cu)_2$ formation. Other experiments should be carried out to confirm this assumption, because with our experiments it is difficult to determine accurately the concentration of the complexes.

Addition of copper solution to HEL solution led to a decrease of the pH. The pH values of the mixtures with

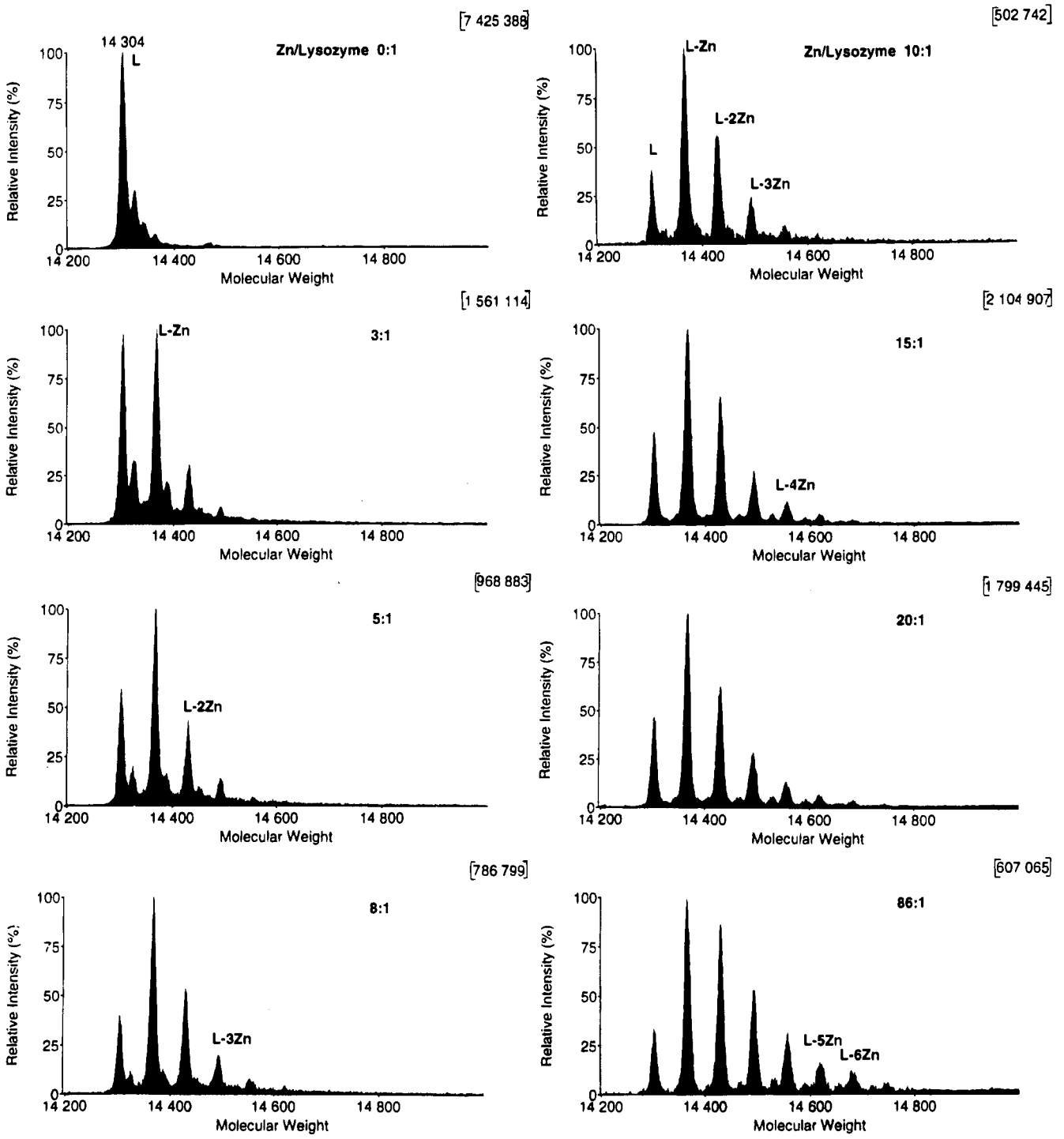

Figure 5. Mass spectra of HEL in the absence and in the presence of zinc based on the ions with nine positive charges with molecular masses on the horizontal axis. The numbers in brackets correspond to the intensities (arbitrary units) of the most abundant complex.

copper/HEL molar ratios of 1, 3, 5, 8, 10, 15, 20, and 86 were 6.9, 6.8, 6.6, 6.6, 6.6, 6.6, 6.6, and 6.4, respectively. The appearance of further peaks, in samples containing an excess of copper (copper/HEL molar ratio = 15–86) may be explained by the occurrence of new sites, due to modification of the protein conformation or modification of amino acid charges. These modifications may be related to the pH or to the ionic strength.

Aliquots of the copper–HEL mixtures were tested for enzyme activity. These mixtures had the same specific activity as native HEL. This suggests that, under our conditions, the interactions between HEL and copper did not alter the enzyme structure and, hence, the catalytic site was preserved. According to Mildvan (1970), a soft acid such as Cu^{2+} can bind soft bases by partially forming covalent bonds. This may not be the case in our experiments. In fact, after dialysis of copper–HEL mixtures, only the uncomplexed HEL peak was detected by ESI-MS analysis (data not shown). This indicates that the interactions between copper and HEL are not covalent interactions. By working at an alkaline pH (9.3), Feeney *et al.* (1956) found that HEL was inactivated by copper. We have conducted our studies at lower pH, and this would certainly explain the differences in our results compared with those of Feeney *et al.* (1956).

Interactions between HEL and Zinc. To our knowledge, no study on the interactions between HEL

Table 3. Mass Assignments for Molecular Ions of HEL and HEL–Zinc Complexes Observed by Electrospray Ionization

no. of Zn atoms (n) bound to molecular ion	[M − 2nH + nZn] calcd	mass obsd	incremental difference in mass[a]
0	14 304.2	14 303.0	
			63.8
1	14 367.6	14 366.8	
			63.5
2	14 431.0	14 430.3	
			63.3
3	14 494.4	14 493.6	
			63.1
4	14 557.8	14 556.7	
			65.2
5	14 621.2	14 621.9	
			60.9
6	14 684.6	14 682.8	

[a] The incremental difference in mass is the difference between two consecutive calculated masses.

and zinc has been reported. Figure 4 shows the mass spectrum of HEL in comparison with mass spectra obtained after the addition of zinc to HEL to obtain a copper/HEL molar ratio of 3, 5, or 86. As with copper, addition of even a large excess of copper (up to 86 mol/mol of HEL) did not affect HEL ionization. As expected, the intensities of the peaks decreased with the increasing concentration of the metal ions. The signals for HEL and 0, 3, 5, 8, 10, 15, 20, and 86 mol of zinc added/mol of HEL are presented with a molecular mass scale on the horizontal axis (Figure 5). These spectra indicate that the number of complexes HEL–$(Zn)_n$ increases with increasing molar ratio of zinc to HEL. At a molar ratio of 20, six HEL complexes with n = 1–6 can be identified in the spectrum. The observed and calculated molecular masses for HEL and HEL–zinc complexes are summarized in Table 3. The incremental difference of 63.42 between two neighboring molecular masses suggests that binding of one atom of zinc releases two protons, as with copper.

Figure 6 presents the percentage of HEL–$(Zn)_n$ complexes as a function of zinc/HEL ratio. It shows clearly that in the case of an excess of zinc (up to 86 mol of zinc/mol of HEL), the HEL was never totally bound in a complex. In mixtures with zinc/HEL ratios = 10–86, it appears that the percentage of uncomplexed HEL is almost identical. From the initial HEL present in the mixture at a zinc/HEL ratio of 86, approximately 10.2% remains uncomplexed. As with copper, we suppose that an equilibrium may be reached between the complexed and uncomplexed forms.

Figure 6 also indicates that HEL presents two major complexes corresponding to the first two additional peaks (Figure 5). The percentage of the two major complexes corresponding to HEL–$(Zn)_1$ and HEL–$(Zn)_2$ varied from 43.7 to 44 and from 13 to 24.5, where the zinc/HEL molar ratio varied from 3 to 10 (Figure 6). Although requiring experimental verification, this observation suggests the presence of two main binding sites.

The pH values of zinc–HEL mixtures were 6.9, 6.8, 6.7, 6.6, 6.6, 6.5, 6.5, and 6.4 for zinc/HEL molar ratios of 1, 3, 5, 8, 10, 15, 20, and 86, respectively. As we have suggested for copper, the appearance of further peaks, in samples containing an excess of zinc (zinc/HEL molar ratio = 20–86) may be explained by the occurrence of new sites, due to modification of the protein conformation or modification of amino acid charges.

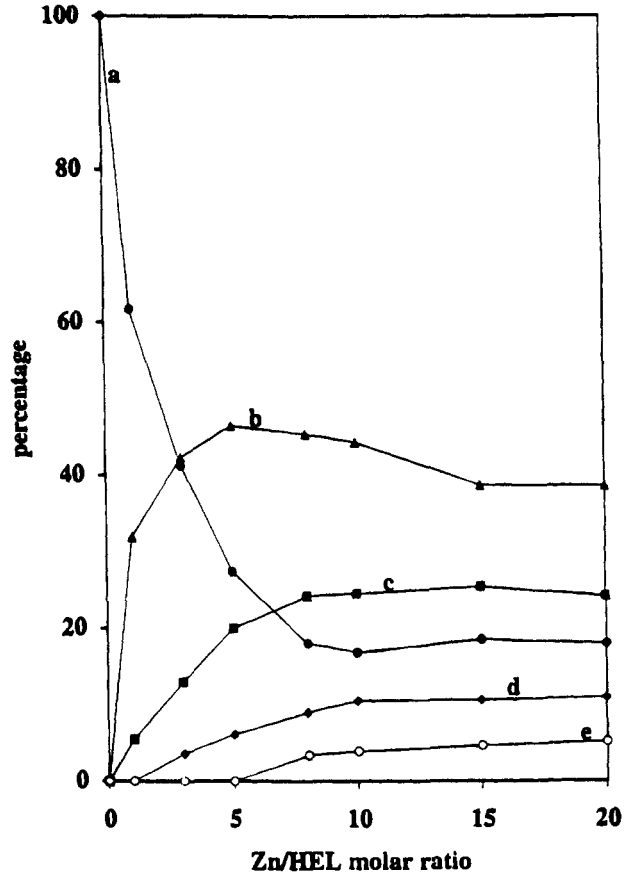

Figure 6. Percentage of HEL–$(Zn)_n$ complexes as a function of zinc/HEL molar ratio. This figure was drawn from the results shown in Figure 5: (a) uncomplexed HEL; (b) HEL–$(Zn)_1$; (c) HEL–$(Zn)_2$; (d) HEL–$(Zn)_3$; (e) HEL–$(Zn)_4$.

Aliquots of the zinc–HEL mixtures were tested for enzyme activity. These mixtures had the same specific activity as native HEL. This suggests that, as with copper, the interactions between HEL and zinc did not alter the catalytic site.

Conclusions. This work presents the first use of a powerful technique, ESI-MS, to investigate interactions between HEL and metal ions. Studies were carried out with Cu^{2+} and Zn^{2+} in different conditions to determine HEL binding capacity. Up to 8 mol of Cu^{2+} or 6 mol of Zn^{2+} could bind 1 mol of HEL. Two major complexes, corresponding to the binding of 2 mol of copper or zinc, were detected under our conditions.

Concerning the amino acids involved in the metal binding sites, a hypothesis could be proposed on the basis of previous studies. Freeman, in 1973, reported that one imidazole can binding one or two metal ions. Histidine, which presents an imidazole group, could be a metal-binding site because imidazole–metal bonds are flexible and imidazole is an excellent electron donor, available for action at physiological pH (which corresponds to our conditions). Other amino acids with carboxylic groups could bind metal ions. In fact, as stated by Rebek *et al.* (1985, 1986), the carboxylic group may interact with several cations; it may share cations between both of its oxygen atoms, depending on the geometry of the position of the hydrogen atom in the carboxyl group. In HEL, histidine is also probably involved in one of the sites. Because HEL possesses only one histidine, according to the lysozyme model of Blake *et al.* (1965), His 15, Asp 18, Met 12, and Trp 123 could be involved in one of the probable two main sites. Trp 62, Trp 63, Cys 64, Asp 66, and Asp 48 might be

implicated in the second main site. Further investigations should be undertaken to confirm these suggestions.

ABBREVIATIONS USED

HEL, hen egg white lysozyme; ESI-MS; electrospray ionization mass spectrometry.

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