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Iron and citrate interactions with hen egg white lysozyme

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Abstract

The binding of ions to proteins is of great interest in biological science (catalytic function, structural stability) and a good understanding of this relationship is needed for the control of the structure and functionality of proteins. The interaction of hen egg white lysozyme with calcium, magnesium, iron, citrate, phosphate and sulphate are studied by using the Scatchard representation or ESI–MS for iron-binding studies. This is the first time that anions have been considered and the results indicate that citrate interacts with lysozyme through electrostatic bonds. A pH of 6 favours citrate interaction to lysozyme, whereas no interaction appears at pH 9. ESI–MS studies also highlight that iron co-ordinates lysozyme, inducing the release of three protons, including one arising from an amine lateral chain.

Keywords: Lysozyme; Ion–protein interactions; ESI–MS; Scatchard representation

1. Introduction

Lysozyme, a protein first discovered by Fleming in 1922, is found widely distributed in the natural environment. It is present in most physiological liquids (milk, blood, tears, urine, etc) and in different plants. Several lysozyme types have been discovered; the c-type, similar to egg white lysozyme, is the most common. These enzymes share common properties, including killing of bacteria (Ibrahim, 1996; Wang, Murao & Arai, 1990), and are therefore used in the pharmaceutical and food industries.

Hen lysozyme is a monomeric globular protein with 129 amino acids (MW = 14 300 Da) and its isoelectric point (pHi) is 10.7 due to the high proportion of lysine and arginine. From the high degree of homology in the amino acid sequences of α-lactalbumins and lysozymes (Brew, Vanaman & Hill, 1967), X-ray and modelling studies (Warme, Momany, Rumball, Tuttle & Scheraga, 1974) and the intron-exon arrangement of their genes (Qasba & Safaya, 1984), it has been postulated that these proteins have evolved from a common ancestral molecule. In spite of the 50% homology between bovine α-lactalbumin and c-type lysozyme (Browne, North & Phillips, 1969), these proteins are functionally different.

The lysozyme/α-lactalbumin family show an affinity for ions. Early studies on the interactions between ions and lysozyme took place in 1952 with the Cu^{2+} affinity study. Interactions of lysozyme with several ions, studied by various techniques, included Co^{2+} and Mn^{2+} by electronic paramagnetic resonance (Gallo, Swift & Sable, 1971) and circular dichroism (Ikeda & Hamagushi, 1973) and Cu^{2+} by spectrofluorimetry and X-ray crystallography (Teichberg, Sharon, Moult, Smilansky & Yonath, 1974). Since the beginning of the 80s and the discovery of Ca^{2+} affinity for α-lactalbumin (Hiraoka, Segawa, Kuwajima, Sugai & Murai, 1980), binding studies between ions and the lysozyme/α-lactalbumin family have been intensified. Except for traces of Mn^{2+} and Zn^{2+}, Ca^{2+} is the only ion discovered in native bovine α-lactalbumin (Kronman, 1989). In the presence of Ca^{2+}, hen egg white lysozyme conserves only 26% of the free enzyme activity. Thus, calcium was predicted to

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bind near the catalytic site to cause inhibition of lysozyme activity (Imoto, Ono & Yamada, 1981). Later, one calcium binding site was discovered in donkey (Godova-Zimmerman, Conti & Napolitano, 1988), pigeon (Nitta, Tsuge, Shimazaki & Suga, 1988) and equine (Desmet, Van Dael, Van Cauwelaert, Nitta & Sugai, 1989) lysozymes.

The use of new investigative methods for the studies of protein/ion interactions, such as mass spectrometry (Loo, 1997), demonstrated hen egg white lysozyme affinity for zinc and copper (Moreau, Awadé, Mollé, Le Graët & Brulé, 1995). Some studies proved that ions influence lysozyme structure and functionality (Feeney, MacDonnell & Ducay, 1956; Imoto et al., 1981). For a better understanding of ion–protein relations in a biological medium, we investigate the interactions of hen egg white lysozyme with calcium, magnesium, iron, citrate, phosphate and sulphate.

2. Materials and methods

2.1. Sample preparation for ion–lysozyme complex analysis by ultrafiltration

Na_2SO_4, Na_2HPO_4 and $Na_3C_6H_5O_7$ (tri-sodium citrate) (purity > 99%, Sigma, St Quentin Fallavier, France) were dissolved in bidistilled water, to give stock solutions with final concentrations of 60, 10 and 90 mg l^{-1}, respectively. Stock solutions and lysozyme (Ovonor, Annezin les Bethunes, France) were mixed to give ion/lysozyme solutions with molar ratios of 0.5, 0.75, 1, 1.5, 2, 3, 5, 10. For cation analysis, increasing quantities of $CaCl_2$ and $MgCl_2$ were added to a stock lysozyme solution at a concentration of 4 g l^{-1} to give ion/lysozyme solutions with molar ratios of 0.5, 0.75, 1, 1.5, 2, 3 and 5. The ionic strength of ion/lysozyme solutions were adjusted to 30 or 120 mM by NaCl (Merck, Darmstadt, Germany). After mixing for a few minutes, the pH was adjusted to 6 and 9 with NaOH or HCl. A 30 min time delay, determined by previous studies, was respected in order to reach a complete equilibrium.

2.2. Scatchard representation

Scatchard plot, r/L versus r, where r is the fractional binding ratio (moles of ion bound/mol of protein) and L is the free ion concentration, is the most frequently used method for evaluating affinity between protein and ions (Scatchard, 1949). This enables the determination of the number of binding sites and the affinity constant. The method requires only quantification of the ion fraction bound to the protein and the free ion fraction. Several ion concentrations must be studied to obtain the complete binding isotherm. Ion distribution was followed on both sides of an ultrafiltration membrane. Complete binding isotherms were performed three times, as soon as an interaction appeared, and the mean of affinity constants, site numbers and standard deviations recorded. The differences between the mean of affinity constants and site numbers were statistically analysed using Student's t-test.

2.3. Ion–lysozyme complex analysis by ultrafiltration

Ion separation between bound protein form and free form was performed at room temperature by ultrafiltration for Scatchard representation [except for the iron–lysozyme interaction studies (electrospray ionisation–mass spectrometry analysis, ESI–MS)]. Ultrafiltration was performed on a Amicon CH2 system (Millipore SA, St Quentin en Yvelines, France) equipped with a hollow fibre cartridge having a nominal cut-off of 3000 Da (H1P3-20, Millipore SA, St Quentin en Yvelines, France). Retention of lysozyme in the retentate was checked by analysis of the permeate using reverse phase high performance liquid chromatography (RP–HPLC, column Vydac C4 214 TP, 5 μm, 300 Å, Touzart et Matignon, Vitry sur Seine) as described by Nau, Mallard, Pages, and Brulé (in press); the retention rate was always higher than 99%. One ml aliquots of permeate and retentate were taken, 30 min after every modification of the solution. The concentration of bound ions was calculated by the difference between retentate and permeate concentrations.

2.4. Citrate and sulphate anion quantification using HPLC

Sulphate and citrate were quantified by using a DX-500 high performance liquid chromatograph (Dionex, Jouy en Josas, France) equipped with an anion-exchange column (AS11 IonPac column 4×250 mm, Dionex, Jouy en Josas, France) fitted with a AG11 guard column. Sodium hydroxide from a fresh bottle of 50% NaOH solution that was low in carbonate concentration, (JT Baker, Deventer, The Netherlands) was used as eluent under linear gradient elution conditions. The linear gradient elution program was initially 0.2 mM NaOH and reached 30 mM in 23 min. Separations were carried out at 20°C and at a flow-rate of 2 ml min^{-1}. Before detection by the ED40 conductivity detector maintained at 35°C, an auto-suppression external water mode was used with an anion self-regenerating suppressor (ASRS-I, 4 mm). Standard solutions of sulphate and citrate with concentrations of 1, 2, 5, 10, 20 mg kg^{-1} were prepared from a 1000 mg l^{-1} commercial solution of sulphate and from a citric acid monohydrate salt (Merck, Darmstadt, Germany). Injection (25 μl volume) was carried out using an auto-sampling device.

2.5. Phosphate quantification

Phosphorus concentration was determined according to the IDF standard 33B method, 1987 (molecular adsorption spectrometry of phosphomolybdate compounds) in which the mineralising step was eliminated, because only the initial phosphorus solution and solution from permeate compartment were analysed. A conversion factor of 95/31 was used to convert the phosphorus concentration into phosphate concentration.

2.6. Calcium and magnesium quantification

Ca^{2+} and Mg^{2+} concentrations were quantified by using an atomic absorption spectrometer (Varian AA300 Sunnivale, USA), according to the method described by Brulé, Maubois, and Fauquant (1974).

2.7. Iron–lysozyme complex analysis by electrospray ionisation mass spectrometry (ESI–MS)

Lysozyme (4 g l^{-1}) and iron stock solution prepared from $FeCl_2$ (Sigma, St Quentin Fallavier, France) was used to prepare the Fe/lysozyme solutions with molar ratios of 1, 2, 3, 5 and 10. A pH of 4, was maintained for all preparations. All experiments were performed with a quadrupole mass spectrometer (API,III, Sciex, Toronto, Canada). Solutions were sprayed through a stainless steel capillary held at 4.8 kV, generating multiply charged ions. Positive ionisation was used. The liquid nebulization was assisted with coaxial air flow along the sprayer. The nebuliser pressure was usually adjusted within the range 0.4–0.45 MPa. The solution was delivered to the sprayer by a syringe infusion pump (through a fused silica capillary of 0.75 μm i.d.) at 5 μl min^{-1}. The interface between the sprayer and the mass analyser consisted of a conical orifice of 100 μm diameter. To enhance ion signals, the potential on the orifice was 110 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 (m/z) scale was calibrated with the ammonium adduct of propylene 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 2.5 and masses were calculated with Biomultiview 1.2.

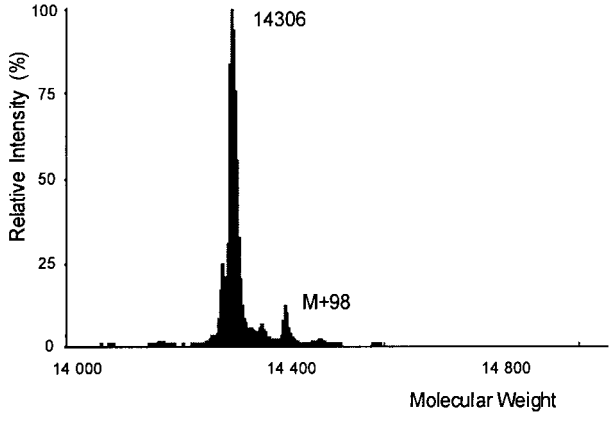

Fig. 1. Mass spectrum of commercial hen egg white lysozyme with molecular masses on the horizontal axis.

3. Results and discussion

3.1. Lysozyme characterisation

Native hen egg white lysozyme was diluted in bidistilled water (MilliQwater, Millipore, St Quentin-Yvelines, France). Mass spectrum indicated the multiply charged ions were in the range from 1000 to 2500 amu (atomic mass unit). The average molecular mass, M, was calculated as 14 306 Da (Fig. 1), consistent with the value of 14 304.2 Da obtained from the primary sequence of the protein.

The mass spectrum also showed one compound with a mass of M + 98 Da, the area of which was 10% of the total lysozyme area (Fig. 1). This peak was reduced with dialysis against TFA, pH = 2.5, suggesting an electrostatic interaction between lysozyme and a compound of 98 g mol^{-1}. As lysozyme was extracted from egg white by using ion-exchangers, the presence of a phosphate, or a sulphate anion bound to lysozyme, seemed to be the most likely explanation for the mass M + 98 Da.

3.2. Lysozyme–anion interactions

The above hypothesis was explored by studying interactions between phosphate and sulphate anions with the lysozyme molecule by using the Scatchard representation at pH 6 or 9 and ionic strengths of 30 or 120 mM. Ionic strength and pH conditions have been chosen as follows: pH 9 and ionic strength 120 mM, which are egg white characteristics. A lot of food products have a pH of 6. Thirty mM was the lowest ionic strength used for studying ion/lysozyme interactions with an ultrafiltration membrane, to avoid ionic disturbance by the Donnan effect (Donnan, 1911). Citrate anion, an authorised additive used in the food industry, was also studied under the same conditions.

When the Scatchard method was applied to sulphate and phosphate anions, no binding site appeared whatever the pH (6 or 9) and the ionic strength (30 and 120 mM). Thus, either suggested anions were not included in mass 98 or they were bound to the positively charged

residues of the protein as counter ions. In fact, ESI–MS analysis needs an ionic strength lower than 1 mM, whereas the ionic strength was set at 30 and 120 mM for Scatchard analysis. Competition to neutralise protein charge between Cl^- and $H_2PO_4^-/HPO_4^{2-}$ or SO_4^{2-} was in favour of Cl^- which was in great excess.

At pH 6, the Scatchard representation revealed 0.76 ± 0.038 and 0.28 ± 0.025 citrate binding sites on lysozyme for ionic strengths of 30 and 120 mM, respectively (Fig. 2). An affinity constant of 3.88×10^3 $M^{-1} \pm 0.38 \times 10^3$ M^{-1} was calculated at an ionic strength of 30 mM, and 4.17×10^3 $M^{-1} \pm 0.80 \times 10^3$ M^{-1} for an ionic strength of 120 mM. With a pH increase to 9, we witnessed the disappearance of citrate fixation whatever the ionic strength.

The ionic strength increase significantly ($p < 0.01$) reduced the number of citrate binding sites on lysozyme. These citrate–lysozyme bonds were probably electrostatic bonds and they were of low affinity. For the two ionic strengths, affinity constants were not statistically different.

Citrate behaviour was different for the two pH conditions. Experiments revealed that citrate anions bound to lysozyme only at pH 6. For electrostatic attractions, two distinct parameters act simultaneously. The first one is the citrate charge and the second one is the lysozyme charge.

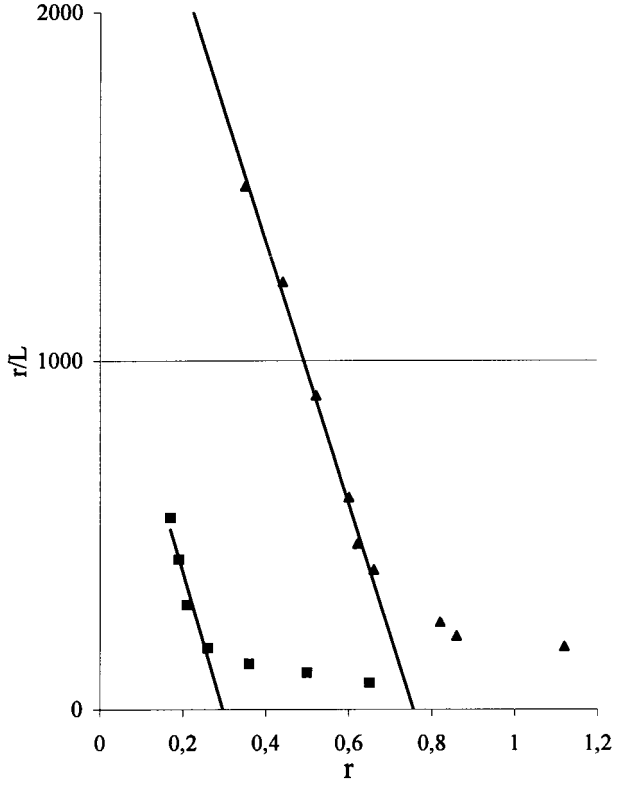

Fig. 2. Scatchard plot of r/L versus r for binding of citrate to lysozyme at pH 6 and ionic strengths 30 mM (▲) and 120 mM (■). r is the binding ratio and L is the free citrate concentration.

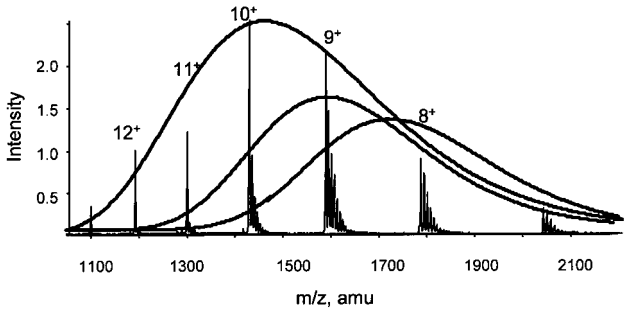

Fig. 3. Electrospray ionisation mass spectrum at pH 4 of iron–lysozyme complexes at iron/lysozyme molar ratio of 10. The protonation states are indicated above the peaks.

Lysozyme charge seemed to be the most influential factor explaining citrate anion binding. The citrate pKa_3 is 6.4. At pH 9, citrate with a triple negative charge was not bound to lysozyme, whereas at pH 6, 30% with a triple negative charge and 70% with a double negative charge, had an affinity for lysozyme. Either, only citrate with two negative charges bound to lysozyme or, more probably, the lysozyme electropositivity increase to pH 6, enabled the interaction with citrate. Electrostatic attraction to citrate needs positive charges on the lysozyme molecule. All amino acids which give rise to positive charges on proteins, except histidine ($pKa_3 = 6.1$), have dissociation constants higher than 9. Thus, when the pH decreased from 9 to 6, protonation of the histidine lateral chain was possible. The lysozyme molecule

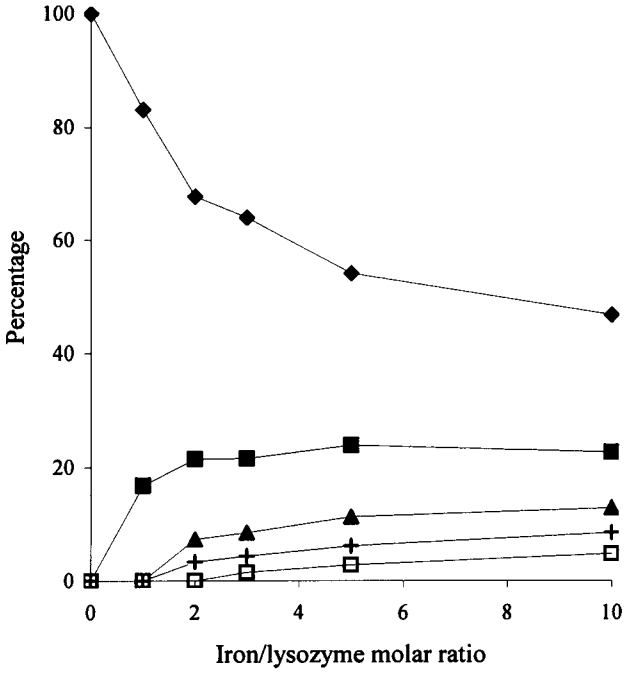

Fig. 4. Percentage of uncomplexed and complexed iron–lysozyme as a function of iron/lysozyme molar ratio. This figure is obtained from the results shown in Fig. 5: uncomplexed lysozyme (◆); lysozyme–$(Fe)_1$ (■); lysozyme–$(Fe)_2$ (▲); lysozyme–$(Fe)_3$ (+); lysozyme–$(Fe)_4$ (□).

has one histidine, of which only a half is protonated at pH 6. Moreover, 0.76 or 0.28 citrate binding sites per lysozyme molecule were calculated using the Scatchard representation at pH 6 with ionic strengths of 30 or 120 mM, respectively. Thus, the hypothesis of electrostatic attractions between citrate anions and positive charges, including one protonated histidine, might be expressed.

3.3. Lysozyme–cation interactions

Calcium, magnesium and iron interactions with lysozyme have been studied. Calcium and magnesium are widely distributed in food products and iron contamination often comes from industrial equipment and water.

Scatchard representation has been used for studies of calcium and magnesium interactions under the same conditions as anions. We were unable to determine any affinity between Ca^{2+} and Mg^{2+} and the lysozyme molecule for ionic strengths, 30 and 120 mM, at pH 6 and 9.

Interaction between the iron cation and the lysozyme molecule has been studied using the ESI–MS method. The mass spectrum indicated a multi-charged ion, having a m/z range from 1000 to 2500 amu. The overall shape of mass spectra was conserved when iron was added. The most intense signals came from ions with 10 positive charges (Fig. 3), irrespective of the presence or absence of iron. The increasing concentrations of iron led to the reduction of the free enzyme concentration due to lysozyme forms which bound one to four iron ions (Fig. 4), thus confirming the results of Ramadan and Porath (1985). However, the free form was always prevalent. With an iron/lysozyme molar ratio greater than 3, iron addition did not increase the proportion of lysozyme–$(Fe)_1$ complex. An equilibrium seemed to have been established between the

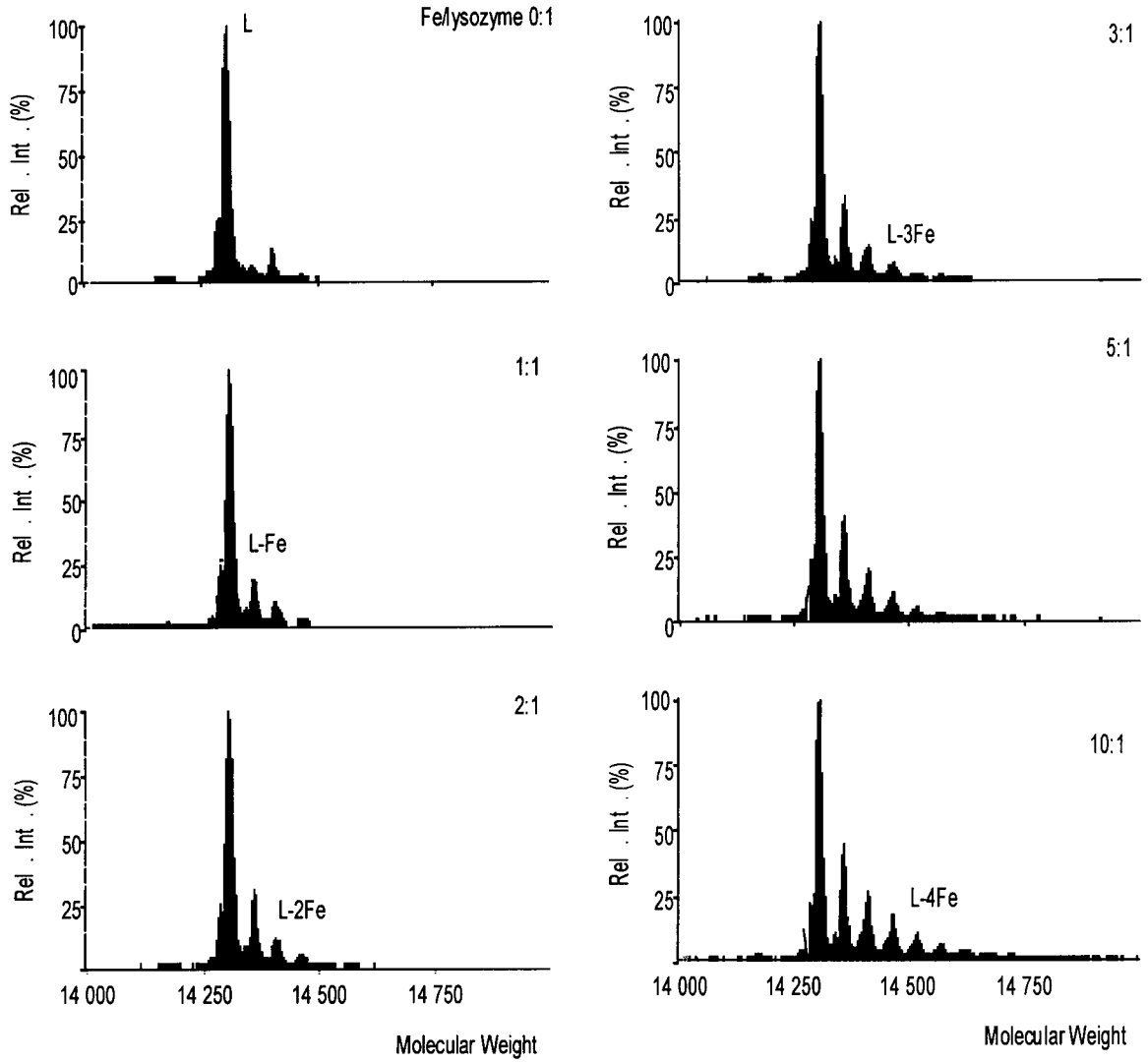

Fig. 5. Mass spectra of lysozyme in the absence and in the presence of iron, based on the ions with 10 positive charges, with molecular masses on the horizontal axis.

uncomplexed lysozyme and the complexed forms. This hypothesis has been suggested for lysozyme binding studies with zinc and copper (Moreau et al., 1995).

After dialysis of iron–lysozyme mixture against water, only the free lysozyme was detected by ESI–MS (data not shown). This indicated that the interactions between iron and lysozyme were of low affinity.

The observed molecular masses for lysozyme and iron lysozyme complexes indicated incremental differences of 53 g between two neighbouring molecular masses (Fig. 5), suggesting that binding of one atom of iron (56 g) released three protons. The proportion of iron-lysozyme complexes increased with the protein m/z ratio (Fig. 3), indicating that the iron binding decreased the protein charge. For example, for one and two iron ions loaded onto the lysozyme molecule, the most intense peaks were, respectively, lysozyme with nine and eight positive charges. Iron binding on the lysozyme would then occur on positively charged sites with proton release. At pH 4, the amino acids, lysine, arginine and histidine, are positively charged. Lysozyme would co-ordinate iron with the aminated groups of one of these three amino acid lateral chains leading to a reduction in the number of ionisable sites of the complexes. The release of the two other protons would come from the glutamic and/or aspartic amino acid lateral chains. FT–IR study of Fe^{3+}-binding to lysozyme could answer this hypothesis by using the same method as Mizuguchi, Nara, Kawano and Nitta (1997). The induced negative charges would co-ordinate the iron cation. Lysozyme oxygen and sulphur atoms and water oxygen atoms would participate in the missing co-ordination links.

ESI–MS analysis revealed that copper and zinc formed complexes with lysozyme (Moreau et al., 1995). In spite of an equivalent number of binding sites (four or five binding sites for a metal/lysozyme molar ratio of 10), the proportion of iron–lysozyme complex was only 53%, whereas the proportions of copper–lysozyme and zinc–lysozyme complexes were 70 and 83%, respectively. But pH values used for the copper–lysozyme and zinc–lysozyme interaction analysis (pH 7) could not be achieved for the iron–lysozyme interaction study. Iron binding on lysozyme produced a noteworthy pH decrease which could not be corrected by NaOH because of the formation of ferric oxide. At pH 4, protons competed more strongly with the metal ions for the protonisable groups of the amino acid lateral chains, whereas some of these functions had already lost protons at pH 7.

4. Conclusion

This work reveals interactions between lysozyme and iron and citrate ions. In the experimental conditions used, the co-ordination bonds between lysozyme and iron and the electrostatic bonds between lysozyme and citrate were of low affinity. For both binding types, the aminated functions of the amino acid lateral chains were involved.

Iron co-ordination needs several electron donor functions near the metal ion. The decrease of lysozyme positive charge, following iron addition, assumes one proton release from a quaternary amine to enable iron co-ordination. The two other protons would come from the glutamic and/or aspartic carboxylic functions. Lysozyme oxygen and sulphur atoms and water oxygen atoms are also able to provide the other electrons for the iron co-ordination.

Our results also indicated that, citrate attraction is dependent on the protein charge. Citrate–lysozyme electrostatic bonds would be situated on the quaternary aminated lateral groups and the hypothesis of a protonated histidine for interaction is not excluded.

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