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ESI-MS measurements for the equilibrium constants of copper(II)-insulin complexes

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ABSTRACT

Trace elements regulate many biological reactions in the body. Copper(II) is known as one of trace elements and capable of binding to proteins. Insulin is a blood glucose-lowering peptide hormone and it is secreted by the pancreatic β-cells. In this study, Cu(II)-insulin complexes were investigated by using ESI-MS method. Insulin molecule gives ESI-MS peaks at +4, +5, +6 and +7 charged states. Cu(II)-insulin complexes can be monitored and quantified on the ESI-MS spectra as the shifted peaks according to insulin peaks. The solutions of Cu(II)-insulin complexes at different pHs and mole ratios of Cu(II) ions to insulin molecule were measured on the ESI-MS. The highest complex formation ratio for Cu(II)-insulin were found at pH 7. The multiple bindings of Cu(II) ions to insulin molecule was observed. The formation equilibrium constants of Cu(II)-insulin complexes were calculated as Kf_1: 3.34×10^4, Kf_2: 2.99×10^4, Kf_3: 7.00×10^3 and Kf_4:2.86×10^3. The specific binding property of Cu(II) ions was controlled by using different spray ion sources including electrospray and nano-electrospray. The binding property of Cu(II) also investigated by MS/MS fragmentation. It was concluded from the ESI-MS measurements that Cu(II) ion has a high affinity to insulin molecules to form stable complexes.

1. Introduction

Insulin is a peptide (protein) hormone consisting of 51 amino acids synthesized and stored within the pancreas in the body. It has two peptide chains, denoted A and B, linked by disulfide (sulfur sulfur) bridges between cysteine residues. The molecular weight of insulin is 5733.49 Da [1–3]. Insulin carries total 51 amino acids and 6 of them positively and 10 negatively charged amino acids. The iso-electric point of insulin is 5.3 [4].

Zinc and calcium ions play important roles in the biosynthesis and storage of insulin in the body. The $(Zn^{2+})_2(Ca^{2+})(Ins)_6$ hexamer complex formed in the pancreatic β-cell. This hexamer complex is an inactive and storage form of insulin. The hexamer complex converted to insulin monomer for biological activity [5,6]. Insulin can form many adduct complexes by binding the electrolyte ions of Ca(II), Mg(II), Na(I), K(I) and many trace elements such as Zn(II) and Cu(II) etc. In our previous works, we found by electrospray ionization mass spectrometry (ESI-MS) method that insulin has high affinity to the electrolyte ions as in the order of Ca(II) > Mg(II) > Na(I) > K(I)) [7,8]. If one of them is absent, the other ion or ions can include in the complex formation with insulin.

Trace elements are very important in the body. They regulate many physiological reactions by forming their complexes (adducts) with peptides, proteins, carbohydrates and other biological small or macromolecules. The average intakes of copper by human adults, vary from 0.6 to 1.6 mg/d [9]. Copper is an essential trace element in living systems, forming a large number of metalloproteins. Examples of some copper protiens are ceruloplasmin (human serum protein); ascorbate oxidase (plants and bacteria); plastocyanin (higher plants and cyanobacteria); superoxide dismutase, tyrosinase, cytochrome oxidase and hemocuprein (animals) [10]. Electron transfer, oxygen transport, oxidation, reduction and disproportionation are the functions of the copper proteins [11]. The copper proteins are divided into mainly three groups as Type I (blue copper proteins, bound to two histidine, one cysteine and methionine), Type II (bound to two histidine), Type III (binuclear Cu) [11–13]. Cu(II)-insulin adduct complexes are possible because of the including histidine and cysteine amino acids of insulin molecule.

The binding of metal ions to peptides or proteins can be monitored by ESI mass spectrometers. Electrospray ionization (ESI) produces multiply charged molecules from solution under mild conditions [14,15]. In the ESI-MS system, the charged peptides or metal-peptide complexes are generated directly from their aqueous solutions in the presence of electric field. The soft nature of ion generation in ESI MS allows their transition to the gas phase preserving weak noncovalent complexes as well. ESI-MS is capable of measuring the compositions and binding stoichiometry of metal–protein complexes to be established directly [16].

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Successful studies by using ESI-MS method are given in the literature [17–22].

In the present work, Cu(II)-insulin adduct complexes were investigated by using ESI mass spectrometer. The effect of pH on the binding of Cu(II) ions to insulin molecule were examined. The dissociation equilibrium constants (K_1, K_2, K_3 and K_4) and formation equilibrium constants (K_{f1}, K_{f2}, K_{f3} and K_{f4}) of Cu(II)-insulin complexes were calculated by considering the binding stoichiometry. The ESI-MS spectra of Cu(II)-ins. complexes were measured by using aqueous solutions of the complexes. The effect of flow rate to the peak intensities and MS/MS spectra were also examined for the stability of Cu(II)-insulin complexes.

2. Experimental

2.1. Materials

In all the experimental studies, Bovine insulin (Mw: 5733.49 Da, Sigma-Aldrich, St Louis, MO, USA) was used. $CuSO_4 \cdot 5H_2O$ was obtained from Merck (Darmstadt, Germany) and its desired solutions were prepared. All the other reagents were of analytical grade. Ultra-pure deionized water (18.2 MΩ) was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard®2 and a QuantumTM EX (Millipore Bedford, MA). The ultra-pure deionized water was used in the preparation or dilution of the solutions.

2.2. ESI-MS instrument

The experimental ESI-MS spectra of the insulin molecule and Cu(II)-insulin complexes were measured on an Esquire 3000 spectrometer with quadrupole ion trap mass analyzer (ESI-QIT-MS) (Bruker Daltonics Inc. USA). In the ESI-MS measurements, the following conditions were performed; the flow rate of the sample solution was 150 μL/h, the nitrogen pressure was 12.0 psi, the capillary voltage was −4.5 kV and the capillary heating temperature was 250 °C. The spray needles having 350 μm o.d. and 100 μm i.d. for electrospray and 150 μm o.d. and 5 μm i.d. for nano-electrospray were used. All the ESI-MS measurements, the spectra were recorded consecutively at the target mass of *m/z*: 1000 Da for one-minute period and 1500 Da for one-minute period. All the collected spectra were averaged in the specified *m/z* region.

2.3. ESI-MS measurement of insulin solution

A stock insulin solution (3.07×10^{-4} M) was prepared by dissolving the insulin powder in 1 mL volume including 950 μL of ultra-pure water and 50 μL of glacial acetic acid (CH_3COOH). The prepared insulin solutions were stored at 4 °C temperature. After testing different insulin concentrations, the best peak intensity of the insulin was obtained by using 1×10^{-6} M insulin concentration.

2.4. Effect of pH on Cu(II)-insulin complexation

The effect of pH on the formation of Cu(II)-insulin complexes was studied at pH 2, 3, 4, 5, 6, 7 and 8. In the first step of the complex formation, the insulin solution was prepared in different pH values and then Cu(II) was added in these solutions. By providing constant molar ratio of Cu(II) to insulin (100/1), the ESI-MS spectra were measured at different pH values. The solution pH values were adjusted by using CH_3COOH and NH_3 solutions. After determining the best pH values for complexation, we used CH_3COOH, NH_4CH_3COO buffer to maintain the constant solution pH and all other solutions were prepared by using the same buffer solution.

2.5. Cu(II)/insulin mole ratios

By keeping constant the concentrations of the insulin (1×10^{-6} M) and buffer (1×10^{-5} M) solutions, the ESI-MS spectra of the complexes were measured at different molar ratios of Cu(II)-ions to insulin molecule. To calculate the equilibrium constants of Cu(II)-insulin complexes, the different concentrations of Cu(II) ions in constant 1×10^{-6} M insulin solutions were prepared at pH 7 and they were measured on the ESI mass spectrometer. After the optimization tests, the measurements were carried out at the Cu(II)/insulin mole ratios of 10/1, 20/1, 40/1, … 250/1. The ESI spectra were taken after 30 min. by the mixing the Cu(II)ions with insulin solutions. The samples were prepared at room temperature. The equilibrium constants of Cu(II)-insulin complexes have been calculated from the peak intensities of the un-complexed and the complexed insulin.

2.6. MS/MS spectra measurements of Cu(II)-insulin

The MS/MS spectra (Tandem spectroscopy) of the Cu(II)-insulin complexes were also examined for the stability of copper binding to insulin molecule. The high intensity peaks of the primary MS spectra of Cu(II)-insulin complexes were selected for the MS/MS measurements. The selected peaks at 956.7 and 1147.8 *m/z* for insulin and 828.9, 967.0 and 1160.1 for $Cu(II)_1$-insulin and 837.8, 977.4 and 1172.6 *m/z* for $C(II)_2$-insulin were fragmented. The MS/MS measurements were taken by applying an ion isolation RF waveform voltage at the endcaps. At this time, the ring electrode voltage is ramped to a new value to trap both precursor and its products. During this time being, CID experiment was done by applying a resonance excitation RF voltage at the endcaps. This activates the ions first and subsequently fragmentation of ions.

2.7. Effect of flow rate with nanoESI-MS

The binding ratios of Cu(II) ions to insulin molecule were examined by changing flow rates in nanoESI-MS system. The spray droplets from the nanoESI system will be lower volume at lower flow rates. Increasing the flow rate will create bigger droplets. Depending on the concentration, number of insulin and metal ion number will increase inside the droplet. Protein-ligand complex desolvation will need longer time for complete desolvation and after desolvation, more metal ion will be exists around the insulin. This may cause some extra nonspecific binding in gas phase during the measurements. Therefore, we examined the binding ratios at different flow rates with nanoESI system. The nanoESI-MS measurements were performed with nano-ESI needles which are made of silicate capillaries with 150 μm o.d. and 5 μm i.d. NanoESI-MS measurements were studied at the flow rates of 833–2500 nL/min for controlling the peak intensity ratios of Cu(II)-insulin to insulin. The Cu(II)-insulin solutions in 50% MetOH +50% water solvent were used in the nanoESI-MS studies.

3. Results and discussion

3.1. Binding properties of Cu(II) ions to insulin molecule

ESI-MS is a powerful instrument for monitoring covalent and non-covalent bindings of metal ions to peptides, proteins and other biomolecules. The bound metal ions to a biomolecule in the solution phase can be maintained during the desolvation and transfer to the gas phase. Thus, the solution phase stoichiometry of complex and the binding ratios can be monitored directly by ESI-MS [23–25]. The equilibrium constants can be obtained from the ESI-MS data [7,8].

The ESI-MS spectra of the insulin and Cu(II)-insulin complex solution in water were compared in Fig. 1. In general, the ESI MS measurements are performed by using the solvent mixture of met50% + wat%50. However, water is main solvent in the biological fluids of the body. Therefore, in this study, the ESI-MS measurements for the