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Probing the toxic mechanism of Ag^+ with lysozyme

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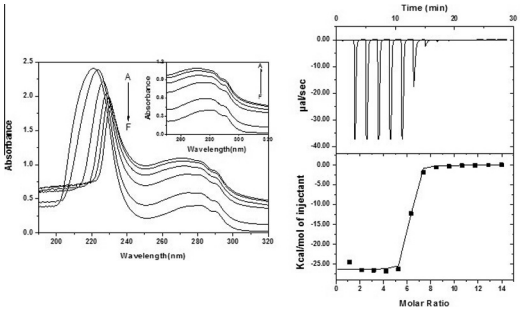
HIGHLIGHTS

- This work investigated the toxic mechanisms of Ag^+ to lysozyme.
- Ag^+ interacted with lysozyme and changed the frame structure of lysozyme to become looser.
- Ag^+ could spontaneously bind with lysozyme through hydrogen bonding and van der Waals forces.
- Ag^+ bound to lysozyme at the active site and furtherly influence the function of lysozyme.

GRAPHICAL ABSTRACT

Ag^+ could spontaneously interact with lysozyme through hydrogen bonds and van der Waals forces with one binding site, resulting in the conformational changes of lysozyme and the decrease of enzyme activity. Thus, Ag^+ may influence the structure and function of proteins.



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ABSTRACT

Silver (Ag) is widely used in human activities, which provides possibilities to distribute in organisms and tissues, resulting in harmful effects on human health. In this work, lysozyme was chosen as the target molecule to study the mechanism of toxic interactions between Ag^+ and protein using fluorescence emission spectra, synchronous fluorescence spectra, UV–vis absorption spectra, circular dichroism (CD) spectra, isothermal titration calorimetry (ITC), and enzyme activity assay. The results of fluorescence emission and synchronous fluorescence showed that there were interactions between Ag^+ and lysozyme by eliminating the inner filter effect (IFE). Data from UV–vis spectra indicated that the frame structure of lysozyme became looser with Ag^+ existent, while the micro-environment of aromatic amino acid residues did not show any significant alteration. CD results suggested that the secondary structure of lysozyme presented a decrease in α-helix contents with the increasing amount of Ag^+. ITC results showed Ag^+ can spontaneously bind with lysozyme through hydrogen bonding and van der Waals forces with one binding site ($K_a = 1.93 \times 10^6$). The lysozyme activity was inhibited by Ag^+ according to the enzyme activity assay, revealing that Ag^+ bound to lysozyme at the active site which resulted in inhibition of lysozyme activity. This work showed that Ag^+ can cause damages to the structure and function of lysozyme.

1. Introduction

Silver is a kind of transition metal, which is rare in the earth's crust [1]. As a widespread substance, silver compound is widely used for photographic materials, artificial rainfall and antibacterial agent [2–4], which causes Ag^+ easily entering into living organisms [5,6]. As one of the trace elements in human being, microscale of silver is harmless to human body, but excessive intake of silver may affect the activity of protease in the body, threatening our health and even endangering the life [7].

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Lysozyme, also known as muramidase, is a 14.6-KDa single chain protein composed of 129 amino acid residues [8,9]. There are 6 tryptophans and 3 tyrosines in lysozyme, and three of the Trp residues are located in the substrate binding sites, two are in the hydrophobic and one is located at the edge of lysozyme. Among them, Trp 62 and Trp 108 which are located in the substrate binding sites play important parts in binding to substrate and stabilizing the structure. As an important molecule in the innate immune system, lysozyme participates in a variety of immune responses. For example, lysozyme plays the role of sterilization by destroying Gram-positive bacterial cell wall to protect the cells from death [10–12]. In addition to the antibacterial, it also can be used as antioxidant, antiseptic and antiviral [13–16]. Lysozyme is widely found in eukaryotes and prokaryotes [17]. In humans, it is mainly distributed in body fluids including blood, urine and tears. Some diseases may occur due to the change of content of lysozyme in body fluids [18]. Therefore, we choose lysozyme as the target molecule to study the mechanism of toxic interactions of Ag^+.

Currently, many scholars have studied the toxicity mechanism of silver ions and nano silver at the cellular and protein molecular level [6,19–21]. However, fluorescence inner filter effect is ignored in many fluorescence experiments, which may affect the accuracy and precision of fluorescence assays. Moreover, little work has been done to study the interaction of Ag^+ and protein by ITC which can provide the precise determination of the binding affinity constant (K_a), binding stoichiometry (n), enthalpy changes (ΔH), entropy changes (ΔS) and Gibbs energy changes (ΔG). We studied the mechanism of toxic interactions between lysozyme and Ag^+ by spectroscopic and ITC method. The fluorescence inner filter effect was corrected using the method of Lakowicz. The study has great significance for understanding the toxicity of silver ions at the molecular level and contributes to improve medical diagnosis and targeted therapy [22].

2. Materials and methods

2.1. Materials

Lysozyme (from chicken egg white, Amersco, USA) was dissolved in ultrapure water to form a 1.0×10^{-4} M solution, and then preserved at 0–4 °C. $AgNO_3$ (Sinopharm Chemical Reagent Co., Ltd) was dissolved in ultrapure water to form a 1.0×10^{-2} M stock solution. HAc-NaAc buffer solution (0.2 M) was used to stabilize pH at 5.5. Micrococcus lysodeikticus powder and standard lysozyme (20,000 U/mg) with a special solvent for M. Lysodeikticus were purchased from Jiancheng Bioengineering Institute (Nanjing, China). All the reagents were analytical reagent grade and ultrapure water was used throughout the experiments.

2.2. Apparatus

In this work, the fluorescence spectra and synchronous fluorescence spectra were recorded on an F-4600 spectrophotometer (Hitachi, Japan). UV–vis absorption spectra were made with a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). CD spectra were collected using a J-810 CD spectrometer (JASCO, Japan). ITC experiments were carried out with a MicroCal ITC 200 microcalorimeter. All the pH measurements were taken with a pHs-3C acidity meter (Pengshun, Shanghai, China).

2.3. Methods

2.3.1. Fluorescence measurements

In a series of 10 mL colorimetric tubes, 1.0 mL HAc-NaAc buffer solution (pH = 5.5) and 1.0 mL lysozyme solution (1.0×10^{-4} mol/L) were added in each tube, and then we added different volume of Ag^+ aqueous solution (1.0×10^{-3} mol/L). After 30 min stabilization, the fluorescence spectra were measured on an F-4600 fluorescence spectrophotometer with a 1.0 cm cell. The excitation wavelength was 278 nm. The scan range was 285–420 nm. Excitation and emission slit widths were 5 nm. PMT (photomultiplier) voltage was set at 650 V, and the scan speed was 1200 nm/min.

The synchronous fluorescence spectra were measured under a fixed wavelength interval between excitation and emission wavelength ($\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm). The scanning speed was set at 1200 nm/min, and the excitation and emission slit widths were 5 nm.

2.3.2. UV–vis absorption measurements

The UV–vis absorption spectra were scanned from 190 nm to 320 nm with 1.0 cm quartz cells under the slit width of 2 nm. The $AgNO_3$ solution of the same concentration without lysozyme was used as the reference in the measurement.

2.3.3. CD measurements

The CD spectra were record on a J-810 CD spectrometer in the range of 190–260 nm, using a 1 mm quartz cell and the scan speed of 200 nm/min. The measurement results were the average of two scans.

2.3.4. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was collected on a MicroCal iTC 200 microcalorimeter. $AgNO_3$ and lysozyme solution were diluted by NaAc-HAc (0.02 M, pH = 5.5), and then filtered with micro-filters (0.22 μm) before measurement. The sample cell was filled with 280 μL lysozyme and the injection syringe was loaded with 40 μL $AgNO_3$. The measurement was performed under a stirring speed of 1000 rpm with equilibration time of each injection fixed at 120 s and the cell temperature kept at 298 K.

2.3.5. Lysozyme activity determination

The activity of lysozyme was measured by the conventional turbidimetric method [23]. The bacterium fluid (2.5 μg/mL) was prepared by M. Lysodeikticus bacterial powder (5 mg) and bacterial powders solvent (20.0 mL). Sample lysozyme (0.2 mL) solution with different concentrations of Ag^+ and the standard lysozyme solution (2.5 μg/mL, 0.2 mL) were respectively placed into a colorimetric tube, and the application bacterium fluid (2.0 mL) were added immediately. We put them into 37 °C water bath for 15 min and immediately transferred them to an ice water bath for 3 min. Then the transmittances were collected at 530 nm in a 1 cm quartz cell. The activity of lysozyme (A_{lys}) was calculated by the following equation:

$$A_{lys} = \frac{UT_2 - UT_0}{ST_2 - ST_0} \times 2.5 \times 80 \quad (1)$$

where UT_2 and UT_0 denote the light transmittances of the sample lysozyme at 5 s and 125 s, respectively. ST_2 and ST_0 represent the light transmittances of the standard lysozyme, and 2.5 represents the concentration of the standard lysozyme solution. Then 80 is the dilution multiple of the sample lysozyme before testing.

3. Results and discussion

3.1. The influence of Ag^+ on the fluorescence spectra of lysozyme

In order to study the interactions between small molecules and proteins, the fluorescence emission spectrum was measured under simulated physiological conditions. Lysozyme has intrinsic