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Spectroscopic Investigation on the Interaction of Pb(II) with Keyhole Limpet Hemocyanin

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Abstract

The interaction between Pb^{2+} and Keyhole Limpet Hemocyanin (KLH) was investigated by fluorescence, Ultraviolet-visible (UV-Vis) absorption and Circular Dichroism (CD) spectroscopy. The experimental results showed that Pb^{2+} could quench the intrinsic fluorescence of KLH following static and dynamic quenching process. The number of binding sites n was approximately equal to 1. $\Delta H < 0$ and $\Delta S < 0$ indicated that a Pb-KLH complex was formed. Furthermore, the data of synchronous fluorescence, UV-Vis and CD spectra suggested that Pb^{2+} changed the micro-environment and conformation of KLH.

Keywords: Keyhole limpet hemocyanin; Lead ion; Spectroscopic techniques

Introduction

Lead is a common heavy metal contaminant in the environment, which causes serious damage to the human health when exceeding the tolerable concentrations. Many studies have shown that lead is not an essential element for many organisms, but it can interact with biological macromolecules, and then interfere with the normal physiological functions [1]. A large number of researchers had already studied the interaction of small molecules. For example, Kandagal et al. have studied the interaction between drug doxepin and human serum albumin, and Xu et al. have investigated the binding of pesticide phenthoate to bovine serum albumin [2,3]. Currently, the molecular interactions between proteins and many heavy metal ions have been investigated successfully, for example Lead [4], Chromium [5], Nickel [6] and so on.

Hemocyanin (Hc) plays an important role as dioxygen carriers in the hemolymph of many molluscs and arthropods [7]. Interest in the molluscan Hc is based primarily on the unique immunostimulatory properties of KLH, which is a high molecular weight, copper-containing protein found in the sea mollusk *Megathura crenulata*. KLH is used to be as an immunotherapeutic agent for the treatment of certain types of cancer and for the diagnosis of Schistosomiasis [8]. Currently, Hc has been extensively studied in the following aspects: the structure, evolution and diversity, the relationship between the structural features [9,10]. But little information is available for the interaction between metal ions and Hc, because of the complexity of Hc structure [11]. Studying the influence of heavy metal ions on Hc would evaluate its potential toxicity to the biological species whose respiratory proteins is Hc.

Therefore, we selected Pb^{2+} and KLH as the heavy metal ion and model protein, respectively, then carried out detailed investigations on the interaction mechanism under simulative physiological conditions by spectroscopic techniques. This work explored the binding constants, binding sites and the thermodynamic parameters at different temperatures. The effect of Pb^{2+} on the microenvironment and conformation of KLH was analyzed by synchronous fluorescence, UV-vis absorption and circular dichroism spectra.

Experimental

Materials

Keyhole Limpet Hemocyanin (KLH) was purchased from Sigma Chemical Co., USA and used as received. The stock solution of KLH was prepared by dissolving appropriate solid KLH into ultrapure water and stored at 0-4°C, then diluted to 1.0×10^{-7} mol/L using 0.05 mol/L Tris (3-Hydroxymethyl-methane)-HCl buffer (pH 7.4). Lead (II) nitrate (analytical reagent) was obtained from Sinopharm Chemical Reagent Co., Ltd. (China) was dissolved with ultrapure water as a 0.1 mol/L stock solution. All other reagents were of analytical purity without further purification and ultrapure water (18.25 MΩ cm) was used throughout the experiment.

Apparatus

All fluorescence spectra were recorded on LS-55 fluorescence spectrophotometer (Perkin-Elmer, USA). The UV-Vis absorption spectra were collected on a double beam TU-1901 spectrophotometer (Puxi Analytical Instrument Ltd., China). Circular Dichroism (CD) spectra were gauged by a Chirascan Spectropolarimeter (Applied Photophysics Ltd., UK) under constant nitrogen flush. The ultrapure water as obtained from Synergy UV Ultrapure Water System (Millipore Corporation, USA).

Spectroscopic measurements

The fluorescence spectra were performed at different temperatures (298 K, 303 K and 308 K) in the range of 300-450 nm. The excitation wavelength was fixed at 280 nm while the widths of both the excitation and emission slit were set to 10.0 nm. Scan speed was 500 nm/min, PMT (Photo Multiplier Tube) voltage was 725 V. Data presented are from the average of three experiments.

Synchronous fluorescence spectra of KLH in the absence and presence of Pb^{2+} were measured ($\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm, respectively) in range of 200-450 nm.

The UV-vis absorption spectra were recorded at room temperature with slit width was set at 2.0 nm and the wavelength range was 200-390 nm while the Tris-HCl buffer solution as reference solutions. Data presented are from the average of three experiments.

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CD measurements were operated over the range of 200-260 nm by 1.0 nm intervals in a 1.0 mm cell at room temperature. The scanning rate was adjusted to 120 nm/min and bandwidth was 1.0 nm.

Results and Discussion

Fluorescence spectra of the Pb-KLH systems

Fluorescence emission spectra of KLH in the presence of different Pb^{2+} concentrations at 298°K were shown in Figure 1. The fluorescence intensity of KLH decreased gradually with the increasing Pb^{2+} concentration, and the maximum emission wavelength (λ_{max}=340 nm) has no obvious shift. These results indicated that the Pb^{2+} could quench the intrinsic fluorescence of KLH.

The fluorescence quenching mechanism studies

To confirm the quenching mechanism between the interaction of Pb^{2+} and KLH, we analyzed the fluorescence spectra at different temperatures with the well-known Stern-Volmer equation $F0/F=1+Ksv[Q]=1+kq\tau0[Q]$ [12]. Fluorescence intensity data were analyzed according to F0/F versus [Q] (F0 and F are the steady state fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the quencher concentration.) at 298°K, 303°K and 308°K (Figure 2). The value of Ksv (the quenching constant) and kq (the quenching rate constant) were calculated (Table 1).

The quenching mechanisms are usually classified into dynamic quenching and static quenching or both [13,14]. As shown in Table 1, all the values of kq were greater than 2.0×10^{10} L/mol/s [12]. Meanwhile, the Ksv values decreased with increasing temperature (298°K and 303°K) showed that the quenching mode was a static mechanism. On the contrary, the results of 303°K and 308°K indicated that the quenching mode is a dynamic quenching mechanism. This means that the quenching was not only initiated by dynamic collision but also from the formation of a Pb-KLH complex. This experimental result was similar with the previous literature report [6].

Binding constants and the number of binding sites of Pb-KLH

According to the literature [6], the double-logarithm equation [12] can be used to calculate the binding constant (Ka) and the number of binding sites n. Values of n and Ka (Table S1) were calculated according to the curve lg [(F0-F)/F] vs. lg [Q]. From Table S1, Ka values are relatively low (<10^3), which indicates the binding between Pb^{2+} and KLH is not strong. That is, the Pb-KLH complex is not stable. The n is
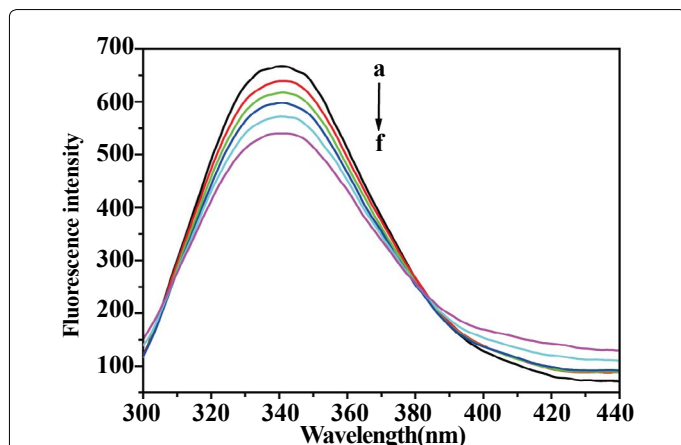

Figure 1: Fluorescence spectra of KLH in the presence of various concentrations of Pb^{2+} (T=298 K, λ_{ex}=280 nm). C_{KLH}=1.0 × 10^{-7} mol/L; C_{Pb}^{2+}/ (10^{-5} mol/L) (a-f): 0, 0.1, 0.5, 1.0, 5.0, 10.0 respectively.
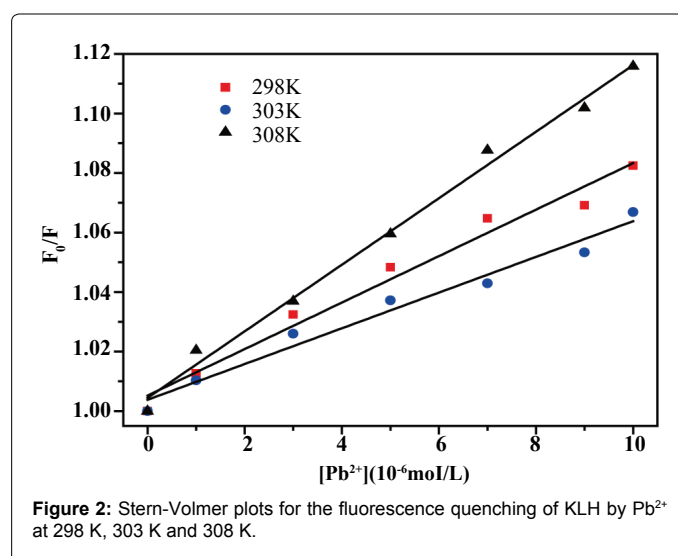

Figure 2: Stern-Volmer plots for the fluorescence quenching of KLH by Pb^{2+} at 298 K, 303 K and 308 K.

pH	T (K)	K_{sv} (10^3 L/mol)	k_q (10^{11} L/mol/s)	R^a	SD^b
7.4	298	7.81	7.81	0.9889	0.0055
7.4	303	5.85	5.85	0.9818	0.0032
7.4	308	11.18	11.18	0.9965	0.0113

[a]R is the correlation coefficient
[b]SD is the standard deviation for the K_{sv} values

Table 1: Stern-Volmer quenching constants for the interaction of Pb^{2+} with KLH at different temperatures.

approximately equal to 1, indicating that the ratio between Pb^{2+} and KLH was 1:1.

The interaction forces between Pb^{2+} and KLH

There are main four types of interactions between small molecules and biological macromolecules [15]. In order to deduce the interaction of Pb^{2+} with KLH, the thermodynamic parameters (ΔG, ΔH, ΔS) were calculated from the Van't Hoff equation lnK=−ΔH/(RT)+ΔS/R and ΔG=ΔH-TΔS [15]. The thermodynamic values of ΔH and ΔS for temperature range of 298-303°K, were calculated according to solve the equation of lnK=−ΔH/(RT)+ΔS/R (Ka is the binding constant, T is the temperature at 298 and 303°K). Next, the equation of ΔG=ΔH-TΔS was used to obtain the ΔG.

As shown in Table S1, ΔG<0 means that the binding process was spontaneous. Moreover, ΔH<0 shows that the reaction is exothermic reaction, increasing temperature will not benefit to generate Pb-KLH complex, thus the Ka values (Table S1) decreased with increasing temperature. Further quenching reason is mainly dominated by a static quenching mechanism, confirming that the formation of a Pb-KLH complex. Refer to the literature [16] ΔH<0 and ΔS<0 indicated that the reaction was mainly enthalpy-driven.

Conformational investigations

To explore the structural changes of KLH with addition of Pb^{2+}, the synchronous fluorescence spectra, UV-vis absorption spectra and CD spectra were measured in this system.

When the D-value (Δλ) is set at 15 nm or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine (Tyr) residues or tryptophan (Trp) residues, respectively [17]. For Tyr and Trp residues, the changes of fluorescence emission peak reflect the polarity of its environment [17].

The effect of Pb^{2+} on KLH synchronous fluorescence spectroscopy was depicted in Figure 3. The synchronous fluorescence intensity for Tyr (Figure 3a) increased with the increasing Pb^{2+} concentration indicated that Tyr was buried more compactly in the internal hydrophobic region, which is similar to the micelle-sensitized fluorimetry [18]. It is apparent that the maximum emission wavelength of the Tyr residue had a blue-shift, indicating the hydrophobicity around the Tyr residue was increased and the polarity was decreased in the presence of Pb^{2+}. In contrast, the synchronous fluorescence intensity for Trp decreased with the increasing Pb^{2+} concentration and the maximum emission wavelength of tryptophan kept its position (Figure 3b), which indicated that the interaction of Pb^{2+} with KLH does not affect the microenvironment of the Trp. Moreover, the addition of the Pb^{2+} resulted in the fluorescence intensity of Tyr (Figure 3a) changed more sharp than that of Trp (Figure 3b). Thus, it is concluded that Pb^{2+} is closer to Tyr residues compared with Trp residues during the binding process.

The UV-vis absorption spectra of the KLH and Pb-KLH system are displayed in Figure 4a and 4b, respectively. As shown in Figure 4a, KLH alone has three absorption peaks at about 210 nm, 280 nm and 344 nm in KLH solution. The absorption peak at about 210 nm reflects the framework conformation of the protein (mainly the peptide strands and the helix structure), at 280 nm peak stands for aromatic residues absorbance [19] the band at 344 nm due to the active site copper(II)-peroxide complex, responsible for the characteristic blue color of oxygenated hemocyanin [20]. As shown in Figure 4b, the absorbance of KLH at 210 nm and 280 nm increased with the addition of Pb^{2+}
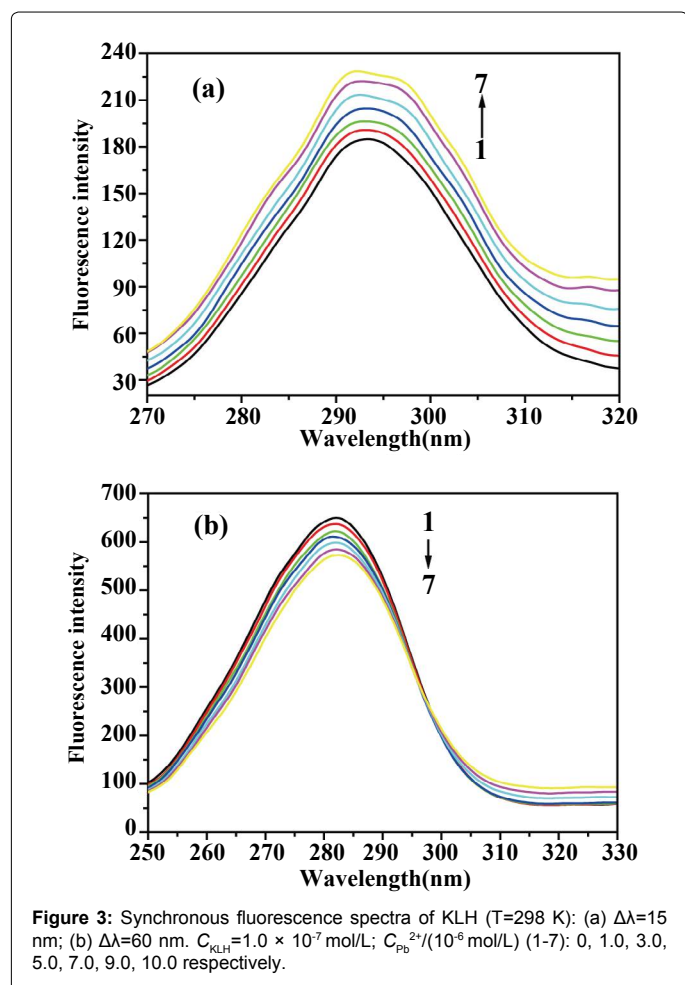

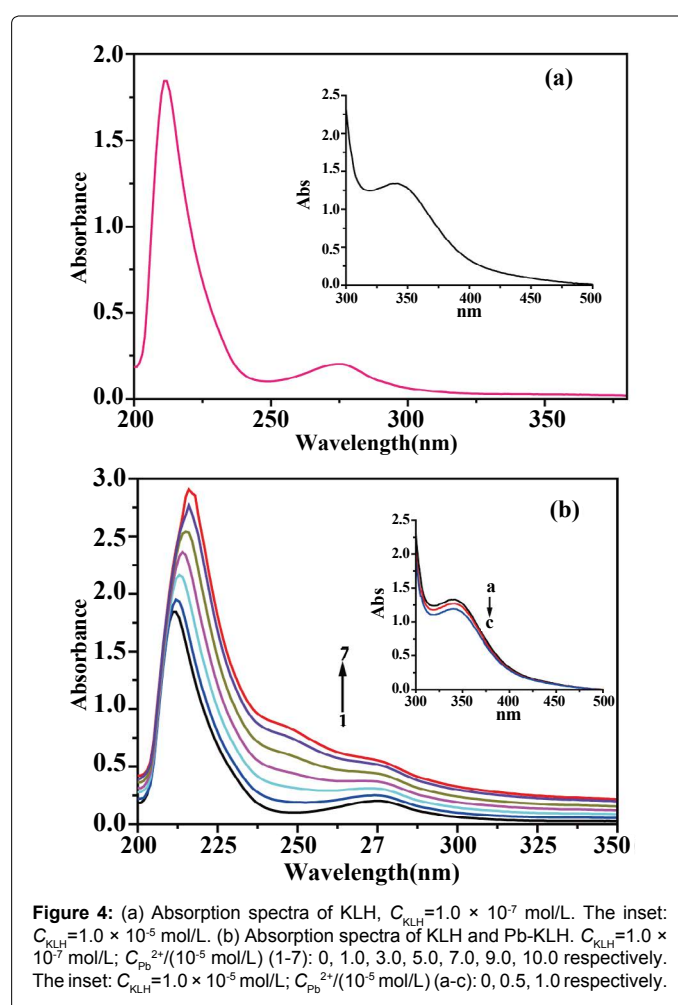**Figure 4:** (a) Absorption spectra of KLH, C_{KLH}=1.0 × 10^{-7} mol/L. The inset: C_{KLH}=1.0 × 10^{-5} mol/L. (b) Absorption spectra of KLH and Pb-KLH. C_{KLH}=1.0 × 10^{-7} mol/L; C_{Pb}^{2+}/(10^{-5} mol/L) (1-7): 0, 1.0, 3.0, 5.0, 7.0, 9.0, 10.0 respectively. The inset: C_{KLH}=1.0 × 10^{-5} mol/L; C_{Pb}^{2+}/(10^{-5} mol/L) (a-c): 0, 0.5, 1.0 respectively.

and the maximum position of the peak at 210 nm had a red-shift. The results indicated that Pb^{2+} interacted with the groups which maintain the skeleton structure of KLH. Pb^{2+} loosens the protein skeleton and promotes the unfolding process, exposing Trp and other aromatic amino acid residues in the internal hydrophobic region to the medium [21]. The peaks at 344 nm have no obvious changes with the addition of Pb^{2+}, because the active site copper(II)-peroxide complex is surrounded by amino acid residues of hydrophobic side chain, usually O_2 and a few small molecules can free access to the active site. These results also confirmed that the potential fluorescence quenching mechanism of KLH with Pb^{2+} is mainly a static quenching procedure [22].

CD spectra of KLH in the absence and presence of Pb^{2+} was shown in Figure S1. The double-peaked band with minima at 210 nm and 222 nm for curve a can be observed, which represents the transition of $n \rightarrow \pi^*$ of peptide strands and α-helical structure [23]. The program package CDPro (Applied Photophysics Ltd., UK) with the prediction algorithms of CDNN was utilized to analyze the detailed secondary structure changes and the results were listed in Table 2. The α-helix content decreased indicated that Pb^{2+} bound to the amino acid residues of KLH, and influenced the secondary structure of KLH [24].

Conclusions

The interaction between Pb^{2+} and KLH was investigated by spectroscopic methods including fluorescence, UV-Vis absorption and CD spectra. The experimental results demonstrated that Pb^{2+} effectively quenched the fluorescence of KLH by the static and dynamic

Figure 3: Synchronous fluorescence spectra of KLH (T=298 K): (a) Δλ=15 nm; (b) Δλ=60 nm. C_{KLH}=1.0 × 10^{-7} mol/L; C_{Pb}^{2+}/(10^{-6} mol/L) (1-7): 0, 1.0, 3.0, 5.0, 7.0, 9.0, 10.0 respectively.

Sample	Content (%)			
	α-helix	β-sheet	β-turn	Random coil
a	25.6	22.5	18.4	38.5
b	25.6	22.5	18.4	38.5
c	24.4	23.5	18.6	39.9

Table 2: Secondary structure fractions of KLH altered by Pb^{2+}.

mode. There was a formation of Pb-KLH complex in the quenching process, but the binding between Pb^{2+} and KLH was not strong. The thermodynamic parameters $\Delta G<0$, $\Delta H<0$, and $\Delta S<0$ indicated that the binding process was spontaneous and exothermic interaction. The binding of Pb^{2+} to KLH induced conformational changes of KLH, which was confirmed by synchronous fluorescence spectra, UV-vis absorption spectra and CD spectra. The results described herein may well be of help to extend our understanding of the Pb-KLH interaction and study the toxicological effects of heavy metal ions on Hc.

Acknowledgements

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