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Fluorescence Spectroscopy of Interaction between Hg(II) and Keyhole Limpet Hemocyanin

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The interaction between Hg(II) and keyhole limpet hemocyanin (KLH) under simulated physiological conditions was investigated by fluorescence spectroscopy. The keyhole limpet hemocyanin fluorescence quenching mechanism and the interacting mode of the Hg(II)-KLH system were studied. The experimental data showed that the intrinsic fluorescence of keyhole limpet hemocyanin was quenched with Hg(II) through a static quenching process, which indicates that a Hg(II)-KLH complex was formed. The thermodynamic parameters, binding constants and number of binding sites were calculated at different temperatures. These experimental results show that the hydrogen bonds and van der Waals forces are attributed to stabilization of the Hg(II)-KLH system. Synchronous fluorescence spectra indicated that Hg^{2+} lead to conformational changes of keyhole limpet hemocyanin.

Keywords: Synchronous fluorescence spectroscopy, Interaction, Keyhole limpet hemocyanin, Hg(II).

INTRODUCTION

Mercury is a widely distributed, non-essential heavy metal with intrinsic toxicity to living organisms that has toxicity to the human body *via* the inhalation, dermal and oral modes of exposure and absorbed into the blood. Inorganic mercury is the most common form in nature and has impacted human health extensively and directly. Mercury in blood may bind to sulfhydryl groups and then be transported to many other tissues. Acute and prolonged exposure to mercury may produce injury to many organs, such as the lung, kidney, brain and liver [1]. Mercury is a significant environmental pollutant that originates from industry.

Keyhole limpet hemocyanin (KLH) which is copper-containing protein found in the sea mollusk *Megathura crenulata* [2], is used to be as an immunotherapeutic agent for the treatment of diseases (such as bladder carcinoma, schistosomiasis and acquired immunodeficiency syndrome (AIDS)) [3]. Consequently, the study of the interaction between heavy metals and protein is of fundamental importance for providing more information to understand their transport, distribution and toxicity mechanism. However, few papers have focused on the interactions of Hg(II) with hemocyanin. In this paper, the mechanism of Hg(II)-KLH interaction is explored and it will provide references for evaluating the effect of Hg(II) on keyhole limpet hemocyanin.

EXPERIMENTAL

Keyhole limpet hemocyanin (> 99 %, lyophilized power) was obtained from Sigma (USA). $HgCl_2$ had a purity of no less than 99.5 % (Tianjin Damao Reagent). A keyhole limpet hemocyanin solution was stored in a *Tris*-HCl buffer solution (0.05 mol L^{-1} *Tris*, 0.1 mol L^{-1} NaCl, pH 7.4).Other chemicals were used as received without further purification. Deionized water was used in all experiments.

Methods: Fluorescence spectra were measured on an LS-55 spectrometer (Perkin-Elmer, USA). The fluorescence-quenching spectra were measured at 298, 303 and 308 K. The slit were set to 10 nm for both width of the excitation and the emission. The excitation wavelength was set to 280 nm and the fluorescence spectra were recorded from 300 to 450 nm. To correct the background fluorescence, the appropriate solvent blank (*Tris*-HCl buffer) were used as reference. The synchronous fluorescence spectra were performed at room temperature. The D-value ($\Delta\lambda$) was adjusted to 15 nm or 60 nm.

RESULTS AND DISCUSSION

Fluorescence-quenching mechanism: Typical fluorescence-quenching mechanisms contain fluorescence dynamic quenching, fluorescence static quenching and mixed quenching [4]. The classical Stern-Volmer equation was used to describe the quenching process [5]:

$$F_0/F=1+K_{SV}[Q]=1+k_q\tau_0[Q] \quad (1)$$

where steady state fluorescence intensities with and without quencher were described by F_0 and F. K_{sv} is the quenching constant and [Q] is the quencher concentration. k_q is the quenching rate constant of bimolecular and τ_0 (~10^{-8} s) is the average lifetime of molecule in the absence quencher [6].

In the present study, upon addition of various concentrations of Hg^{2+} to a solution of keyhole limpet hemocyanin, the fluorescence spectra at three temperatures (298, 303 and 308 K) were determined to study the mode of quenching process. The influence of Hg^{2+} on the fluorescence intensity of keyhole limpet hemocyanin at 298 K was shown in Fig. 1.

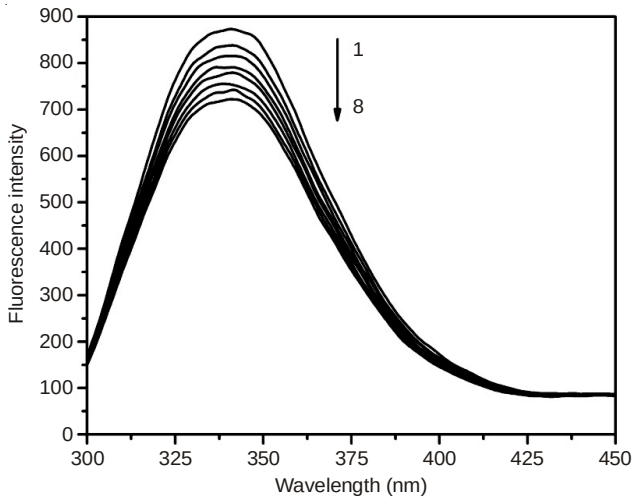

Fig. 1. Fluorescence spectra of keyhole limpet hemocyanin in the presence of various concentrations of Hg(II) (T=298 K; λ_{ex} = 280 nm). C_{KLH} = 1.0 × 10^{-7} mol L^{-1}; $C_{Hg^{2+}}$/(10^{-5} mol L^{-1}) (1→8): 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, respectively

As shown in Fig. 1, keyhole limpet hemocyanin had a strong emission peak at approximately 340 nm when excited at 280 nm. The fluorescence intensity progressively decreases when increased amounts of Hg^{2+} were added, suggesting that Hg^{2+} could quench the intrinsic fluorescence of keyhole limpet hemocyanin. The values of K_{sv} and k_q are calculated by the plots corresponding to eqn. 1 for the Hg(II)-KLH at corresponding temperatures of F_0/F *versus* [Q] (figure not shown) and summarized in Table-1. The result reveals that the K_{sv} was inversely correlated with temperature, which indicates that a Hg(II)-KLH ground-state complex was formed [7]. It is suggested that the static-quenching procedure was the main reason for the quenching mechanism of keyhole limpet hemocyanin by Hg^{2+}.

TABLE-1
QUENCHING CONSTANTS FOR THE INTERACTION OF Hg^{2+} WITH KEYHOLE LIMPET HEMOCYANIN

pH	T (K)	K_{sv} (10^3 L mol^{-1})	k_q (10^{11} L mol^{-1} s^{-1})	R	S.D.
7.4	298	6.87	6.87	0.9910	0.0033
	303	4.84	4.84	0.9956	0.0016
	308	4.07	4.07	0.9923	0.0018

Number of binding sites: For static quenching, when Hg(II) bind independently to a set of equivalent sites on keyhole limpet hemocyanin, the apparent binding constant K_a and the number of binding sites n can be obtained from the well-known equation [8]:

$$\log [(F_0–F)/F] = n \log [Q] + \log K_a \quad (2)$$

K_a and n of the Hg(II)-KLH system at 298 K were calculated from the plot of $\log(F_0–F)/F$ *versus* log[Q]. The linear correlation coefficient R (0.9971) indicates that the use of eqn. 2 is reasonable. The values of K_a and n (Table-2) were 954.73 L mol^{-1} and 0.8, respectively. There was one type of binding site for Hg^{2+} in keyhole limpet hemocyanin because the value of n was approximately equal to one [9].

TABLE-2
BINDING CONSTANTS AND RELATIVE THERMODYNAMIC PARAMETERS OF Hg(II)-KLH

T (K)	K_a (L mol^{-1})	n	R	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})
298	954.73	0.800	0.9971			-16.88
303	561.71	0.786	0.9972	-53.72	-123.63	-16.26
308	473.12	0.784	0.9990			-15.64

Binding model and thermodynamic parameters: The thermodynamic parameters obtained from the van't Hoff equation were applied to deduce the interaction of Hg^{2+} with keyhole limpet hemocyanin. The main force contributing to protein stability in protein reactions is thermodynamic parameters. The enthalpy change (ΔH) can be considered as a constant if the temperature does not vary significantly in the equation below [10]:

$$\ln K = –\Delta H/(RT) + \Delta S/R \quad (3)$$

$$\Delta G = \Delta H – T\Delta S \quad (4)$$

where K is similar to the binding constants K_a at 298, 303 and 308 K. ΔS is the entropy change, ΔG is the free energy change.

The binding constants and relative thermodynamic parameters of Hg(II)-KLH were listed in Table-2. According to the association of the thermodynamic parameters with various interactions reported by Ross and Subramanian [11]. For the Hg(II)-KLH system, the $\Delta G < 0$ means that the interaction process was spontaneous. The results of $\Delta S < 0$ and $\Delta H < 0$ reveal that the binding is mainly enthalpy driven with an unfavourable entropy, which is an exothermic reaction. The stabilization of the Hg(II)-KLH system mainly depends on hydrogen bonds and van der Waals forces.

Synchronous fluorescence spectroscopy: In order to investigate the micro-environment of amino acid residues, synchronous fluorescence which is a simple and effective method was introduced to measure the emission wavelength shift [12]. The characteristic information of tyrosine (Tyr) residues or tryptophan (Trp) residues was obtained by the synchronous fluorescence with the D-value (Δλ) between the excitation wavelength and the emission wavelength fixed at 15 nm or 60 nm [13].

As shown in Fig. 2, when Δλ = 15 nm, the λ_{max} had a slight blue-shift. While, a slight red-shift was observed when Δλ = 60 nm. Moreover, the degree of the fluorescence quenching of the tyrosine residues was less than that of the tryptophan residues at the same concentration of Hg^{2+}. These phenomena imply that Hg^{2+} was close to the tryptophan

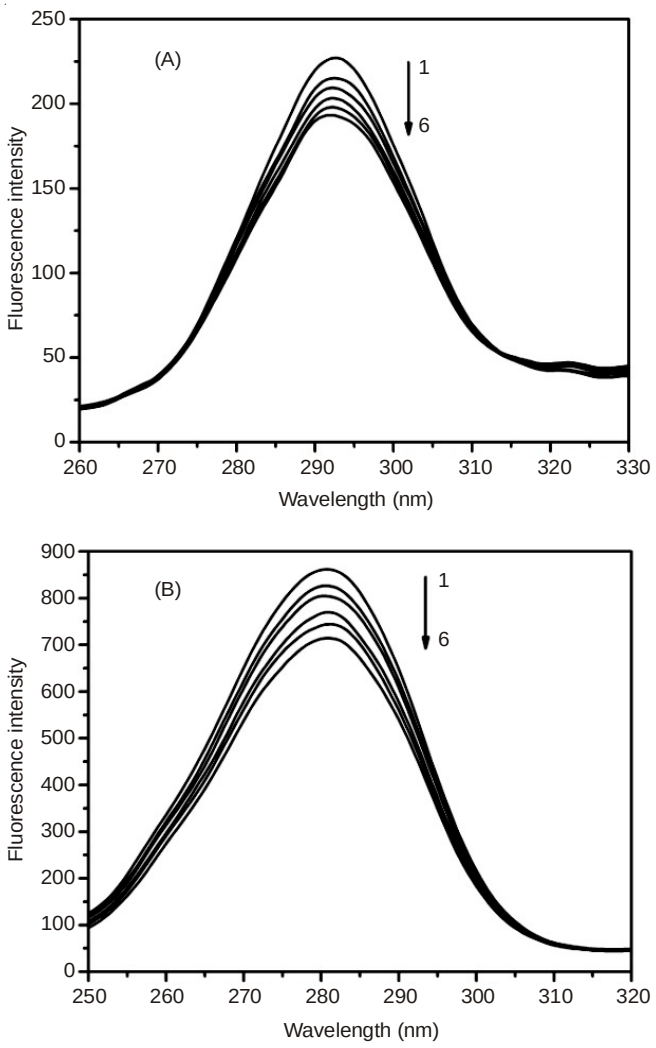

Fig. 2. Synchronous fluorescence spectra of keyhole limpet hemocyanin (T = 298 K): (A) $\Delta\lambda$ = 15 nm; (B) Δ = 60 nm; $C_{KLH} = 1.0 \times 10^{-7}$ mol L^{-1}; $C_{Hg^{2+}}/(10^{-5}$ mol $L^{-1})(1 \rightarrow 6)$: 0, 0.5, 1.5, 2.5, 3.5, 4.0, respectively

residues, resulting in the decrease of hydrophobicity near the tryptophan residues in the presence of Hg^{2+} [14].

Conclusion

This paper provides a new strategy to investigate the interaction mechanism of Hg^{2+} to keyhole limpet hemocyanin *in vitro*. In this experiment, the results obtained from fluorescence methods demonstrated that a static-quenching mechanism was a main factor for the fluorescence quenching of keyhole limpet hemocyanin. There was a single class of binding sites on the keyhole limpet hemocyanin with Hg^{2+}. The interaction was mainly enthalpy-driven due to the hydrogen bonding and van der Waals forces in the reaction. From synchronous fluorescence spectra, it is seen that Hg^{2+} is located in close proximity to tryptophan residues of the main polypeptide chain of keyhole limpet hemocyanin, leading to conformational changes to the keyhole limpet hemocyanin. Such conformational changes may induce further toxic effects.

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