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Spectroscopic Techniques Study on the Quenching Mechanism of Cytochrome B5 Affected by Cu(II)

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The interaction between Cu^{2+} and cytochrome *b*5 (cyt*b*5) has been investigated by multi-spectroscopic techniques. The results show that Cu^{2+} could interact with cytochrome *b*5 and induce fluorescence of the latter to be quenched. The quenching mechanism is dominated by dynamic quenching. Synchronous fluorescence and circular dichroism spectra demonstrate that Cu^{2+} could also influence the micro-circumatance of aromatic amino residues and secondary structure of the protein.

Key Words: Cytochrome B5, Quenching mechanism, Spectrosciopic techniques.

INTRODUCTION

Fluorescence quenching is an important method to study the interaction of substances with protein because it is sensitive and relatively easy to use. Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore, which distinguishes it from generalized techniques, such as calorimetry, far-ultraviolet circular dichroism and infrared spectroscopy[1-3].

Cytochrome *b*5 (cyt*b*5), a component of endoplasmic reticulum membranes, is a hemeprotein with molecular weight 16.8 kD. Cyt*b*5 plays an important role in physiologically important reactions. Different kinds of trace metal elements, such as Cu^{2+}, Zn^{2+} *etc.*, exist in the organisms and their pharmacological functions shouldn't be neglected. As a common metal element, copper ion plays an important role in organism's growth, cell division, protein synthesis and the activity of many metalloenzymes[4]. It is important for biology to research the interaction between Cu^{2+} and biological moleculars *in vitro*. Recently, the conformation change of nucleic acid and protein induced by metal ions has become a hot subject[5-8]. Investigating the interaction of ligands (including metal ions, fatty acid, steroids and a variety of drugs) with proteins can elucidate the properties of complex between ligand and protein, as it may provide useful information of the structural features that determine the therapeutic effectiveness of drugs[9]. Although a few papers deal with the interaction of biomacromolecules with metal ions[10,11], many fundamental studies are still needed.

In view of above, in this paper we firstly investigate the direct interaction of cyt*b*5 with Cu^{2+} by fluorescence, circular dichroism, synchronous flurescence, time-resolved fluorescence techniques. The fluorescence quenching mechanism for cyt*b*5 affected by Cu^{2+} was proposed and found that conformation of cyt*b*5 was changed by interaction with Cu^{2+}. This paper can help us to know the interaction *in vivo* clearly.

EXPERIMENTAL

The plasmid for trypsin-solubilized bovine liver microsomal Cyt*b*5 is a gift from Professor Brian Hoffman of North-Western University. Cultures of *E. coli* containing this plasmid were grown, cells were isolated and cyt*b*5 was extracted and purified according to the procedures described by Mauk and co-workers[10]. Protein concentrations were determined assuming absorption coefficients for ferricytochrome *b*5 of 117 mM^{-1} cm^{-1} at 412.5 nm[11]. All other chemicals were analytical grade. Water (specific resistance 18 MΩcm) was obtained from Millipore Q.

Fluorescence measurements: Fluorescence measurements were performed on an FP-6500 fluorescence spectrophotometer (Jasco, Japan) equipped with a 150 W xenon lamp source and the temperature was controlled by JULABO refrigerating and heating circulator (JULABO F-12 ME, Germany). The fluorescence emission spectra were recorded with 1.0 cm quartz cell in the wavelength of 290-400 nm upon excitation at 280 nm at a scanning speed of 500 nm/min under 298, 310 and 320 K, respectively and bandwidth for both

excitation and emission was 3 nm. The preparation of sample was as follows: stock solution of Cu^{2+} (1×10^{-3} mol/L, water as solvent) was added into 2.5 mL of a 1×10^{-7} mol L^{-1} cyt*b*5 in 50 mM, Na_2HPO_4-NaH_2PO_4 buffer solution gradually and samples with the concentration of Cu^{2+} varying from 0 to 17×10^{-6} mol/L were yielded. At the same time the synchronous fluorescence spectra were obtained.

Absorption spectroscopy measurements: The absorption spectra were recorded at room temperature (25 ºC) on a V-560 spectrophotometer (Jasco, Japan). The absorbance spectra were recorded in the wavelength of 220-400 nm with 1.0 cm quartz cells. The concentration of cyt*b*5 (0.5μM cyt*b*5 in 50 mM Na_2HPO_4-NaH_2PO_4 buffer) was kept constant while varying the concentration of Cu^{2+} by adding stock solution of Cu^{2+} (50 mM NaH_2PO_4-Na_2HPO_4 buffers at pH 7.4) and different concentration of Cu^{2+} solutions were used as blank, respectively.

Time-resolved fluorescence measurements: Time-resolved fluorescence measurements were carried out on a FLS920 fluorescence spectrophotometer (Edinburgh instruments, Edinburgh) equipped with a hydrogen lamp source, its EHT is 7.10 Kv, frequency is 40.010-40.000 KHz, pressure is 0.30 bar.

Circular dichroism measurements: Circular dichroism spectra were run on a J-810 spectrophotometer (Jasco, Japan) using a 0.1 cm path length cell with scanning speed of 50 nm/min at 0.1 nm intervals, 3 times of scan averaged for each circular dichroism spectrum in the range of 190-250 nm at 298 K.

RESULTS AND DISCUSSION

Fluorescence measurements: Fluorescence spectra can provide the information about the molecular environment vicinity of the chromophore molecules. The conformational changes of cyt*b*5 were evaluated by measuring the intrinsic fluorescence intensity of cyt*b*5[12] before and after the addition of Cu^{2+} in the range of 290-400 nm upon excitation at 280 nm.

The effect of Cu^{2+} on cyt*b*5 fluorescence intensity is shown in Fig. 1. When different amounts of Cu^{2+} solution was titrated into a fixed concentration of cyt*b*5, a remarkable decrease in the fluorescence intensity of cyt*b*5 was observed, which indicated that Cu^{2+} interacted with cyt*b*5. This result is similar to Jinsheng Wang's condition that when Cu^{2+} was added into the cytc system, the fluorescence emission peaks of cytc at 290 nm(Ex) and 330 nm (Em) were both quenched apparently when Cu^{2+} was added into the cytochrome C system[13], which indicated the reactions between Cu^{2+} and the protein compound had taken place. Furthermore, the decrease of fluorescence intensity of cyt*b*5 also revealed that the energy transfer of Cu^{2+} and cyt*b*5 is non-radiation energy transfer[14].

Mechanisms of fluorescence quenching: The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. The static quenching is due to the formation of ground-state complex between fluorophores and quencher. While dynamic quenching, also called collisional quenching, results from collision between fluorophores and a quencher[15,16]. The equation for dynamic quenching is presented by reference[16,17].

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

where, F and F_0 are the fluorescence intensity with and without quencher, respectively. K_q, K_{SV}, τ_0 and [Q] are the quenching rate constant of the biomolecule, the dynamic quenching constant, the average fluorescence lifetime of the fluoroohore without quencher and the concentration of quencher, respectively.

The process of static quenching is described in formula (2).

$$F_0/F = 1 + K[Q] \quad (2)$$

where, K is the forming constant of ground-state complex.

To compared formula (1) with (2), it is showed that the linearity correlation exists in F_0/F and [Q] for both static quenching and dynamic quenching. Therefore, it is impossible to judge that the quenching mechanism belongs to dynamic or static quenching only through the determination of the spectral data. The methods how to distinguish dynamic or static quenching are described thereinafter[18].

(1) The dynamic quenching, because diffusion coefficient of molecules is enhanced with the increase of temperature. (2) The dynamic quenching's absorption spectroscopy is not changed. (3) For dynamic quenching, quencher can shortened fluorescence lifetime, that is $\tau_0/\tau = F_0/F$, which is the most right way to distinguish static quenching and dynamic quenching.

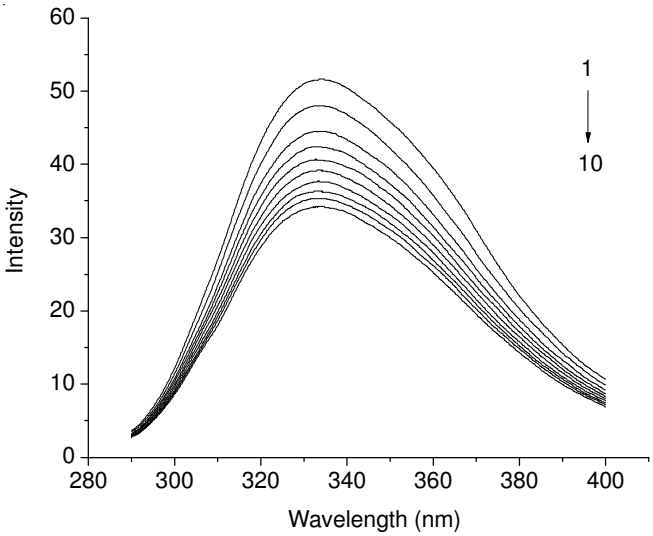


Fig. 1. Fluorescence spectra of cyt*b*5 containing different concentration of Cu^{2+} (298 K, pH 7.4); $C_{cytb5} = 1 \times 10^{-7}$ mol/L, from a to j, $C_{Cu^{2+}}$ = 0, 1, 3, 5, 7, 9, 11, 13, 15 and 17×10^{-6}mol/L

Effect of temperature: In order to clarify the fluorescence quenching mechanism induced by Cu^{2+}, the well-known Stern-Volmer equation was utilized. These K_{SV} values were observed to be 3.2×10^4, 4.14×10^4, 5.21×10^4 L mol^{-1} from the slopes at 298, 310 and 320 K (Fig. 2), respectively. Fig. 2 showed the slopes increased with increasing temperature. Dynamic quenching depended on diffusion, the increase of temperature can make molecule diffuse more quickly. Consequently, the quenching rate constant of the biomolecule was also increased with increasing temperature. Therefore, it indicated that the fluorescence quenching process of cyt*b*5 might be mainly governed by a dynamic quenching mechanism.

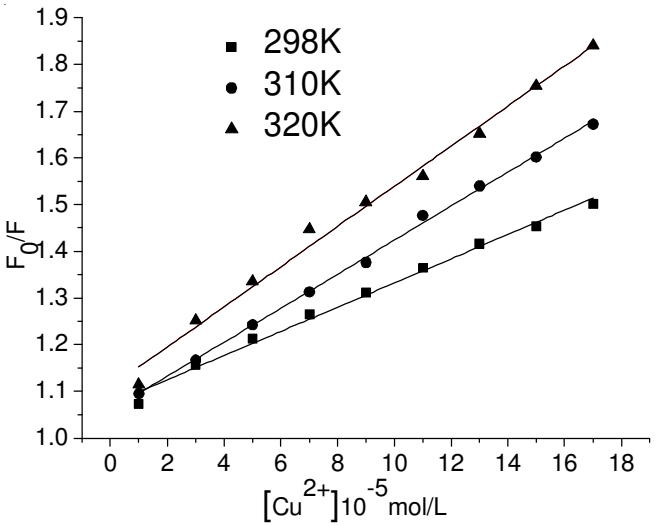


Fig. 2. Stern-Volmer plots for the quenching of cyt*b*5 by Cu^{2+} at different temperatures. pH =7.4, λex = 280 nm

Absorption spectroscopy: UV-VIS absorption measurement is a simple method and is applicable to explore the structural change and to know the complex formation[19]. In this study, we had recorded the absorption spectra of cyt*b*5 in the presence of different amounts of Cu^{2+} (Fig. 3). It was evident that the absorption intensity of cyt*b*5 was invariable at 280 nm with the addition of Cu^{2+}. It showed that UV absorbance spectra hadn't been changed by added Cu^{2+}. The dynamic quenching only affected excited state of fluorophores, so its absorbance spectra hadn't changed. Therefore fluorescence quenching of cyt*b*5 induced by Cu^{2+} was the interaction result of Cu^{2+} and excited state cyt*b*5.

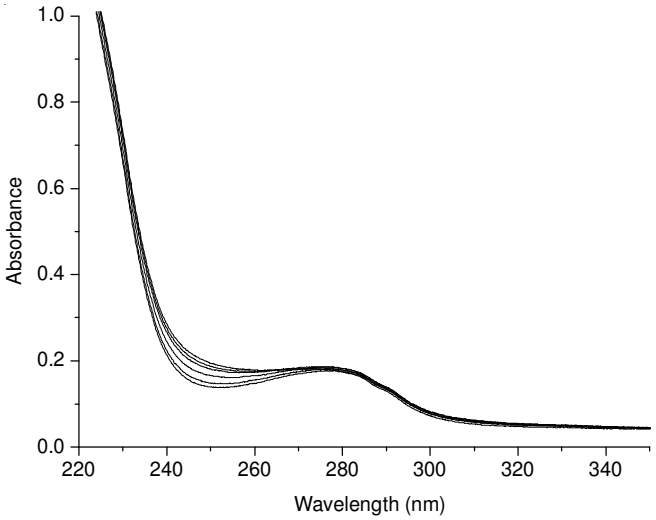


Fig. 3. UV absorption spectra of cyt*b*5 containing different Cu^{2+} concentration. $C_{cytb5} = 0.5 \times 10^{-6}$ mol/L, from 1 to 6, $C_{Cu^{2+}} = 0, 0.5, 1.5, 2.5, 3.5, 4.5 \times 10^{-5}$ mol/L

Affirmance of fluorescence quenching mechanism: Fluorescence lifetime is the most right way to distinguish static quenching and dynamic quenching. We have measured the fluorescence lifetime of cyt*b*5 with or without Cu^{2+} (Fig. 4). Fig. 4 showed that τ_0 was 5.42 ns for cyt*b*5 without Cu^{2+} and that τ was 5.01 for cyt*b*5 with Cu^{2+}. Table-1 showed that the value of τ_0/τ was proximate the same to the one of F_0/F. So, the quenching mechanism of cyt*b*5 was dynamic quenching affected by Cu^{2+}. The quenching mechanism of cyt*b*5 is different to quenching mechanism of myoglobin and cytochrome C affected by Cu^{2+}, which is interesting problem to study. They have different coordination and connecting manners. Whether or no, it is harmful to organism when the concentration of Cu^{2+} is superfluous.

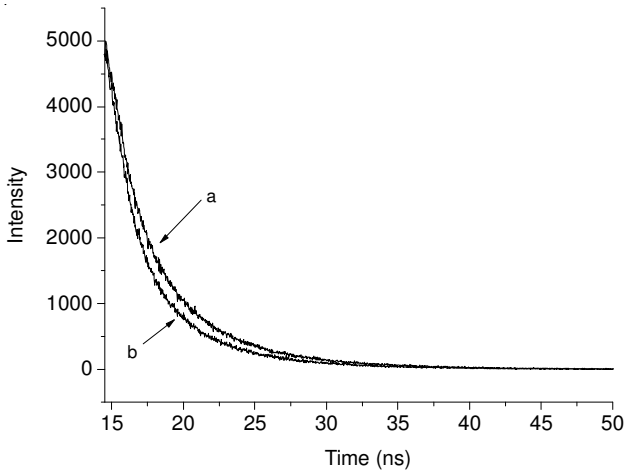


Fig. 4. Decay curve of the time-resolved fluorescence spectra of cyt*b*5 without Cu^{2+} (a) and with Cu^{2+} (b)

TABLE-1
τ_0/τ AND F_0/F

Sample	Steady fluorescence ($\times 10^3$)	Fluorescence lifetime τ(ns)
Without Cu^{2+}	1.828	5.42
With Cu^{2+}	1.668	5.01
Ratio	1.099	1.08

Effect on cytochrome *b*5 conformation of Cu^{2+}

Synchronous fluorescence: Synchronous fluorescence spectra shows change of Tyr residues at the wavelength interval (Δλ) of 20 nm and change of Trp residues at Δλ of 80 nm for cyt*b*5[20]. The synchronous fluorescence spectroscopy gives information about the molecular environment in a vicinity of the chromospheres molecules and has several advantages, such as sensitivity, spectrum simplification, spectrum bandwidth reduction and avoiding different perturbing effects[2,21].

Synchronous fluorescence spectra of cyt*b*5 was recorded in Figs. 5 and 6 with various amounts of Cu^{2+}. It was seen that little red shift of Trp and Tyr fluorescence (Fig. 5a,b) occurred with the addition of Cu^{2+}. And the intensity of decreasing for Trp [35.5 % at 311 nm and 33.9 % at 358 nm, (Fig. 6a)] was larger than that for Tyr [32.1 % at 310 nm, (Fig. 6b)]. That indicated that the polarity was increased and the interaction of Cu^{2+} with cyt*b*5 affected the conformation of Tyr residue microenvironment feebly. Fig. 6 showed that the decrease extent of fluorescence intensity of Trp was stronger than that of Tyr, which showed that Cu^{2+} was closer to trytophan residues compared to tyrosine residues. Indeed, the conformation of cyt*b*5 was affected by Cu^{2+}.

Circular dichroism: Circular dichroism is a sensitive technique to monitor the conformational changes in the protein upon interaction with the ligands[22,23]. The circular dichroism

spectra of cyt*b*5 in the absence and presence of Cu^{2+} exhibited two negative peaks at 208 and 222 nm in near-UV region, which were characteristic of an α-helical structure of protein. The banding intensity of these negative bands changed with increasing the concentration of Cu^{2+} (Fig. 7), suggesting the helix structure content and β-sheet of cytochrome *b*5 has been changed.

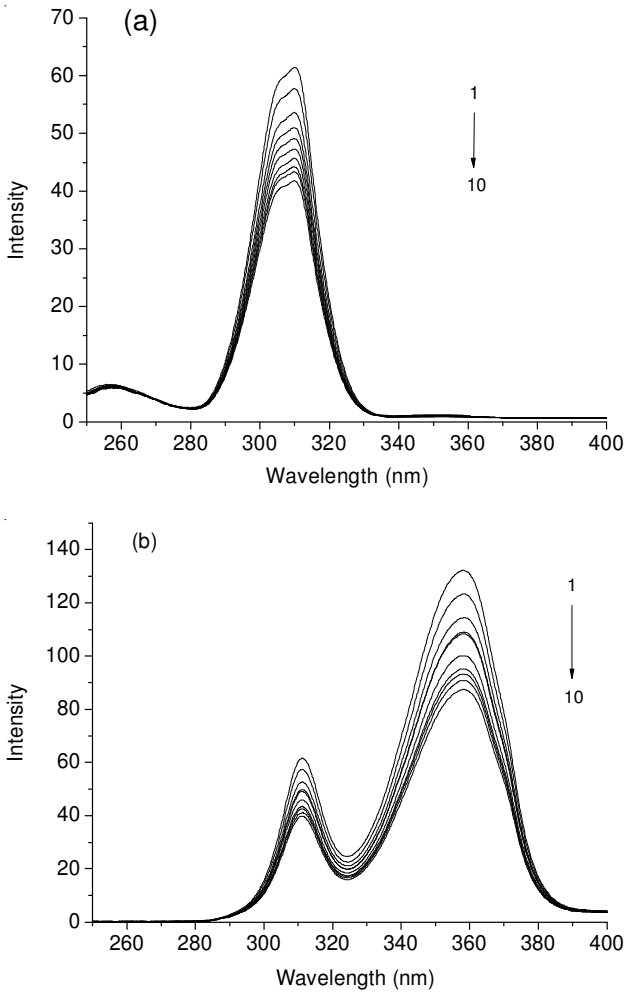
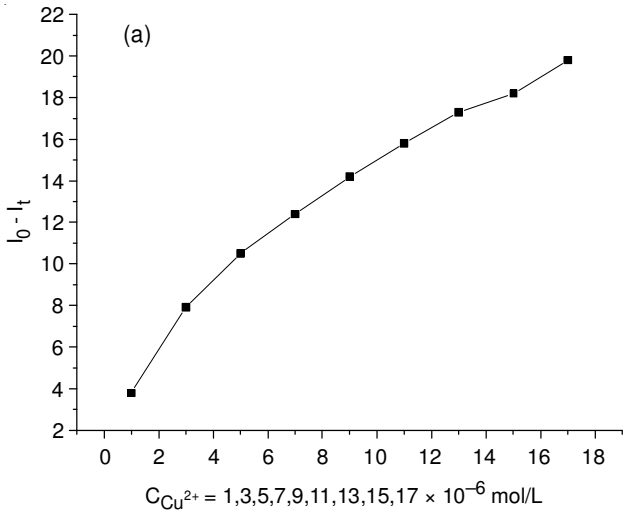


Fig. 5. Synchronous fluorescence spectra of cyt*b*5 in the presence of Cu^{2+}. $C_{cytb5} = 1 \times 10^{-7}$ mol/L, from 1 to 10, C c_u^{2+} = 0, 1, 3, 5, 7, 9, 11, 13, 15 and 17 × 10^{-6} mol/L; (a) Δλ = 20 nm and (b) Δλ = 80 nm



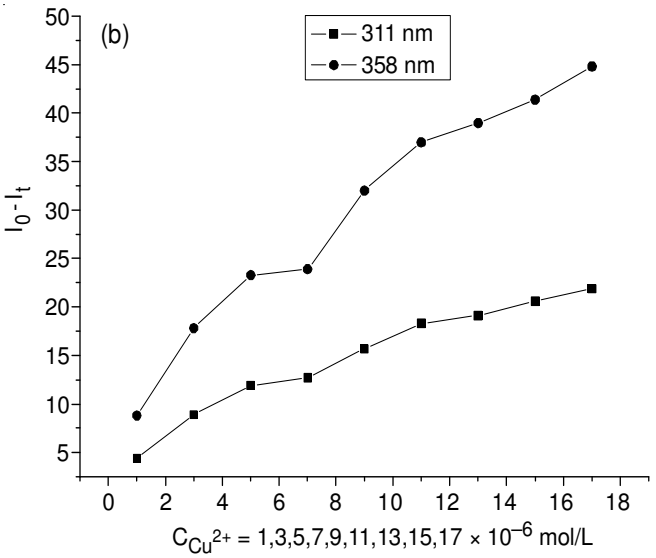


Fig. 6. Synchronous fluorescence spectra of cyt*b*5 in the presence of Cu^{2+}

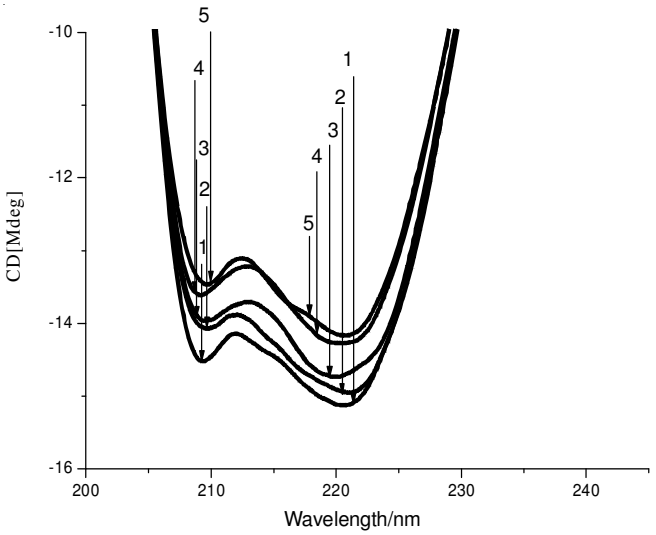


Fig. 7. Circular dichroism spectra of cyt*b*5 affected by Cu^{2+}; C_{cytb5} = 1 × 10^{-6} mol/L, from 1 to 5 $C_{Cu^{2+}}$ =0, 3 × 10^{-5}, 5 × 10^{-5}, 9 × 10^{-5} and 11 × 10^{-5} mol/L

Conclusion

In this paper, the interaction of cyt*b*5 with Cu^{2+} was studied by spectroscopic methods. The experimental results reveal that cyt*b*5 and Cu^{2+} has relatively strong interaction. The fluorescence of cyt*b*5 was quenched by Cu^{2+}. Quenching mechanism is dynamic quenching, increasing temperature can promote the interaction between Cu^{2+} and cyt*b*5. The interaction of cyt*b*5 with Cu^{2+} induced the conformational changes of cyt*b*5. This paper can help us to know the interaction *in vivo* clearly.

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