

Multispectroscopic Study of the Interaction of Chloramphenicol with Human Neuroglobin

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Abstract. The interaction between chloramphenicol (CHL) and neuroglobin (Ngb) has been investigated by using fluorescence, synchronous fluorescence, UV-Vis and circular dichroism (CD) spectroscopy. It has been found that CHL molecule can quench the intrinsic fluorescence of Ngb in a way of dynamic quenching mechanism, which was supported by UV-Vis spectral data. Their effective quenching constants (K_{SV}) are 2.2×10^4 , 2.6×10^4 , and $3.1 \times 10^4 \text{ L}\cdot\text{mol}^{-1}$ at 298 K, 303 K, and 308 K, respectively. The enthalpy change (ΔH) and entropy change (ΔS) for this reaction are $26.42 \text{ kJ}\cdot\text{mol}^{-1}$ and $171.7 \text{ J}\cdot\text{K}^{-1}$, respectively. It means that the hydrophobic interaction is the main intermolecular force of the interaction between CHL and Ngb. Synchronous fluorescence spectra showed that the microenvironment of tryptophan and tyrosine residues of Ngb has been changed slightly. The fluorescence quenching efficiency of CHL to tyrosine residues is a little bit more than that to tryptophan residues of Ngb. Furthermore, CD spectra indicated that CHL can induce the formation of α -helix of Ngb.

Keywords: Neuroglobin, chloramphenicol, fluorescence spectroscopy, circular dichroism, thermodynamic parameters.

1. Introduction

Chloramphenicol (CHL, 2,2-dichloro-N-((1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl) propan-2-yl)acetamide, a chemical structure shown in Figure 1) is a broad bacteriostatic antimicrobial; it exhibits antibacterial activity against both gram-positive and gram-negative bacteria, as well as other groups of microorganisms [1, 2]. CHL has also been proven to be effective against epidemic meningococcal meningitis [3]; it exerts its antimicrobial effect by inhibiting bacterial protein synthesis through binding to the 50S ribosomal subunit to interfere with the requisite peptidyl transferase [4]. CHL is widely used in the treatment of domestic animals, aquaculture species, livestock husbandry and human consumption. However, CHL has shown several harmful side effects in humans, such as fatal aplastic anemia, bone marrow suppression, and gray syndrome. The incidence of aplastic anaemia in Europe is 0.2 cases per 100,000 inhabitants, whereas, in China, the figure reaches to 2 cases per 100,000 [2, 5]. Chloramphenicol is found to have an extensive distribution in humans, such as in heart, lung, kidney, liver, spleen, pleural fluid, seminal fluid, ascitic fluid, and saliva, regardless of the ways of administration [6, 7]. CHL can

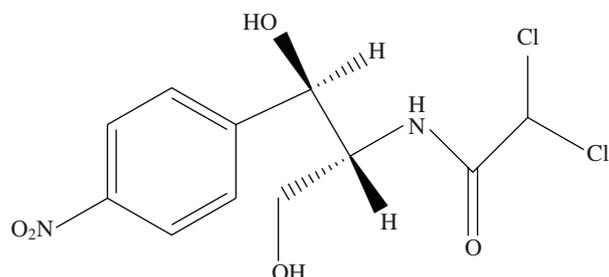


Figure 1: Chemical structure of chloramphenicol.

penetrate the blood-brain barrier and thus has a relatively high concentration in cerebrospinal fluid, which can reach to around 60% of that in plasma [8], whereas in brain tissue, the concentration is equal to or even higher than that in plasma [9].

Neuroglobin (Ngb), a novel member of the globin superfamily, is recently discovered by Burmester et al. in human and mouse [10, 11]. It is predominantly expressed at micromolar concentration in several areas of the central nervous system and even more abundantly in the retina [10, 11]. Although its physiological function is still uncertain, a number of hypotheses concerning its role in body have been proposed for Ngb: (i) it serves as an O_2 -transport protein that facilitates O_2 diffusion to cells that require aerobic mitochondrial respiration [10, 12, 13]; (ii) in the absence of O_2 , it oxygenates NADH as the end oxidase and enhances the production of ATP via glycolysis [14]; (iii) it acts as an O_2 sensor and activates other proteins that have specific regulatory functions [15]; (iv) it may be involved in the metabolism of nitric oxide; (v) it can protect neuron from the injure of hypoxia and ischemia [13].

It is noteworthy that Ngb is predominantly expressed in central nervous system, meanwhile we notice the fact that CHL can penetrate the blood-brain barrier and its concentrations in brain tissue equal or even exceed those in plasma. Considering all of those factors, it is necessary to study the interaction between CHL and Ngb to better understand their molecular interaction mechanism and possible harmful side effects of CHL, as well as the physiological roles of Ngb. In this paper, the interaction between CHL and Ngb at different temperatures has been studied by fluorescence, Uv-vis, and CD spectroscopy, aiming to better understand the toxicological effects of CHL and to explore its binding mechanisms at molecular level.

2. Experimental

2.1. Materials and Instruments

Chloramphenicol was purchased from Sangon (Shanghai, China). The pET3a plasmid DNA containing the gene of human Ngb was a present from Professor Burmester, Johannes Gutenberg University of Mainz, Germany. *E. coli*. BL21 (DE3) plysS cells were conserved in our laboratory. Yeast extract and Tryptone were purchased from Oxoid Ltd. (England). Isopropyl-1-thio-D-galactopyranoside (IPTG) and dithiothreitol (DTT) were obtained from Sigma (USA). All the other reagents were of analytical or biochemical grade.

AKTA purifier 100 fast protein liquid chromatography (Amersham Biosciences, Sweden), HP8453A diode array spectrophotometer (USA), LS-55 spectrofluorimeter (Perkin Elmer, USA) and Jasco J-810 spectropolarimeter (Japan) were used.

2.2. Preparation of Neuroglobin

The pET3a plasmid with the human Ngb gene was transformed into *E. coli*. BL21 (DE3) plysS cells, the Ngb expression, and purification were carried out as previously described [16, 17]; finally, a red soluble electrophoresis purified protein was obtained. The concentration of Ngb stock solution was determined spectrophotometrically by using an absorption coefficient at 532 nm of $10.7 \text{ mmol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$ [18].

2.3. Spectral Measurements

Dilutions of the Ngb stock in $20 \text{ mmol} \cdot \text{L}^{-1}$ Na_2HPO_4 - NaH_2PO_4 (pH 7.0) buffer solution were prepared immediately before use. The stock solution ($5 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$) of CHL was prepared in $20 \text{ mmol} \cdot \text{L}^{-1}$ Na_2HPO_4 - NaH_2PO_4 (pH 7.0) buffer solution.

Fluorescence spectra were recorded on LS-55 spectrofluorimeter (Perkin Elmer, USA) at different temperatures (298 K, 303 K, and 308 K), with bandwidths of 10/5 nm in excitation/emission channels. The excitation wavelength was 280 nm, and the emission was read at 290–420 nm. Appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence.

Synchronous fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromators in the range of 250–600 nm with constant wavelength interval ($\Delta\lambda = 20, 60,$ and 80 nm , resp.). Scan speed of 300 nm/min and bandwidths of 10/5 nm in excitation/emission channels were adopted. Fluorescence intensities were plotted versus the excitation wavelength.

The absorption spectra were recorded at room temperature on Hewlett-Packard 8453A diode array spectrometer (Hewlett-Packard, USA).

Circular dichroism spectra were measured with Jasco J-810 spectropolarimeter (Jasco, Japan) under nitrogen at a constant flow rate of 5 L/min. Technical parameters settings are wavelength range 250–190 nm, scan rate 100 nm/min, pathlength cuvette 0.1 cm, resolution ratio 0.1 nm, time constant 0.1 s and number of scans 2.

3. Results and Discussion

3.1. Fluorescence Spectra

The intrinsic fluorescence of aromatic amino acids in proteins has long been used as an effective method to study protein partial conformation. The fluorescence of tryptophan residues is exquisitely sensitive to perturbations of protein structure, whereas the low quantum yields of phenylalanine and tyrosine render these probes somewhat less useful for such studies. Three tryptophan and four tyrosine residues are located in different domains of Ngb [19]. In order to investigate the binding of CHL to Ngb, the fluorescence emission spectra of Ngb in the presence of CHL at different concentrations were recorded upon excitation at 280 nm; data on three temperatures of 298 K, 303 K, and 308 K were collected, respectively. Figure 2 shows the fluorescence emission spectra of Ngb in the presence of CHL at different concentrations at 303 K. It can be seen from Figure 2 that the fluorescence intensity of Ngb decreased

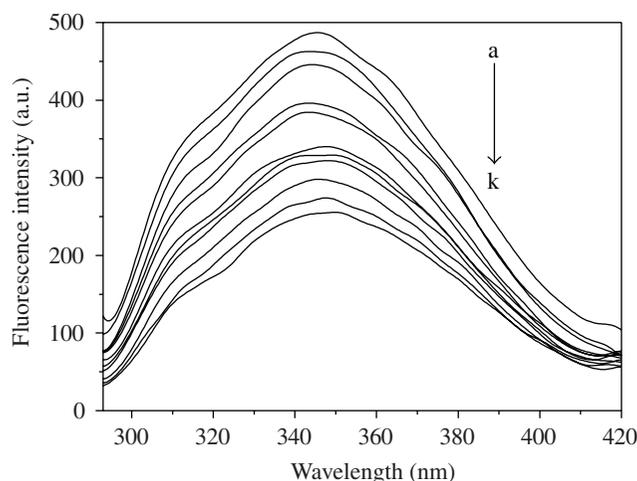


Figure 2: Fluorescence emission spectra of Ngb at 303 K, in the absence of (a) and presence of CHL $3 \mu\text{mol}\cdot\text{L}^{-1}$ (b), $6 \mu\text{mol}\cdot\text{L}^{-1}$ (c), $9 \mu\text{mol}\cdot\text{L}^{-1}$ (d), $12 \mu\text{mol}\cdot\text{L}^{-1}$ (e), $15 \mu\text{mol}\cdot\text{L}^{-1}$ (f), $18 \mu\text{mol}\cdot\text{L}^{-1}$ (g), $21 \mu\text{mol}\cdot\text{L}^{-1}$ (h), $27 \mu\text{mol}\cdot\text{L}^{-1}$ (i), $33 \mu\text{mol}\cdot\text{L}^{-1}$ (j), and $36 \mu\text{mol}\cdot\text{L}^{-1}$ (k); concentration of protein used was $5 \mu\text{mol}\cdot\text{L}^{-1}$.

without changing the emission wavelength and the peak shape with the increasing of CHL concentration, which suggested that CHL can interact with Ngb and quench its intrinsic fluorescence.

The fluorescence quenching processes can happen in different mechanisms, which are usually classified as dynamic quenching and static quenching. This could be distinguished by their variable dependence on temperature and viscosity [20]. Since at higher temperature the molecules have bigger diffusion coefficients for dynamic quenching, the quenching constant is expected to increase with temperature increasing. In contrast, a higher temperature will decrease its stability of the complex, resulting in a lower quenching constant for the static quenching [2]. The fluorescence quenching is usually analyzed using the Stern-Volmer equation [21]

$$\frac{F_0}{F} = 1 + K_{SV} [Q] = 1 + k_q \tau_0 [Q]. \quad (3.1)$$

In this equation, F_0 and F are the steady-state fluorescence intensities in the absence and presence of quenching substance, respectively. K_{SV} is the Stern-Volmer quenching constant, k_q is the bimolecular quenching constant, $[Q]$ is the concentration of quencher, and τ_0 is the lifetime of the fluorophore in the absence of quencher. The fluorescence lifetime of the biopolymer is 10^{-8}s [22]. The Stern-Volmer curves of F_0/F versus $[Q]$ at different temperatures were shown in Figure 3, and the calculated K_{SV} values were presented in Table 1. The plots show that within the investigated concentrations, the results matched well with the Stern-Volmer equation. It can also be shown from Table 1 that the quenching constant increased with temperature increasing, which implies that the probable quenching mechanism of Ngb by CHL is a dynamic quenching.

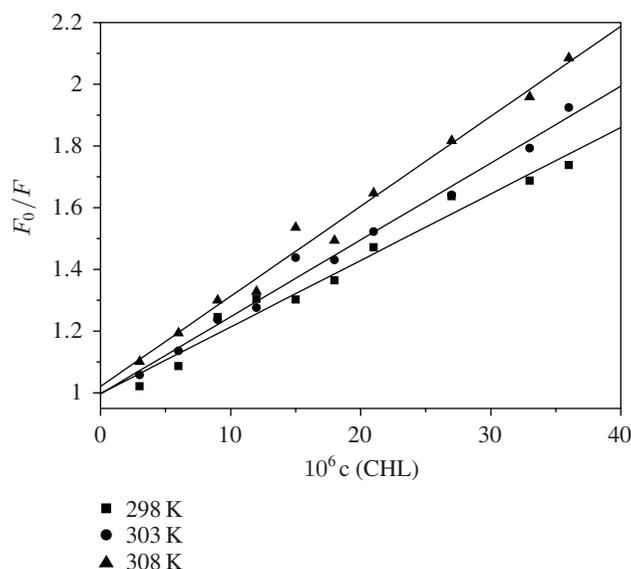


Figure 3: Stern-Volmer plots for the quenching of Ngb by CHL at different temperatures.

Table 1: Stern-Volmer quenching constant K_{SV} and thermodynamic parameters of CHL-Ngb system.

T (K)	K_{SV} ($L \cdot mol^{-1}$)	ΔG ($kJ \cdot mol^{-1}$)	ΔS ($J \cdot mol^{-1} \cdot K^{-1}$)	ΔH ($kJ \cdot mol^{-1}$)
298 K	2.2×10^4	-24.8		
303 K	2.6×10^4	-25.6	171.7	26.42
308 K	3.1×10^4	-26.5		

3.2. Type of Interaction Force between CHL and Ngb

The interaction forces between drugs and biomolecule include hydrophobic force, hydrogen bond, Van der Waals force, and electrostatic interactions [23]. The binding mode of CHL-Ngb interaction can be elucidated by thermodynamic parameters of the interaction. The values of thermodynamic parameters such as ΔH , ΔG , and ΔS can be estimated from the following van't Hoff equation [24]. If the temperature changes only a little, the enthalpy change (ΔH) can be regarded as a constant:

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}, \quad (3.2)$$

$$\Delta G = \Delta H - T\Delta S.$$

In (3.2), K is analogous to the effective quenching constant K_{SV} , and R is the gas constant. The enthalpy change (ΔH) and entropy change (ΔS) can be obtained from the linear van't Hoff plot (Figure 4).

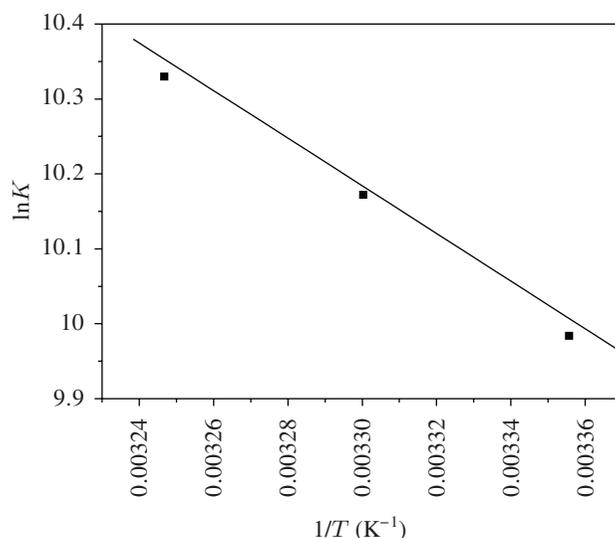


Figure 4: Van't Hoff plot for the interaction of Ngb and CHL.

Ross and Subramanian [24] have characterized the sign and magnitude of the thermodynamic parameters associated with various individual kinds of interaction that may take place in protein association process as follows:

$\Delta H > 0, \Delta S > 0$ hydrophobic force,

$\Delta H < 0, \Delta S < 0$ hydrogen bond or van der Waals force,

$\Delta H \leq 0, \Delta S > 0$ electrostatic force.

It can be seen from Table 1 that the free energy changes are negative ($\Delta G < 0$), which means that the interaction between CHL and Ngb is a spontaneous process. And the values of enthalpy change (ΔH) and the entropy change (ΔS) were found to be $26.42 \text{ kJ}\cdot\text{mol}^{-1}$ and $171.7 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, respectively, which indicated that this reaction is an endothermic interaction and that the hydrophobic interaction is the main binding force of the interaction. Because of $\Delta S > 0$, we can also speculate that the binding is an entropy-driven spontaneous process.

3.3. Analysis of Binding Equilibrium

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant K and the number of binding sites n can be calculated by using the following equation [25]:

$$\lg \frac{F_0 - F}{F} = \lg K + n \lg [Q], \quad (3.3)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, and $[Q]$ is the total quencher concentration. Figure 5 is the plot of $\lg(F_0 - F)/F$ versus $\lg[Q]$, and the number of

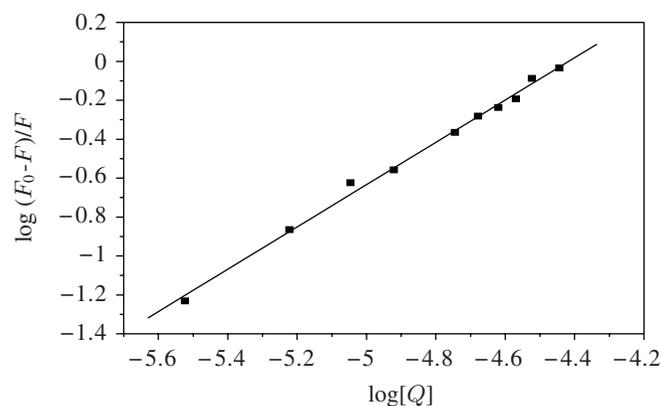


Figure 5: Double-log plots of CHL quenching effect on Ngb fluorescence at 298 K.

binding sites n and the binding constant K at 298 K were found to be 1.08 and $6.3 \times 10^4 \text{ L}\cdot\text{mol}^{-1}$, respectively, which indicates that there is only one class of binding sites to CHL in Ngb, and the correlation coefficient R (0.997) implies that the interaction between CHL and Ngb matches well with the site-binding model as described in (3.3).

3.4. Synchronous Fluorescence Spectra

Synchronous fluorescence spectroscopy is a very useful method to investigate the microenvironment of amino acid residues [26]. By selecting different wavelength intervals, the characteristic fluorescence peaks of tryptophan and tyrosine residues overlapped in the conventional fluorescence spectrum can be isolated [27]. As shown in Figure 6, when $\Delta\lambda$ is 20 nm, the fluorescence emission peak of Ngb is at 300 nm, which is the emission peak of tyrosine residues [28]. When $\Delta\lambda$ is 60 nm, a small peak at 295 nm and a big peak at 340 nm in the fluorescence spectrum were observed, which gave the characteristic information for both tyrosine and tryptophan residues. Furthermore, when $\Delta\lambda$ was increased to 80 nm, the intensity of these two peaks increased, and the peak wavelength shifted to 311 nm and 355 nm, respectively; the latter was regarded as the emission peak of tryptophan residues [28]. In addition, there was no obvious change of emission wavelength and peak shape in these synchronous fluorescence spectra, which supported the dynamic quenching mode of the interaction between Ngb and CHL. Moreover, we have compared the fluorescence quenching efficiency of CHL to individual tyrosine and tryptophan residues of Ngb (Figure 7); results showed that the fluorescence quenching efficiency of CHL to tyrosine was a little more obvious than that to tryptophan.

3.5. Uv-Visible Spectra

Uv-vis spectra study is a very simple method to explore the structural change of a protein sample. Figure 8 shows the influence of CHL on the Uv-vis spectra of Ngb. The ferric forms of Ngb display large amplitudes of the α band (533 nm) and the Soret band (413 nm) [16]. The 280 nm centered band can be assigned to π - π^* electronic transition of tryptophan and tyrosine residues. Under the same experimental conditions, no absorbance band of CHL was observed. It was shown that the absorbance at 413 nm only

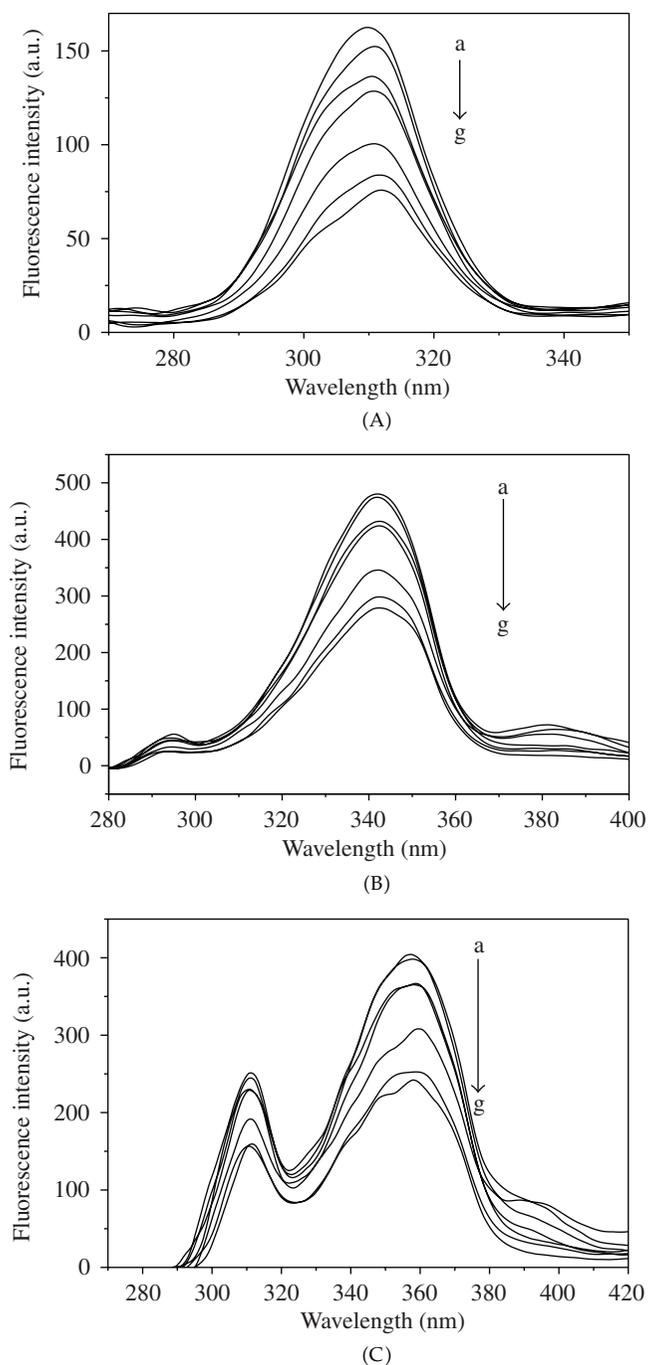


Figure 6: Synchronous fluorescence spectra of Ngb (pH = 7.0, T = 298 K.) in the absence of (a) and presence of CHL $5 \mu\text{mol}\cdot\text{L}^{-1}$ (b), $8 \mu\text{mol}\cdot\text{L}^{-1}$ (c), $10 \mu\text{mol}\cdot\text{L}^{-1}$ (d), $20 \mu\text{mol}\cdot\text{L}^{-1}$ (e), $30 \mu\text{mol}\cdot\text{L}^{-1}$ (f), and $40 \mu\text{mol}\cdot\text{L}^{-1}$ (g); concentration of protein used was $5 \mu\text{mol}\cdot\text{L}^{-1}$. (A) $\Delta\lambda = 20 \text{ nm}$, (B) $\Delta\lambda = 60 \text{ nm}$, and (C) $\Delta\lambda = 80 \text{ nm}$.

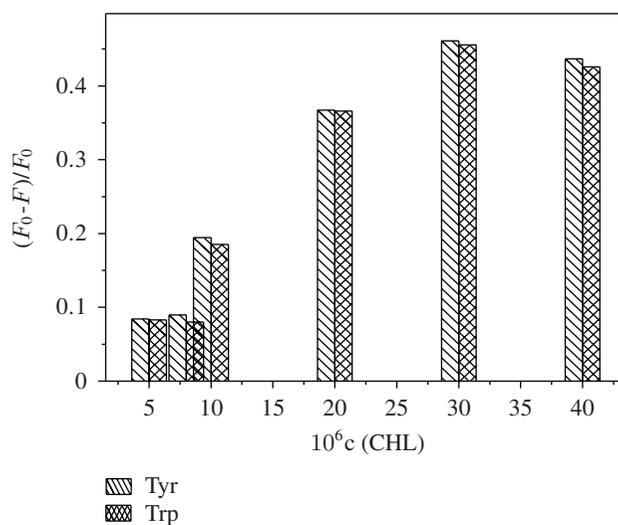


Figure 7: Fluorescence quenching efficiency of CHL to tryptophan and tyrosine residues of Ngb.

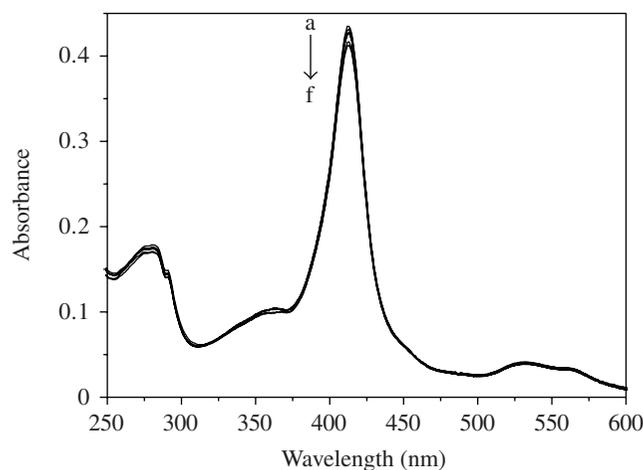


Figure 8: Uv-vis spectra of Ngb in the absence of (a) and presence of CHL $36 \mu\text{mol}\cdot\text{L}^{-1}$ (b), $60 \mu\text{mol}\cdot\text{L}^{-1}$ (c), $84 \mu\text{mol}\cdot\text{L}^{-1}$ (d), $120 \mu\text{mol}\cdot\text{L}^{-1}$ (e), and $180 \mu\text{mol}\cdot\text{L}^{-1}$ (f). Concentration of protein used was $5 \mu\text{mol}\cdot\text{L}^{-1}$, pH 7.0, 298 K.

decreased slightly, but there was no peak shift, which indicated that CHL only has a little influence on the microenvironment of heme center in Ngb molecule and maintains its hexacoordinated configuration. In addition, no obvious change was observed in the absorbance at 280 nm with the addition of increase amounts of CHL. The Uv-vis spectral results support the dynamic quenching mechanisms gained by fluorescence spectroscopy.

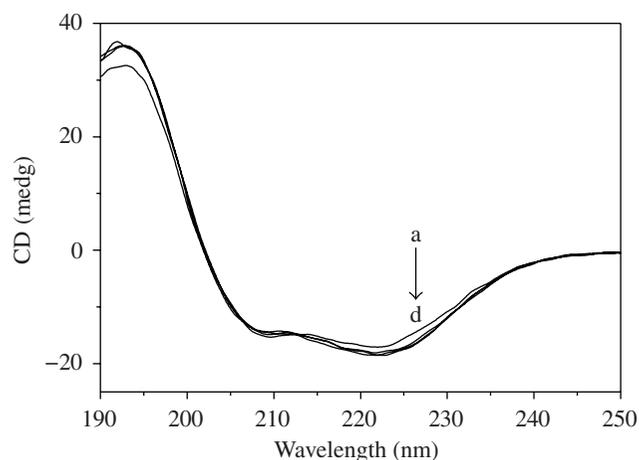


Figure 9: CD spectra of Ngb at 298 K, in the absence of (a) and presence of CHL $10 \mu\text{mol}\cdot\text{L}^{-1}$ (b), $30 \mu\text{mol}\cdot\text{L}^{-1}$ (c), and $40 \mu\text{mol}\cdot\text{L}^{-1}$ (d); concentration of protein used was $5 \mu\text{mol}\cdot\text{L}^{-1}$.

3.6. Circular Dichroism Spectra

Circular dichroism spectroscopy is a unique optical technique to investigate the change of the secondary structure of protein. In order to study the effect of CHL on the secondary structure of Ngb, CD spectra were employed. Figure 9 shows the CD spectra of Ngb in the absence and presence of CHL at different concentrations. The CD spectra of Ngb exhibited two negative bands in the far-UV region at 208 and 222 nm, which are contributed to the $n \rightarrow \pi^*$ transfer for the bond and are characteristic of α -helix structure of protein [25, 29]. As shown in Figure 9, the peak intensity at 222 nm increased with the increasing concentration of CHL, but it did not cause a significant shift of the peaks. The calculated result showed an increase of α -helix content from 52.6% to 64.9% at a molar ratio of Ngb to CHL of 1 : 8, which indicated that the interaction between Ngb and CHL can affect the intramolecular hydrogen bonding interaction of Ngb by hydrophobic force, consistent with the result of fluorescence.

4. Conclusions

The interaction of CHL with Ngb has been studied by different spectroscopic methods including fluorescence spectroscopy, synchronous fluorescence spectroscopy, Uv-vis absorption spectroscopy, and CD spectroscopy. The fluorescence spectral results showed that CHL can quench the intrinsic fluorescence of Ngb through dynamic quenching mechanism, which was also supported by Uv-vis spectral results, and the thermodynamic parameters of the interaction indicated that hydrophobic force plays a major role in the process. Furthermore, the synchronous fluorescence spectra indicated that the microenvironment of tryptophan and tyrosine residues changed slightly, and the fluorescence quenching efficiency of CHL to tyrosine residues was little more obvious than to tryptophan residues of Ngb. Moreover, CD spectroscopy indicated that CHL can induce the formation of α -helix of Ngb, which is consistent with the result of hydrophobic interaction between CHL and Ngb.

Acknowledgments

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