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Interaction between Minodiaq and bovine hemoglobin: Fluorescence study

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ABSTRACT

The interaction between Bovine Hemoglobin (BHb) and Minodiaq was investigated at different temperatures by fluorescence spectroscopy, as well as the effect of some metal ions (Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, V^{5+}, Mo^{6+}, Cr^{6+}) on the BHb-Minodiaq system. Results showed that Minodiaq could quench the intrinsic fluorescence of BHb, and the quenching mechanism was a dynamic quenching process. The hydrophobic force played an important role on the conjugation reaction between BHb and Minodiaq. Synchronous spectra and CD spectra revealed that the microenvironment and the conformation of BHb were changed during the binding reaction. Studies on the interaction between BHb and drug will facilitate interpretation of the drug's metabolism and transporting process in the blood, and will help to explain the relationship between structures and functions of BHb.

KEYWORDS

Bovine hemoglobin;
Minodiaq;
Metal ions;
Interaction.

INTRODUCTION

With the change of the human condition and dietary patterns, diabetes has become "the third biggest killer" to harm human health next to cancer and AIDS[1]. Minodiaq is one kind of diabetes medications which are belonged to sulfonylureas (the structure shown in Figure 1)[2,3]. Sulfonylureas help the pancreas make more insulin, they also help the cells to respond better to insulin and this benefits lower blood sugar and keep it under better control. Hemoglobin (Hb) is one of the important proteins in the human body, main ingredient in the erythrocyte. It is well known for its function in the vascular system, being a carrier of oxygen[4]. Bovine hemoglobin (BHb), which shares 90% amino acid sequence homology with human hemoglobin, has a few advantages over its human counterpart. Compared with human hemoglobin, BHb plays a better oxygen carrier role[5]. Studies on the binding of drug with protein will facilitate interpretation of the metabolism and transporting process of drug and will help to explain the relationship between structure and function of protein.

At present, the molecular interaction between BHb and many drugs has been investigated successfully in bio-medical domain[6-8]. However, the interaction between Minodiaq and BHb has not been investigated, especially the effects of some metal ions on the binding of drugs to BHb. In this report, the interaction between

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Minodiaq and BHb was investigated by fluorescence spectroscopy, UV-vis spectroscopy and CD spectra, as well as the effects of some metal ions (Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, V^{5+}, Mo^{6+}, Cr^{6+}) on the BHb-Minodiaq system. There has been increasing attention on the study of molecular interactions between protein and many drugs. It is a great significance to study the binding mechanism for life science, chemical, pharmaceutical and clinical medicine.

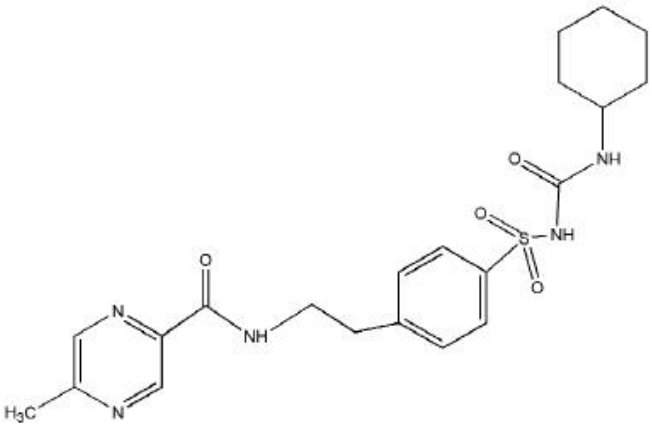

Figure 1 : Chemical structure of Minodiaq

EXPERIMENTAL

Apparatus

All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectro- fluorophotometer. Absorption was measured with an UV-vis recording spectrophotometer (UV-265 Shimadzu, Japan). CD spectra were recorded on a MOS-450/SFM300 circular dichroism spectrometer (Bio-Logic, France). All pH measurements were made with a pHS-3C precision acidity meter (Leici, Shanghai). All temperatures were controlled by a CS501 super-heated water bath (Nantong Science Instrument Factory).

Materials

Minodiaq (CAS#, 29094-61-9) was obtained from Monitor of Chinese Veterinary Medicine (the purity grade inferior 99%). Bovine Hemoglobin (CAS#, 9008-02-0) was purchased from Sigma Company (Sigma–H3760; the purity grade inferior 99%). Stock solutions of BHb (2.0×10^{-4} mol·L^{-1}), Minodiaq (5.6×10^{-5} mol·L^{-1}) and metal ions (1.0×10^{-3} mol·L^{-1}) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris-HCl (0.05 mol·L^{-1}) buffer solution containing NaCl (0.15 mol·L^{-1}) was used to keep the pH of the solution at 7.40, and NaCl solution was used to maintain the ionic strength of the solution. All other reagents were analytical grade and all aqueous solutions were prepared with newly double-distilled water and stored at 277 K.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter effect using the following relationship[9]:

$$\mathbf{F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2}} \quad \textbf{(1)}$$

Where, F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively. A_{ex} and A_{em} are the absorbance values of Minodiaq at excitation and emission wavelengths, respectively. The fluorescence intensity used in this paper was corrected.

Procedures

In a typical fluorescence measurement, 1.0 mL of Tris-HCl buffer, pH 7.4, 0.5 mL of 4.0×10^{-5} mol·L^{-1} BHb solution and different concentrations of Minodiaq were added into a 10 mL colorimetric tube successively. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 30 minutes at different temperatures (293, 303 and 310 K). Excitation wavelength for BHb was 280 nm (or

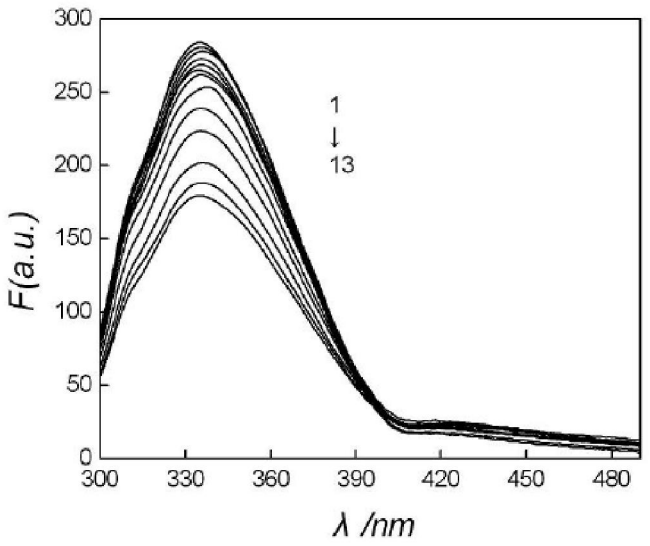

Figure 2 : Fluorescence spectra of BHb-Minodiaq system (T = 293 K λ_{ex} = 280 nm); C_{BHb} = 2.0×10^{-6} mol·L^{-1}, 1~13 $C_{Minodiaq}$ = (0, 0.056, 0.112, 0.224, 0.448, 0.672, 0.896, 1.34, 1.79, 2.24, 3.36, 3.92, 4.48) $\times10^{-5}$ mol·L^{-1}

295 nm), with excitation and emission slit at 5 nm. Then the ûuorescence spectra were then measured (emission wavelengths of 290~500 nm) at 293, 303 and 310 K. Besides, we recorded the fluorescence spectra when the value between the excitation and emission wavelengths, $\Delta\lambda$ was 15 and 60 nm. CD spectra were performed between 200 and 300 nm using a 1.0 mm path length quartz cuvette at 293 K.

RESULT AND DISCUSSION

Fluorescence quenching spectra of BHb-Minodiaq system

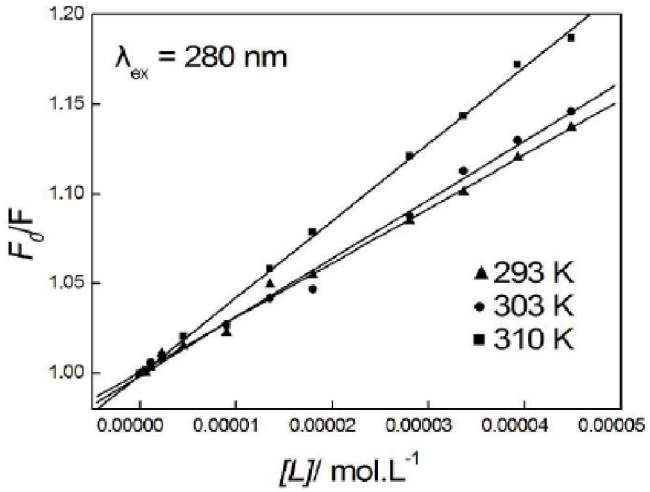

Figure 3 : Stern-Volmer plots for the quenching of BHb by Minodiaq at different temperatures; $C_{BHb} = 2.0\times10^{-6}$ mol·L^{-1}, $C_{Minodiaq} = 5.6\times10^{-7}$ mol·L^{-1} and 4.48×10^{-5} mol·L^{-1}.

Proteins were considered to have intrinsic fluorescence due to the presence of amino acids, mainly tryptophan (Trp), tyrosine (Tyr). When the excitation wavelengths were at 280 nm and 295 nm, the emission peaks for BHb were both located at 335 nm. The fluorescence spectrum of BHb-Minodiaq system was shown in Figure 2. As shown in Figure 2, the fluorescence intensity of BHb decreased regularly and there was almost no shift of the emission wavelength with the addition of Minodiaq. This result showed that Minodiaq could quench the intrinsic fluorescence of protein and there was a interaction between Minodiaq and BHb.

To interpret the data from fluorescence quenching studies, it is important to understand what kind of interaction takes place between the fluorophore (BHb) and the quencher (Minodiaq). If it is assumed that the fluorescence quenching of BHb induced by Minodiaq is a dynamic quenching process, fluorescence quenching is described by the Stern–Volmer equation[10].

$$F_0 / F = 1 + K_q \tau_0 [L] = 1 + K_{sv} [L] \quad (2)$$

Where, F_0 and F are the fluorescence intensities of BHb before and after the addition of the Minodiaq, respectively. τ_0 is the average lifetime of fluorescence without ligand, which is about 10^{-8} s. K_{sv} is the Stern-Volmer quenching constant. K_q is the bimolecular quenching constant, and [L] is the concentration of the ligand. Figure 3 displays the Stern–Volmer plots of the quenching of BHb fluorescence by Minodiaq. It shows that the curves have linear relationships with increasing the concentrations of Minodiaq. Based on the linear fit plot of F_0/F versus [L], the K_q values can be obtained. The calculated results were shown in TABLE 1. Different mechanisms of quenching are usually classified as the dynamic quenching and the static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature. The quenching rate constants decrease with the rising temperature for the static quenching, but the reverse effect is observed for the dynamic quenching[11]. In TABLE 1, the Stern–Volmer quenching constants at different temperatures (293, 303 and 310 K) were presented. The results showed that K_q increased with temperature rising in the concentration range of Minodiaq from 0 to 4.48×10^{-5} mol·L^{-1}, it indicated that the quenching mechanism of BHb by Minodiaq was a dynamic type. In addition, all the values of K_q were much greater than the maximum scatter collision quenching constant of various quenchers (2×10^{10} L·mol^{-1}·s^{-1}). This may be the effect of the ionic strength, which makes K_q too large[12].

In order to confirm the quenching mechanism was a dynamic process, the UV-vis absorption of BHb in the absence and presence of Minodiaq were determined at pH 7.40 Tris-HCl buffer solution. The result indicated that Minodiaq did not change the ultraviolet absorption spectrum of BHb, which showed that the quenching process was not a static quenching but dynamic quenching. For the quenching process, the relationship between the fluorescence intensity and the concentration of quencher can be usually described by Eq. (3)[13] to obtain the binding constant (K_a) and the number of binging sites (n) in most paper:

$$\lg\{(F_0 - F)/F\} = n\lg K_a + n\lg[D_t] - n(F_0 - F)/F_0[B_t]\} \quad (3)$$

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Where, $[D_t]$ and $[B_t]$ are the total concentrations of Minodiaq and BHb, respectively. On the assumption that n in the bracket is equal to 1, the curve of $\lg(F_0\text{-}F)/F$ versus $\lg\{[D_t]\text{-}[B_t](F_0\text{-}F)/F_0\}$ is drawn and fitted linearly, then the value of n can be obtained from the slope of the plot. If the n value obtained is not equal to 1, then it is substituted into the bracket and the curve of $\lg(F_0\text{-}F)/F$ versus $\lg\{[D_t]\text{-}n[B_t](F_0\text{-}F)/F_0\}$ is drawn again. The above process was repeated again and again till n obtained was only a single value or a circulating value. Based on the n obtained the binding constant K_a can be also obtained. In the work, a calculation program was developed. Figure 4 shows plots of $\lg(F_0\text{-}F)/F$ versus $\lg\{[D_t]\text{-}n[B_t](F_0\text{-}F)/F_0\}$ for the BHb-Minodiaq system at different temperatures. The calculation process can be finished with calculator based on the simple program and the calculating result can be obtained by inputting F, $[D_t]$ and $[B_t]$. The calculated result was shown in TABLE 1. The fact was that the values of n were all equal to 1 approximately imply that just one binding site for Minodiaq existed in BHb. Meanwhile, the increasing trend of K_a with the increasing temperature, further suggested that the quenching was a dynamic process.

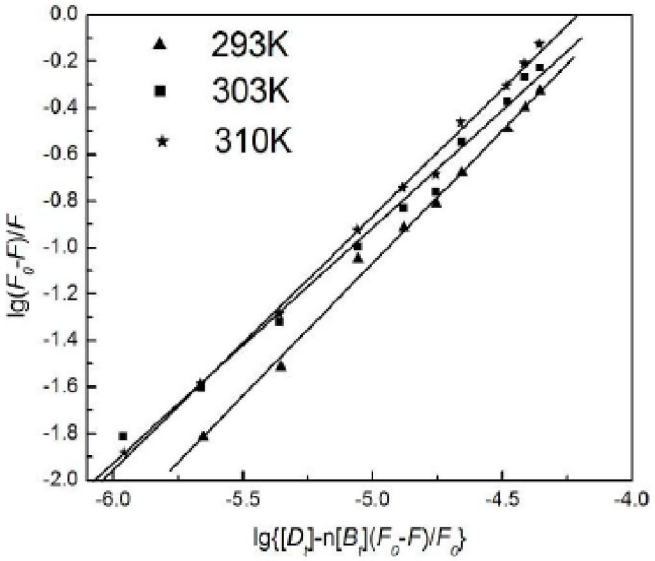

Figure 4 : Plots of $\lg(F_0\text{-}F)/F$ versus $\lg\{[D_t]\text{-}n[B_t](F_0\text{-}F)/F_0\}$ for the BHb-Minodiaq system at different temperatures. C_{BHb} = 2.0×10^{-6} mol·L^{-1}, $C_{Minodiaq}$ = 1.12 ×10^{-6} mol/L and 3.92 ×10^{-5} mol·L^{-1}.

Participation of tyrosine (Tyr) and tryptophan (Trp) groups in the drug-serum albumin complex formation is confirmed by quenching of protein fluorescence excited at different wavelengths[14]. When the wavelength is 280 nm, the Trp and Tyr residues in protein are excited, whereas the 295 nm wavelength excites only Trp residues[15]. BHb is a tetrameric protein composed of two α and two β subunits. There are three Tyr (α-24Tyr, α-42Tyr, α-140Tyr) and one Trp (α-14Trp) residues in α subunit, three Tyr (β-34Tyr, β-144Tyr, β-145Tyr) and two Trp (β-15Trp, β-37Trp) residues in β subunit, respectively[16]. α-14Trp and β-15Trp expose to the subunit surface. However, β-37Trp, α-42Tyr, α-140Tyr, and β-145Tyr are located at the α1β2 subunit interface remained invariant throughout the evolution of α and β subunits, they are indispensable for the maintenance of structure and function of BHb and also considered as the main binding sites for small drug molecule[17]. Based on the Stern-Volmer equation, comparing the fluorescence quenching of protein excited at 280 nm and 295 nm allows to estimate the participation of Trp and Tyr groups in the system[18]. As we can see in Figure 5, in the presence of Minodiaq, the quenching curves of BHb excited at 280 nm and 295 nm no overlap, and the quenching curves of BHb at 280 nm was much greater than 295 nm. This phenomenon showed that Trp and

TABLE 1 : Quenching related parameters of BHb and Minodiaq at different temperatures

λ_{ex}/(nm)	T/K	K_{sv}/(L·mol^{-1})	K_q/(L·mol^{-1}·s^{-1})	r_1	K_a/(L·mol^{-1})	n	r_2
280	293	1.05×10^4	1.05×10^{12}	0.9972	1.11×10^4	1.15	0.9918
	303	1.34×10^4	1.34×10^{12}	0.9964	1.24×10^4	0.97	0.9981
	310	1.42×10^4	1.42×10^{12}	0.9937	1.53×10^4	1.11	0.9906
295	293	3.01×10^3	3.01×10^{11}	0.9980	2.99×10^3	0.99	0.9975
	303	4.46×10^3	4.46×10^{11}	0.9975	3.42×10^3	0.92	0.9943
	310	5.46×10^3	5.46×10^{11}	0.9988	4.28×10^3	0.92	0.9979

K_q is the quenching rate constant; K_a is the binding constant; n is the number of binding site. r_1 is the linear relative coefficient of F_0/F~[L]; r_2 is the linear relative coefficient of $\lg(F_0\text{-}F)/F$~$\lg\{[D_t]\text{-}n[B_t](F_0\text{-}F)/F_0\}$.

Tyr residue played important role in the interaction between Minodiaq and BHb.

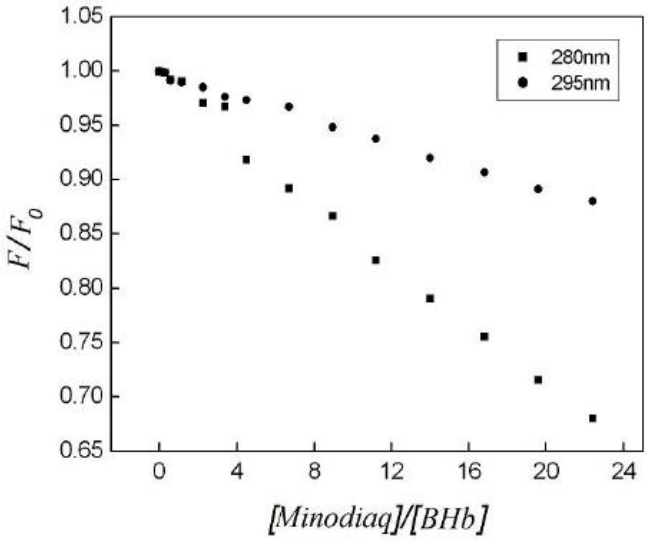

Figure 5 : Quenching curves of BHb-Minodiaq system at λ_{ex} = 280 nm and 295 nm; C_{BHb} = 2.0×10^{-6} mol·L^{-1}; $C_{Minodiaq}$ = (0, 0.056, 0.112, 0.224, 0.448, 0.672, 0.896, 1.34, 1.79, 2.24, 3.36, 3.92, 4.48)×10^{-5} mol·L^{-1}

Type of interaction force of BHb-Minodiaq systems

Generally, the interaction force between the small drug molecule and biological macromolecule include hydrogen bond, Van der Waals force, electrostatic interactions and hydrophobic force, etc. Ross and Subramanian[19] have characterized the sign and magnitude of the thermodynamic parameter, enthalpy change (ΔH), free energy (ΔG) and entropy change (ΔS) of reaction, associated with various individual kinds of interaction. When temperature varies in a small range, the ΔH could be considered as a constant. Negative ΔH and positive $\Delta\Delta$S indicate electrostatic interaction plays a major role in the binding reaction. Positive ΔH and ΔS are generally considered as the evidence for typical hydrophobic interactions. In addition, Van der Waals force and hydrogen bonding formation in low dielectric media are characterized by negative ΔH and ΔS[20]. The thermodynamic parameters can be calculated on the basis of the following equation:

$$\mathbf{R \ln K = \Delta S - \Delta H / T} \quad (4)$$

$$\mathbf{\Delta G = \Delta H - T\Delta S} \quad (5)$$

According to the binding constants K_a of Minodiaq to BHb at different temperatures mentioned above (TABLE 1), the thermodynamic parameters were obtained conveniently. The values of H was obtained from the linear van't Hoff plot (Figure 6). Therefore, the values of ΔH, ΔS and ΔG were listed in TABLE 2. The negative value of ΔG clarified an automatic reaction between Minodiaq and BHb, the positive value of ΔS and positive value of ΔH showed that the hydrophobic forces play a major role in the binding process.

Furthermore, the positive value of ΔH indicated that the interaction between BHb and Minodiaq was an endothermic reaction. i.e., increasing temperature promoted the interaction between BHb and Minodiaq and also fluorescence quenching between BHb and Minodiaq. Those conclusions were consistent with the results in TABLE 1, which indicated that increasing temperature could promote fluorescence quenching and the quenching constants.

Hill's coefficient of BHb-Minodiaq system

In biochemistry, the binding of a ligand molecule at one site of a macromolecule often influences the affinity for other ligand molecules at additional sites. This is known as cooperative binding. It is classified into positive cooperativity, negative cooperativity and non-cooperativity according to the promotion or inhibition to the affinity for other ligand molecules. Hill's coeffi-

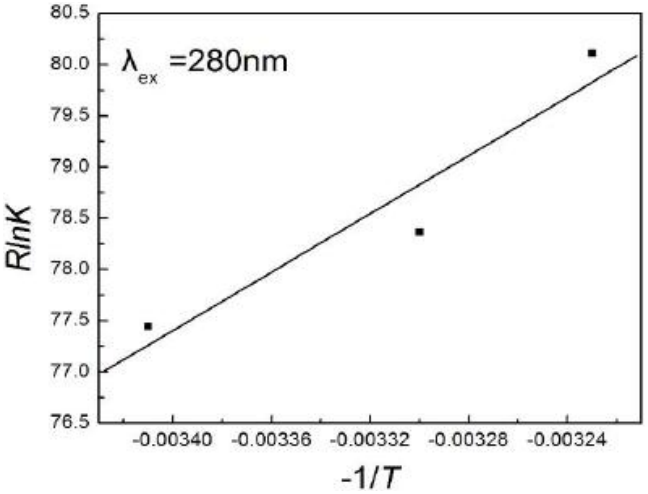

Figure 6 : Van't Hoff plot for the interaction of BHb and Minodiaq in Tris-HCl buffer; pH = 7.4.

TABLE 2 : The thermodynamic parameters

T / K	K_a/(L·mol^{-1})	SD_3	ΔH/(kJ·mol^{-1})	ΔS/(J·K^{-1})	ΔG/(kJ·mol^{-1})
293	1.11×10^4	0.55539	1.335	77.44	-22.69
303	1.24×10^4	0.55539	1.335	78.36	-23.74
310	1.53×10^4	0.55539	1.335	80.11	-24.83

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cient provides a way to quantify this effect and is calculated graphically on the basis of the following equation[21]:

$$\lg\{Y/(1-Y)\} = \lg K + n_H \lg[L] \quad (6)$$

Where, Y is the fractional binding saturation; K is the binding constant and n_H is the Hill's coefficient. Hill's coefficient is greater than 1, which exhibits positive cooperativity. Conversely, Hill's coefficient is less than 1, which exhibits negative cooperativity. A coefficient of 1 indicates non-cooperative reaction.

For fluorescence measurement:

$$Y/(1-Y) = Q/(Q_m - Q) \quad (7)$$

Where, $Q = (F_0 - F)/F_0$; $1/Q$m = intercept of the plot $1/Q$ versus $1/[L]$. Hill's coefficients were presented in TABLE 3. The values of n_H were greater than 1 at different temperatures, these results indicated that there was positive cooperative reaction between BHb and Minodiaq.

TABLE 3 : Hill's coefficients n_H of BHb-Minodiaq systems at different temperatures

T/K	λ_{ex}/280 nm			λ_{ex}/295 nm		
	n_H	r_3	SD_4	n_H	r_3	SD_4
293	1.18	0.993 5	0.08821	1.07	0.996 3	0.05607
303	1.04	0.996 9	0.05098	1.03	0.998 2	0.02348
310	1.30	0.994 8	0.07044	1.23	0.997 8	0.03798

r_3 is the linear relative coefficient of lg[Y/(1–Y)]~lg[L]; SD is the standard deviation.

Binding distances between BHb and Minodiaq

According to Förster's non-radiative energy transfer theory[22], energy efficiency E, critical energy-transfer distance R_0 (E = 50%), the energy donor and the energy acceptor distance r and the overlap integral between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor J can be calculated by the following formulas[23]:

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r^6) \quad (8)$$

$$R_0^6 = 8.78\times10^{-25} K^2 \Phi N^{-4} J \quad (9)$$

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (10)$$

Where, K^2 is the orientation factor, Φ is the fluorescence quantum yield of the donor, N is a refractive index of the medium, $F(\lambda)$ is the fluorescence intensity of the fluorescence donor at wavelength λ and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at this wavelength. The overlap of UV-vis absorption spectra of Minodiaq and the fluorescence emission spectra of BHb (λex =280 nm) were shown in Figure 7. Under these experimental conditions, it has been reported that K^2 =2/3, N =1.336 and Φ =0.062[24]. Thus J, E, R_0 and r were calculated and shown in TABLE 4. The donor-to-acceptor distance r <7 nm indicated that the energy transfer from BHb to Minodiaq occured with high possibility[25]. The energy efficiency E increased and the distance r decreased with increasing temperature (TABLE 4), which resulted in the enhanced stability of the binary systems and the values of K_a. This result coincides with Section fluorescence quenching spectra of BHb-Minodiaq system, namely the increasing trend of K_a with increasing temperature.

Conformation investigation of BHb

Synchronous fluorescence spectra are used to investigate the protein conformational change, as it has

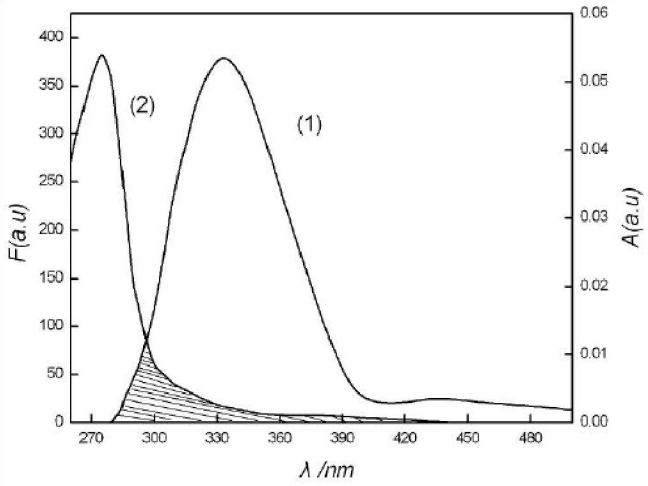

Figure 7 : Fluorescence emission spectra for BHb (1) and UV absorbance spectra for Minodiaq (2); T = 293 K; $C_{Minodiaq}$ = C_{BHb} = 6.0×10^{-6} mol·L^{-1}

TABLE 4 : Parameters of E, J, r, R_0 between BHb and Minodiaq at different temperatures

T/K	E (%)	J/(cm^3·L·mol^{-1})	R_0/nm	r/nm
293	1.19	8.32×10^{-16}	1.38	3.03
303	1.96	1.22×10^{-15}	1.55	2.98
310	2.91	1.67×10^{-15}	1.63	2.93

R_0 is the critical distance when E is 50%; r is the distance between acceptor and donor; J is the overlap integral between the fluorescence emission spectrum of donor and the absorption spectrum of the acceptor.

been shown to give narrow and simple spectra. For the synchronous fluorescence spectra of protein, when the $\Delta\lambda$ value between the excitation and emission wavelengths is fixed at either 15 or 60 nm, the synchronous fluorescence gives characteristic information for Tyr residues or Trp residues[26]. Because of the red shifts of maximum emission wavelengths of both Tyr and Trp with the less hydrophobic environment, blue shifts of maximun emission wavelengths with the more hydrophobic environment occur[27]. These red or blue shifts indicated that the conformation of protein has been changed. In order to further study the effect on the conformation of BHb, the synchronous fluorescence spectra were measured when the $\Delta\lambda$ =15 nm and $\Delta\lambda$ =60 nm as shown in Figure 8. It can be seen from Figure 8 that the fluorescence intensities of Tyr and Trp residues in BHb decreased regularly with increasing concentration of Minodiaq, and the emission maximum of Tyr and Trp residues did red shift of 3 nm which indicated that the conformation of BHb was changed, the polarity around the Tyr and Trp residues was increased and the hydrophobicity was decreased. This may be due to the changes of residue microenvironment with the insertion of Minodiaq. In BHb, aromatic residues such as α-42Tyr, α-140Tyr, β-37Trp and β-145Tyr are located at the α1β2 subunit interface and undergo changing of environment with the quaternary structure transition from T to R[28]. The BHb central cavity contains functional significantly center for binding several class of allosteric effectors, which modulate BHb affinity to oxygen. The red shift implies that Minodiaq has ability to bind into BHb central cavity and induces the structural and functional changes of BHb[29]. In addition, with the increased amount of Minodiaq the extent of the red shift and the decline in fluorescence peak of Trp is larger than the extent of the red shift and the decline in fluorescence peak of Tyr, this indicated that Minodiaq was much closer to Trp than Tyr, and mainly quenched the fluorescence of β-37Trp.

Effect of metal ions on the BHb-Minodiaq system

In the plasma, there are some metal ions that can participate in many important vital actions and affect the reactions of the drugs with the plasma protein. So the effect of metal ions (Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, V^{5+}, Mo^{6+}, Cr^{6+}) on the binding constant of Minodiaq to BHb was also discussed. The concentrations of BHb and metal ions were constant as 2.0×10^{-6} mol·L^{-1} and 1.0×10^{-5} mol·L^{-1}, respectively, and different concentrations of Minodiaq were added in the solution respectively at 303 K. The excitation wavelength was 280 nm and the excitation and emission slits were set at 5 nm each. The solution was subsequently scanned on the fluorophotometer and determined the fluorescent intensity at 335 nm. Based on the Eq. (3), we can obtain the value of K_a of BHb-Minodiaq system with Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, V^{5+}, Mo^{6+}, Cr^{6+}. The calculated results were shown in TABLE 5. From TABLE 5, we

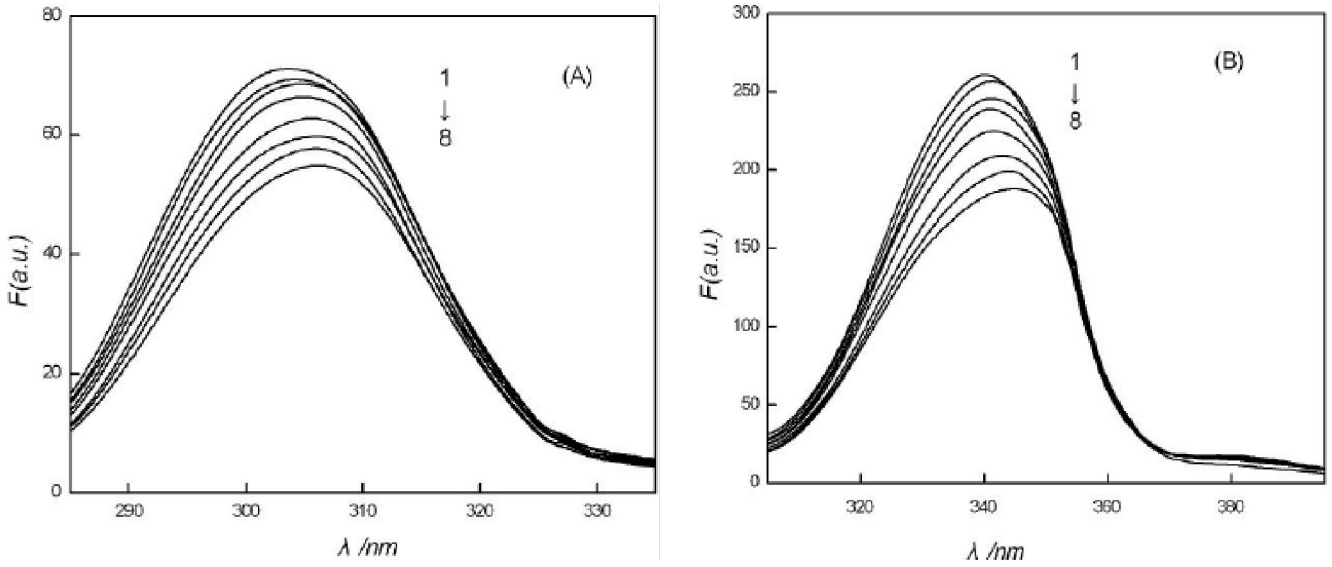

Figure 8 : Synchronous fluorescence spectra of BHb-Minodiaq system (T = 293 K); C_{BHb} = 2.0×10^{-6} mol·L^{-1}, 1 ~8 $C_{Minodiaq}$ = (0, 0.896, 1.34, 1.79, 2.24, 3.36, 3.92, 4.48) $\times10^{-5}$ mol·L^{-1}; (A) $\Delta\lambda$ = 15 nm; (B) $\Delta\lambda$ = 60 nm

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can observe that the existence of Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, V^{5+}, Mo^{6+}, Cr^{6+} decreased the binding constant between BHb and Minodiaq, which resulted from certain competition among metal ions and Minodiaq for the BHb, the formation of metal ions-BHb complexes was likely to affect the conformation of BHb and inhibit the binding of BHb-Minodiaq[30]; The existence of Ni^{2+} enhanced the binding of BHb and Minodiaq. This is due to the interaction between metal ions and Minodiaq, where the metal ions-Minodiaq complex is formed. This complex then interacts with BHb, forming the new complex metal ions-Minodiaq-BHb. The binding of BHb-Minodiaq is thus enhanced due to the existence of metalion bridge[31]. This result indicated that the existence of metal ions would lead to binding constant K_a changed spontaneously, there were possible effects on the stayed-time from the blood, the transportation of Minodiaq, as well as plasma concentration and effective concentration, having effects on the efficacy of drugs[32].

TABLE 5 : Quenching reactive parameters of BHb and Minodiaq (T = 303 K, λ_{ex} = 280 nm)

metal ions	K_a / (L·mol^{-1})	r	SD
--	1.24×10^4	0.9981	0.0409
Mg^{2+}	5.58×10^3	0.9983	0.03169
Zn^{2+}	1.04×10^4	0.9967	0.05100
Cu^{2+}	9.87×10^3	0.9975	0.05157
Mn^{2+}	5.67×10^3	0.9942	0.06559
Ni^{2+}	1.39×10^4	0.9954	0.08986
V^{5+}	3.29×10^3	0.9984	0.02861
Mo^{6+}	6.16×10^3	0.9929	0.07625
Cr^{6+}	7.47×10^3	0.9909	0.06288

K_a is the binding constant; r is the linear relative coefficient of $\lg(F_0\text{-}F)/F \sim \lg\{[D_t]\text{-}n[B_t](F_0\text{-}F)/F_0\}$. SD is the standard deviation.

CD spectra of BHb-Minodiaq system

CD is a sensitive technique to monitor any kind of conformational change in the protein upon interaction with drug molecule. To investigate the possible influence of Minodiaq binding on the secondary structure of BHb, the CD spectra was performed in the absence and presence of Minodiaq as shown in Figure 9. As evident from Figure 9, the CD spectra of BHb exhibited two negative peaks in the UV region at 209 and 222 nm, which are characteristic features of α-helix structure of proteins[33]. The reasonable explanation of the result is that these negative peaks are both contributed to $n \rightarrow \pi^*$ transition for the peptide bond of α-helix[34]. The bands intensities of BHb at 209 and 222 nm decreased with the addition of Minodiaq without causing any significant shift of the peaks, this indicated the loss of α-helix secondary structure content upon the interaction of Minodiaq with BHb. When molar ratio of BHb to Minodiaq were 1:0, 1:2.8, 1:14, the α-helix content were 25.52%, 21.47%, 19.96%, respectively. The reduction of the α-helix structure content was also an indication of a partial unfolding of the BHb structure, in the presence of Minodiaq[35]. The binding of Minodiaqe with BHb could destroy some protein hydrogen bonding networks and induce some secondary structure change in BHb[36].

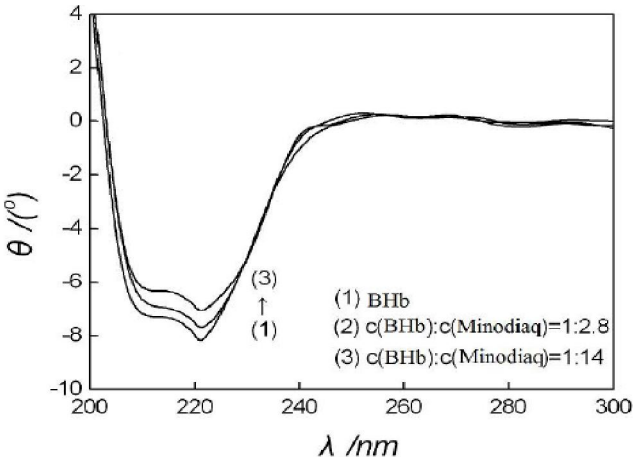

Figure 9 : The circular dichroism spectra of BHb-Minodiaq system (T = 293 K); C_{BHb} = 2.0×10^{-6} mol·L^{-1}, $C_{Minodiaq}$ = (0.56, 2.8) ×10^{-5} mol·L^{-1}.

CONCLUSIONS

In this paper, the interaction of Minodiaq with BHb was studied at different temperatures by fluorescence spectroscopic methods, UV-vis absorption measurement and CD spectroscopy, as well as the effect of some metal ions (Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, V^{5+}, Mo^{6+}, Cr^{6+}) on the BHb-Minodiaq system. The experimental results indicated that Minodiaq molecule had been embedded into the hydrophobic cavity through hydrophobic force and was mainly bound to β-37Trp in BHb. The donor-to-acceptor distance r was less than 7 nm, indicating non-radiation energy transfer. There was positive cooperative reaction in BHb-Minodiaq

system for subsequent ligand. From the synchronous fluorescence and CD spectra, it could be shown that the conformational change of BHb was induced by the interaction of Minodiaq. The existence of metal ions would lead to binding constant K_a of BHb-Minodiaq system changed spontaneously. The study will extend the use of fluorescence spectroscopy, meanwhile, it provides important insights into the future clinical medicine.

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