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Original article

Studies on the interaction of caffeine with bovine hemoglobin

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ABSTRACT

Caffeine (CF) is a member of the methylxanthine family with numerous biological activities, which may contribute to the prevention of human disease but also may be potentially harmful. In the present study, the interaction of CF with bovine hemoglobin (BHb) under physiological condition was studied by fluorescence and UV/vis spectroscopy. Fluorescence data revealed that the fluorescence quenching of BHb by CF was the result of the formed complex of CF–BHb. The binding constants and thermodynamic parameters at three different temperatures, the binding position, and the binding force were determined. The hydrophobic and hydrogen bonds interactions were the predominant intermolecular forces to stabilize the complex. The conformation of BHb was discussed by synchronous fluorescence techniques. The synchronous spectra indicated that the structures of the Tyr and Try residues environments were altered and the physiological functions of BHb were affected by 0. This study provides important insight into the mechanism of erythrocyte sickling, which may be a useful guideline for further toxicology investigation.

1. Introduction

Caffeine (CF, 1′3′-trimethylxanthine, a purine alkaloid, Fig. 1), is a key component of many popular drinks, mainly tea and coffee, but most phytochemists know little about its biochemistry and molecular biology [1]. A number of *in vitro* studies have demonstrated that CF modulates both innate and adaptive immune responses [2]. For instance some studies indicated that CF increases calcium release in the sarcoplasmic reticulum, which has the effect of increasing muscular contraction. Type 2 diabetes is a heterogeneous disorder of glucose homeostasis that develops when the balance among these factors is disrupted in response to genetic and/or environmental influences. The evidence from this study suggests that caffeine consumption has a detrimental impact on glucose regulation in patients with type 2 diabetes. Recent evidence has suggested that caffeine reduces insulin sensitivity in healthy volunteers with normal glucose metabolism [3]. Other studies indicate that CF and its major metabolite paraxanthine suppress neutrophil and monocyte chemotaxis, activate the erythrocyte enzyme glutathione-*S*-transferase (GST), and also suppress production of the pro-inflammatory cytokine tumour necrosis factor (TNF)-α from human blood. Furthermore, CF probably is prosickling also by its interaction and easy passage through biological membranes [4,5]. Recent work showed that exogenous CF increased the intracellular concentration of sodium and calcium ions, and possible changes in ATP level [6,7]. The effect of exogenous CF on human hemoglobin-S (HbS) erythrocytes was investigated *in vitro*, the CF concentration in plasma might proffer marked increase in erythrocyte sickling *in vivo*, and consequently, slow recovery from a sickling crisis [8]. Mathew et al. have reported on the reduction in cerebral blood flow induced by CF, CF was found to be associated with significant reductions in cerebral perfusion thirty and ninety minutes later [9]. CF also influences the heme ligation affinities in the monomeric hemoglobin [10].

Hemoglobin (Hb), the major protein component in erythrocytes, exists as a tetramer of globins chains that is composed of two α and two β subunits; Hb is well known for its function in the vascular system of animals, being a carrier of oxygen. It also aids, both directly and indirectly, the transport of carbon dioxide and regulates the pH of blood [11]. Bovine hemoglobin (BHb), which shares 90% amino acid sequence homology with human hemoglobin, has a few advantages over its human counterpart. BHb is a better oxygen carrier than human hemoglobin. BHb has a less exothermic oxygen binding and delivers oxygen even at low temperatures [12]. The BHb affinity for oxygen is regulated by chlorides rather than 2,3-diphosphoglycerate (DPG), as in human hemoglobin. It does not have to be chemically modified with 2,3-DPG analogues in order to unload oxygen [13]. Determination of the crystal structure of BHb has shown that the most important structural difference with respect to human hemoglobin is a shift, at the level of that crevice which in human hemoglobin constitutes the organic phosphate

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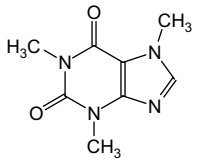

Fig. 1. Molecular structure of caffeine.

binding site, of both the N terminus and A-helix closer to the dyad axis [14]. Research reports on the BHb interaction with some molecules were well published, such as heteropolyacid [15], mercuric acetate [16], herbicide [17], and flavonoids [18]. Since BHb is an important functional protein for reversible oxygen carrying and storage, as well as a mode protein with high α-helical content, the potential changes of conformation and function for BHb after binding of small molecules have been a focus of study.

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. Also, various possibilities of structural rearrangements in the environment of the fluorophore may lead to a similar fluorescence signal; they can complicate interpretation of the experimental result and be exploited to obtain unique structural and dynamic information [19–21]. Bian et al. have studied the interaction of CF with bovine serum albumin, the results indicated that CF could bind with bovine serum albumin strongly at molar ration 1:1 and the combination reaction of CF with bovine serum albumin is a single static quenching process [22]. Kriško et al. have studied the binding of CF to human serum albumin; the results obtained by spectroscopy indicated that an increase in the local protein dynamics and/or polarity change has been introduced upon CF binding [23]. The binding of CF to human plasma albumin (4.5% w/v) *in vitro* was also examined using ultracentrifugation and it was observed to be bound to the extent of 37.8% [24]. CF was found not to bind covalently to liver microsomal proteins from mice, rats and rabbits [25]. Little is known at the molecular level about the interactions of CF with hemoglobin [10]. In this report, we provide investigations on the effect of CF on the structural and spectral properties of BHb, the thermodynamic aspects in the binding process, and characterization of the binding sites.

2. Materials and methods

2.1. Materials and solutions

BHb was purchased from Sigma (St. Louis, MO, USA) and used without further purification. CF was obtained from Fluka (Switzerland). The Tris buffer was purchased from Acros (Geel, Belgium), and NaCl, HCl, etc. were all of analytical purity. BHb solution (5.0 μM) was prepared in pH 7.40 Tris–HCl buffer solution (0.05 M Tris, 0.1 M NaCl). The CF solution (2.5 mM) was prepared by dissolving CF in Tris–HCl buffer solution. Water was purified with a Millli-Q purification system (Barnatead, Dubuque, IA. USA) to a specific resistance >16.4 MΩ cm^{-1}. All solutions were stored in refrigerator at 4 °C in dark.

2.2. Equipments and spectral measurements

The UV/vis spectrum was recorded at room temperature on a SPECORD S 50 (Germany) equipped with 1.0 cm quartz cell. All fluorescence spectra were recorded on LS-50B Spectrofluorimeter (Perkin–Elmer, USA) equipped with 1.0 cm quartz cell and a thermostated bath. The widths of the excitation and the emission slits were set to 10.0 nm/5.0 nm for BHb, respectively.

2.3. Procedures

A 2.5 mL solution, containing appropriate concentration of BHb, was titrated by successive additions of a 2.5 mM stock solution of CF. Titrations were done manually by using trace syringes. The fluorescence spectra were then measured (excitation at 280 nm and emission wavelengths of 290–500 nm) at three temperatures (290 K, 300 K, 310 K).

3. Results and discussion

3.1. UV/vis absorption studies

UV/vis absorption measurement is a very simple method and applicable to explore the structural change and to know the complex formation [26]. Hence, absorption spectra of BHb in presence and absence of CF were recorded (Fig. 2). Fig. 2 showed that the absorption spectral change of BHb in the presence of CF in the wavelength 250–500 nm. The absorption maximum of Soret band is decreased after CF treatment, while the maximum absorption wavelengths remain unchanged. This means that the heme is not exposed from the crevices at the exterior of the subunit and CF is easily integrated into the hydrophobic pocket of BHb. Two isosbestic points were noticed at 340 nm and 425 nm. This indicates the formation of a ground stated complex between BHb and CF [27].

3.2. Fluorescence quenching of BHb by CF

For macromolecules, the fluorescence measurements can give some information of the binding of small molecule substances to protein at the molecular level, such as the binding mechanism, binding mode, binding constants, intermolecular distances, etc. BHb contains three Trp residues in each αβ dimer, for a total of six in the tetramer: two α-14 Trp, two β-15 Trp, and β-37 Trp [28]. Of the three Trp residues, only the β-37 Trp is located at the dimer–dimer interface, wherein the structural difference between quaternary states is largest [29]. The intrinsic fluorescence of BHb primarily originates from β-37 Trp that plays a key role in the quaternary state change upon ligand binding [30]. A valuable feature of intrinsic fluorescence of protein is the high sensitivity of tryptophan to its local environment. Changes in emission spectra of

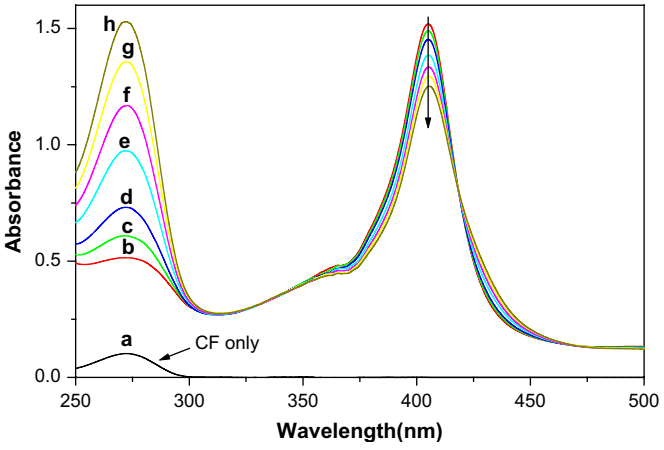

Fig. 2. UV/vis absorbance spectra of BHb in the absence and presence of CF. (a) CF only, c(CF) = 20.0 μM; (b–h) c(BHb) = 5.0 μM, c(CF)/(μM): 20.0, 40.0, 60.0, 80.0, 100.0, 120.0.

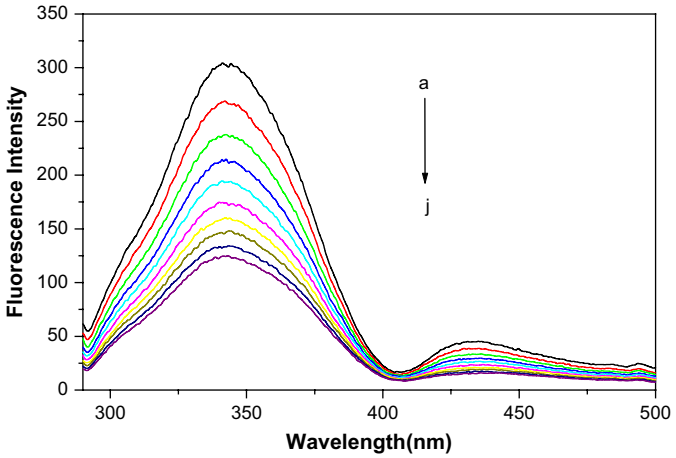

Fig. 3. Effect of CF on fluorescence spectrum of BHb ($T = 290$ K, pH = 7.40, $\lambda_{ex} = 280$ nm). (a–j) c(BHb) = 5.0 μM, c(CF)/(μM): 0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, and 90.0 respectively.

tryptophan are common in response to protein conformational transitions, subunit association, substrate binding, or denaturation [19]. Thus, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics, and it often considered on the study of protein folding and association reactions. The effect of CF on tryptophan residues fluorescence intensity is shown in Fig. 3. The data shows that the fluorescence intensity of BHb decreased regularly with the increasing concentration of CF without changing peak shape. These results indicated that there were interactions between CF and BHb. Because CF could also bind with bovine serum albumin and human serum albumin, CF binding to hemoglobin is not specific.

Generally speaking, the fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule, such as excited-state reaction, molecules rearrangement, energy transfer, ground state complex formation and collision quenching. In all cases, molecular contact between the fluorophore and the quencher is required for fluorescence quenching to occur. It is necessary to know quenching types for researching the mechanism of quenching. In this paper, we have used the binding constants dependence on the temperature to elucidate the quenching mechanism.

For fluorescence quenching spectra and quenching type could be described by the well known Stern–Volmer Eq. (1) [31] and modified Stern–Volmer Eq. (2) [32] to confirm the mechanism.

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

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K_a[Q]} \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_q, K_{SV}, τ_0, f_a, K_a, and [Q] are the quenching rate constant of the biomolecule, the Stern–Volmer dynamic quenching constant, the average lifetime of the biomolecule without quencher ($\tau_0 = 10^{-8}$ s [33]), the fraction of accessible fluorescence, the effected quenching constant for the accessible fluorophores, and the concentration of the quencher, respectively.

Within certain concentration, the curve of F_0/F versus [Q] (Stern–Volmer curve) would be linear if the quenching type is single static or dynamic quenching [34]; similarly, the curve of $F_0/(F_0–F)$ versus 1/[Q] (modified Stern–Volmer curve) would linear for static quenching [35]. If the quenching type is combined quenching (both static and dynamic), the Stern–Volmer plot is an upward curvature [36].

Fig. 4(A) displays the Stern–Volmer plots of the quenching of BHb tryptophan residues fluorescence by CF. The plot shows that within the investigated concentrations, the results agree with the Stern–Volmer Eq. (1). Quenching type should be single static or dynamic quenching. The Fig. 4(B) listed the modified Stern–Volmer curves. From Fig. 4(B), it was known that under certain CF concentration, the curves of $F_0/(F_0–F)$ versus 1/[Q] were linear. All these results showed that there were obviously characters of static quenching.

As a rule, the K_{SV} values decrease with an increase in temperature for static quenching, and the reverse effect would be observed for dynamic quenching [37]; the maximum scatter collision quenching constant, $K_{q,r}$ of various quenchers with the biopolymer was 2×10^{10} L moL^{-1} s^{-1} [35,36]. If the $K_q > K_{q,r}$, the fluorescence quenching of biopolymer surely not come from dynamic quenching. In this paper, the K_{SV}, and K_q at three different temperatures were listed in Table 1. It indicated that the K_{SV} values decreased with an increase in temperature and the K_q was approximately 10^{12} L moL^{-1} s^{-1}. Obviously, this indicated that the quenching was not initiated from dynamic collision but from the formation of a compound.

3.3. *The association constants (K_A) and the number of binding sites* (n)

In this paper, the binding parameters for the CF–BHb system have been derived from fluorescence quenching data obtain at 290,

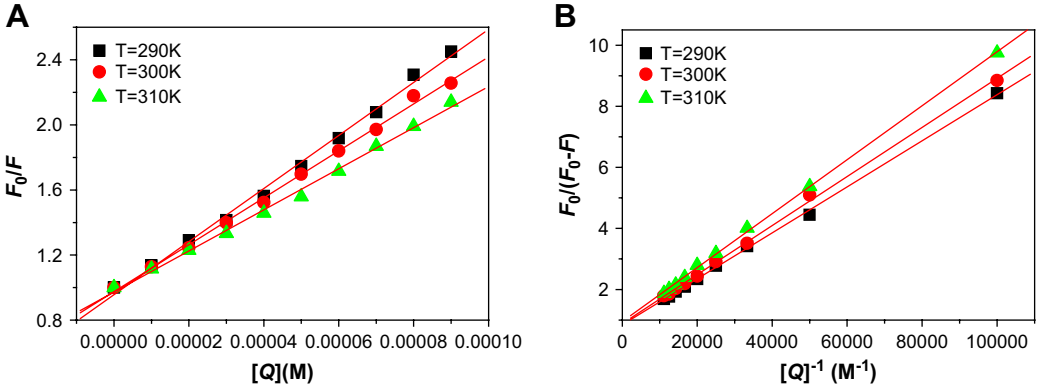

Fig. 4. (A) Stern–Volmer curve and (B) modified Stern–Volmer curve of BHb fluorescence quenching treated with different concentrations of CF: c(BHb) = 5.0 μM, pH 7.40, $T = 290$ K. $\lambda_{ex} = 280$ nm, $\lambda_{em} = 37$ nm.

Table 1
Stern–Volmer quenching constant (K_{SV}) and modified Stern–Volmer association constant (K_a) of the interaction of CF with BHB at three different temperatures.

T (K)	Eq. (1)				Eq. (2)		
	$10^{-4}K_{SV}$ ($L\,mol^{-1}$)	$10^{-12}K_q$ ($L\,mol^{-1}$)	R^a	SD[b]	$10^{-4}K_a$ ($L\,mol^{-1}$)	R^a	SD[b]
290	1.631	1.631	0.9978	0.0347	1.113	0.9995	0.0727
300	1.443	1.443	0.9986	0.0248	1.099	0.9994	0.0855
310	1.263	1.263	0.9980	0.0255	1.073	0.9997	0.0644

[a] The correlation coefficient.
[b] The standard deviation.

300, and 310 K, respectively, according to the modified Scatchard's procedure Eq. (3) [37–39],

$$\frac{F_0}{F} = K_A\left(\frac{[Q]F_0}{F_0 - F}\right) - nK_A[P] \quad (3)$$

where [P] is the molar concentration of the total BHb, [Q] is the molar concentration of total CF, n is the number binding sites on each BHb molecule, and K_A is the equilibrium binding constant.

A plot of F_0/F versus [Q] $F_0/(F_0–F)$ gave a line (Fig. 5) using least-squares analysis. In Table 2, the binding constants K_A and binding sites n were listed for CF associated with BHb. The results showed that the binding constants K_A were decreasing with temperature, which may indicate forming an unstable compound [40]. The unstable compound would be partly decomposed with the rising temperature, therefore, the values of K_A decreased. BHb is one of some ordinary proteins, which has different binding sites for various molecules [15,17,18]. Recent work by Maitra et al. showed that the number of binding sites of alprazolam per Hb molecule was calculated to be 17 [41]. Bian et al. [22] have reported that CF could quench the fluorescence of BSA through the formed complex of CF–BSA; there was only one class of binding sites to CF in BSA. In the present study, the values of n approximately were equal to 5 indicating the existence of more binding sites in BHb for CF with higher binding affinity and selectivity. However, BSA is a single chain protein, whereas hemoglobin is a tetramer. The difference of structure could explain the number of binding sites in BSA less than in BHb.

3.4. Thermodynamic parameters and nature of the binding forces

There are essentially four types of noncovalent interactions that could play a role in ligand binding to proteins. These are hydrogen bonds, van der Waals forces, electrostatic, and hydrophobic interactions. To obtain such information, the implications of the present results have been discussed in conjunction with thermodynamic characteristics obtained for CF binding, and the thermodynamic parameters were calculated from Eqs. (4) and (5).

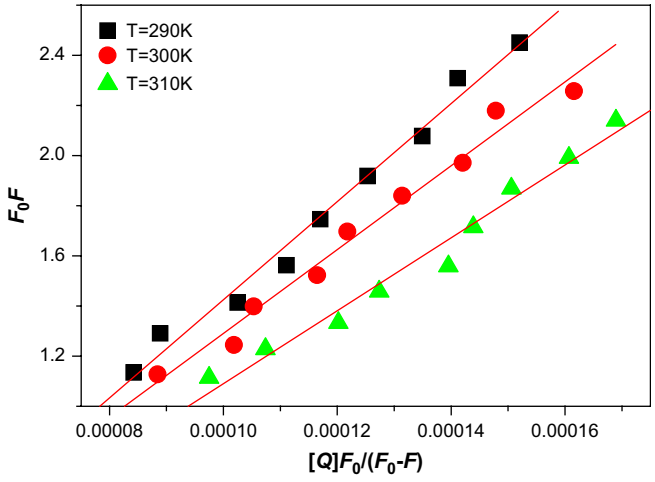

Fig. 5. The plots of F_0/F versus [Q] $F_0/(F_0–F)$. c(BHb) = 5 μM; pH 7.40; λ_{ex} = 280 nm, λ_{em} = 337 nm.

Table 2
Binding parameters and thermodynamic parameters of CF–BHb at pH 7.40.

T	$10^{-4}K_A$ ($L\,mol^{-1}$)	n	R^a	SD[b]	ΔH^0 ($KJ\,mol^{-1}$)	ΔG^0 ($KJ\,mol^{-1}$)	ΔS^0 ($J\,mol^{-1}\,K^{-1}$)
290	1.956	5.432	0.9928	0.059		−23.820	
300	1.672	4.567	0.9908	0.058	−11.086	−24.259	43.912
310	1.454	4.548	0.9878	0.058		−24.700	

[a] The correlation coefficient.
[b] The standard deviation.

$$\ln K_A = -\Delta H^0/RT + \Delta S^0/R \quad (4)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (5)$$

As can be seen from Table 2, the negative sign for ΔG^0 indicates the spontaneity of the binding of CF with BHb. ΔH^0 was a negative value and ΔS^0 was a positive value. Ross and Subramanian [38] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes. From the point of view of water structure, a positive ΔS^0 value is frequently taken as evidence for hydrophobic interaction, because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic interaction. Furthermore, Negative ΔH^0 value cannot be attributed to electrostatic interactions since for electrostatic interactions, ΔH^0 is very small, almost zero [42,43]. Accordingly, it is not possible to account for the thermodynamic parameters of CF–BHb coordination compound on the basis of a single intermolecular force model [44]. It is more likely that hydrophobic and hydrogen bonds interactions were involved in its binding process.

3.5. Conformation investigation

To explore the structural change of BHb by addition of CF, we measured synchronous fluorescence spectra of BHb (Fig. 6) with various amounts of CF.

Synchronous fluorescence spectroscopy technique was introduced by Lloyd in 1971 [45]. It involves simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval between them. The synchronous fluorescence spectroscopy gives information about the molecular environment in a vicinity of the chromospheres molecules and has several advantages, such as sensitivity, spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects [46]. Yuan et al. [47] suggested a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum λ_{max}, the shift in position of emission maximum is corresponding to the changes of the polarity around the chromospheres molecule. When the D value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 nm or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine or tryptophan residues.

It was apparent from Fig. 6 that the emission maximum of tyrosine and tryptophan residues did significant red shift which indicated that the conformation of BHb was changed, the polarity around the tyrosine and tryptophan residues was increased and the hydrophobicity was decreased [48]. This may be due to the changes

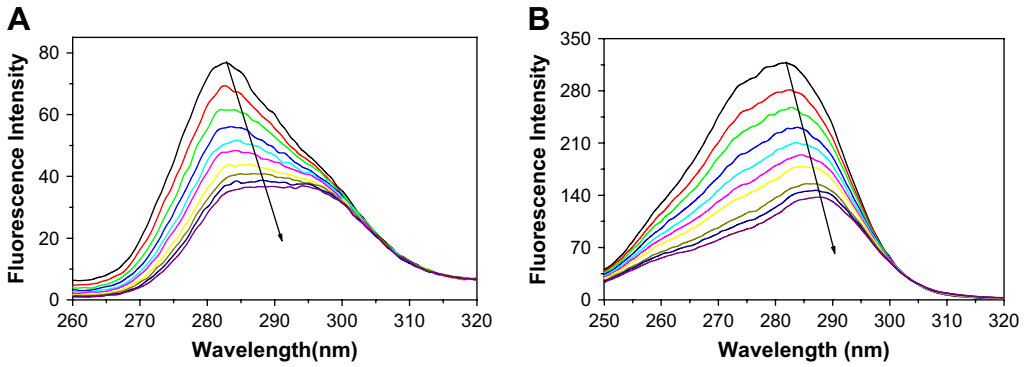

Fig. 6. Synchronous fluorescence spectrum of BHb ($T = 290$ K, pH = 7.40), c(BHb) = 5.0 μM; c(CF)/(μM), from up to down: 0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, and 90.0 respectively. (A) $\Delta\lambda = 15$ nm and (B) $\Delta\lambda = 60$ nm.

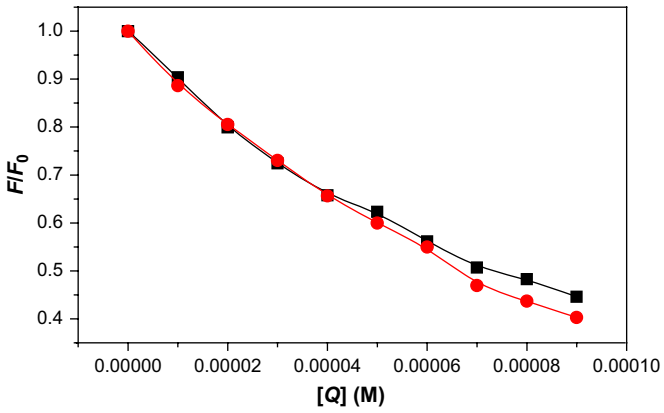

Fig. 7. The quenching of BHb synchronous fluorescence by CF. c(BHb) = 5.0 μM. (■) $\Delta\lambda = 15$ nm and (●) $\Delta\lambda = 60$ nm.

of residue micro-environment with the insertion of CF. BHb contains three Trp residues in each αβ dimer, for a total of six in the tetramer: two α-14 Trp, two β-15 Trp, and β-37 Trp, The α-14 Trp and β-15 Trp residues are outside the subunit interface [28]. The β-37 Trp residue is located at the $\alpha_1\beta_2$ interface, which has been assigned as the primary source of fluorescence emission [49]. The aromatic residues of α-42 Tyr, α-140Tyr, and β-145Tyr are also located at the $\alpha_1\beta_2$ interface [50]. The red shift observed in Fig. 6(A) is consistent with a rearrangement of tertiary structure of Tyr residues during the binding process. The hemoglobin central cavity contains functionally significantly centers for binding several classes of allosteric effectors, which modulate hemoglobin affinity to oxygen [51]. It has been also shown in Fig. 7 that the slope was similar when $\Delta\lambda$ was 15 nm or 60 nm indicating that CF could involve some sites with higher binding affinity and the formation of complex. The CF has an ability to bind into hemoglobin central cavity.

4. Conclusions

This paper presents spectroscopic studies on the interaction of CF with BHb using fluorescence emission spectrum, synchronous fluorescence spectrum and UV/vis spectrum. It was showed that the fluorescence of BHb has been quenched while reacting with CF and forming a certain kind of new compound. The quenching belonged to static fluorescence quenching. The results revealed the presence of more class of binding sites at the interface of BHb; hydrophobic and hydrogen bond interactions played a major role in stabilizing the complex. The results of synchronous fluorescence spectroscopy indicated that the structures of these Tyr and Try residues environments were altered by CF. Studies on the interaction of drugs with proteins are of great important in pharmacy, pharmacology and biochemistry. Because CF may increase some diseases, this study is expected to provide important insight into the mechanism of erythrocyte sickling and the interactions of the physiologically important hemoglobin with purine alkaloids, which may be a useful guideline for further toxicology investigation.

Acknowledgements

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