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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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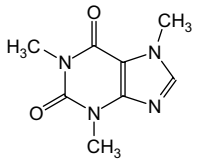

Fig. 1. Molecular structure of caffeine.

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

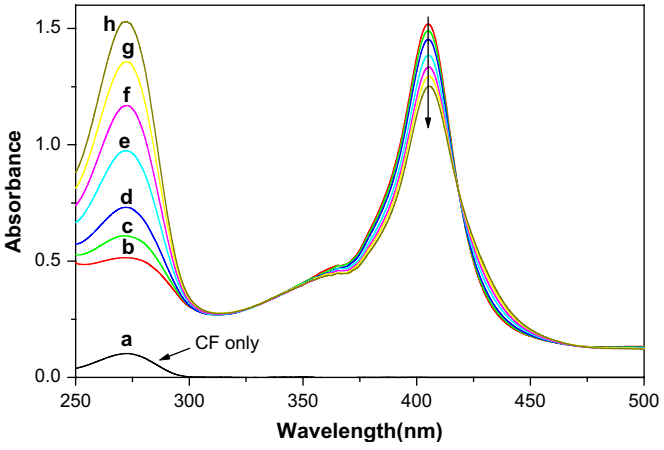

Fig. 2. UV/vis absorbance spectra of BHb in the absence and presence of CF. (a) CF only, c(CF) = 20.0 μM; (b–h) c(BHb) = 5.0 μM, c(CF)/(μM): 20.0, 40.0, 60.0, 80.0, 100.0, 120.0.