Journal of Hazardous Materials 180 (2010) 741–747

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Toxic interaction mechanism between oxytetracycline and bovine hemoglobin

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ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form 25 April 2010
Accepted 26 April 2010
Available online 4 May 2010

Keywords:
Oxytetracycline
Bovine hemoglobin
Fluorescence quenching
UV–vis spectroscopy
CD spectroscopy
Molecular modeling

ABSTRACT

Oxytetracycline (OTC) is a kind of widely used veterinary drugs. The residue of OTC in the environment is potentially harmful. In the present work, the interaction between OTC and bovine hemoglobin (BHb) was investigated by fluorescence, synchronous fluorescence, UV–vis absorption, circular dichroism and molecular modeling techniques under physiological conditions. The experimental results showed that OTC can bind with BHb to form complex. The binding process is a spontaneous molecular interaction procedure, in which van der Waals and hydrogen bonds interaction play a major role. The number of binding sites were calculated to be 1.12 (296 K), 1.07 (301 K) and 0.95 (308 K), and the binding constants were of $K_{296\,K} = 9.43 \times 10^4\,L\,mol^{-1}$, $K_{301\,K} = 4.56 \times 10^4\,L\,mol^{-1}$ and $K_{308\,K} = 1.12 \times 10^4\,L\,mol^{-1}$ at three different temperatures. Based on the Förster theory of nonradiative energy transfer, the binding distance between OTC and the inner tryptophan residues of BHb was determined to be 2.37 nm. The results of UV–vis absorption, synchronous fluorescence and CD spectra indicated that OTC can lead to conformational and some microenvironmental changes of BHb, which may affect physiological functions of hemoglobin. The synchronous fluorescence experiment revealed that OTC binds into hemoglobin central cavity, which was verified by molecular modeling study. The work is helpful for clarifying the molecular toxic mechanism of OTC in vivo.

1. Introduction

The annual output of antibiotics in china was estimated to be about 210,000 tons in 2005, and about 90,000 tons are used as animal feed additives or for therapeutic purposes [1]. Tetracyclines are one of the most extensively used antibiotics in animal feeding operations. Similar to other antibiotics, they are excreted mostly as the parent compound, representing 50–80% of the applied dose [2]. Oxytetracycline (OTC) is a broad-spectrum bacteriostatic antibiotic that belongs to the tetracycline antibiotics [3,4]. It has been widely used as feed additive for therapy of systemic bacterial infections in farmed fish, as a growth stimulator in livestock and as prophylactic treatment of bacterial diseases in plants [4]. The Joint FAO/WHO Expert Committee of Food Additives and Contaminants (JECFA), at its 50th Meeting in 1998, established a group acceptable daily intake (ADI) of 0–0.03 mg kg^{-1} body weight for the tetracyclines (oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC)), alone or in combination. The committee also recommended maximum residue limits (MRLs) of 100 μg L^{-1} in milk and muscle of all food-producing species [5,6]. OTC can enter the environment via wastewater effluent discharges, agricultural runoff et al., being detected at nanogram to low-microgram per liter levels in wastewater effluents and natural waters [7]. When OTC enter the organism, it can inhibit the antibody levels in fish [8], induce DNA damage in carp kidney cells [9], interact with cytoplasmic protein synthesis [10] and induce blood disorder in Juvenile Nile Tilapia *Oreochromis niloticus* [11].

Hemoglobin (Hb) is a protein responsible for oxygen carrying in the vascular system of animals. It can also aid the transport of carbon dioxide, regulate the pH of blood and remove hydrogen ions in the capillaries and carries them to the lungs [12]. Except for albumin, as a kind of intracellular protein, hemoglobin can also function as binders of drugs [13]. When the residue of the veterinary drugs in the environment enter organism, they may penetrate erythrocytes and interact with Hb [13]. OTC can significantly reduce the erythrocyte count and Hb value [11]. However, the interaction mechanism between OTC and bovine hemoglobin has not been reported.

In the present work, we studied in vitro interaction of OTC with bovine hemoglobin (BHb) under the simulative physiological conditions by using fluorescence quenching, UV absorption spectrometry, synchronous fluorescence, circular dichroism and molecular modeling techniques. The binding mechanism of OTC with BHb (association constants, thermodynamic parameters, the number of binding sites, the binding forces, the specific binding site, and the energy transfer distance between OTC and BHb) was estimated. The effect of OTC on BHb conformation was also discussed.

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doi:10.1016/j.jhazmat.2010.04.110

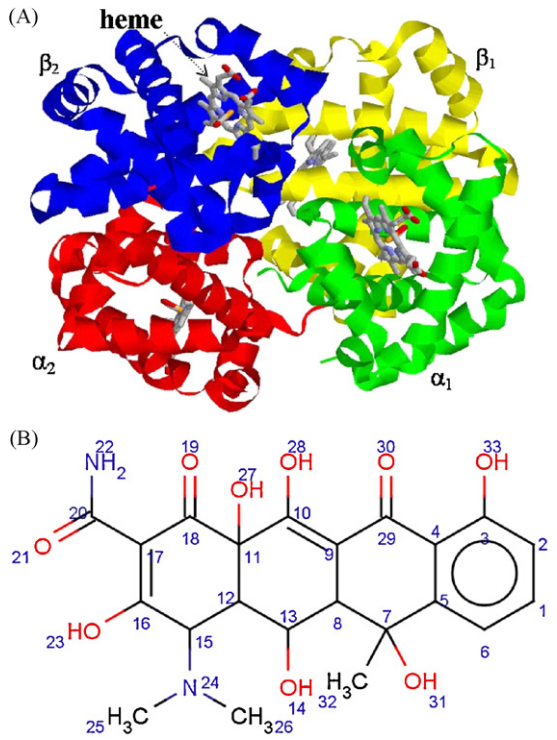

Fig. 1. (A) Molecular structure of BHb (PDB code 1G09) and (B) molecular structure of OTC with with atom numbers.

The study can provide the basic data for understanding the toxicity mechanism of OTC in vivo.

2. Materials and methods

2.1. Reagents

BHb (Beijing Biodee Biotechnology, Co., Ltd.) was dissolved in ultrapure water to form 3.0×10^{-5} mol L^{-1} solution, then preserved at 0–4 °C and diluted as required. The molecular structure of BHb (PDB code 1G09) is shown in Fig. 1(A) [14].

A stock solution of OTC (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 0.0461 g OTC (Beijing Shanglifang Joint Chemical Technology Research Institute) in 100 mL of water. Hydrochloric acid solution (1:1) was used to promote dissolution. The molecular structure of OTC is shown in Fig. 1(B).

A 0.2 mol L^{-1} of phosphate buffer (mixture of $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$, pH 7.4) was used to control pH. $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$ were of analytical reagent grade, obtained from Tianjin Damao Chemical Reagent Factory.

2.2. Apparatus and methods

All fluorescence spectra were recorded on an F-4600 Spectrofluorimeter (Hitachi, Japan). The excitation and emission slit widths were set at 5.0 nm. The scan speed was 1200 nm/min. PMT (Photo Multiplier Tube) voltage was 700 V.

The UV–vis absorption spectra were measured on a UV-2450 spectrophotometer (SHIMADZU, Kyoto, Japan). CD spectra were recorded on a J-810 circular dichroism spectrometer (JASCO). The pH measurements were made with a pHs-3C acidity meter (Pengshun, Shanghai, China).

2.3. Experimental procedures

The fluorescence measurements were carried out as follows: to each of a series of 10 mL test-tube, 1.0 mL of 0.2 mol L^{-1} phosphate buffer (pH 7.4) and 1.0 mL of 3.0×10^{-5} mol L^{-1} BHb were added, followed by different amounts of 1.00×10^{-3} mol L^{-1} stock solution of OTC. The fluorescence spectra were then measured (excitation at 280 nm and emission wavelengths range 290–420 nm). The synchronous fluorescence spectra were obtained through simultaneous scanning of the excitation (λ_{ex} = 250 nm) and emission monochromators while maintaining a constant wavelength interval between them ($\Delta\lambda$, 15 nm and 60 nm). CD spectra were collected from 200 to 260 nm with three scans averaged for each CD spectrum.

In order to eliminate the inner filter effects of protein and ligand, absorbance measurements were performed at excitation and emission wavelengths of the fluorescence measurements. The fluorescence intensity was corrected using the equation [15]:

$$F_{cor} = F_{obs} 10^{(A_1+A_2)/2} \quad (1)$$

where F_{cor} and F_{obs} are the fluorescence intensity corrected and observed, respectively; A_1 and A_2 are the sum of the absorbances of protein and ligand at excitation and emission wavelengths, respectively.

2.4. Molecular modeling study

Docking calculations were carried out using DockingServer. The MMFF94 force field [16] was used for energy minimization of ligand molecule (OTC) using DockingServer. PM6 semiempirical charges calculated by MOPAC2009 [17] were added to the ligand atoms. Nonpolar hydrogen atoms were merged, and rotatable bonds were defined.

Docking calculations were carried out on BHb protein model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [18]. Affinity (grid) maps of 100 Å × 100 Å × 100 Å grid points and 0.375 Å spacing were generated using the Autogrid program [18]. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively.

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis and Wets local search method [19]. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each run of the docking experiment was set to terminate after a maximum of 250,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

3. Results and discussion

3.1. Fluorescence quenching of BHb by OTC

The fluorescence technique can be used to investigate the binding information of small molecules to protein such as the binding mechanism, binding mode, binding constants and intermolecular distances. We utilized the technique to study the interaction between BHb and OTC.

BHb contains three Trp and five Tyr residues in each αβ dimer, for a total of six Trp and ten Tyr residues in the tetramer [20]. Though all the Trp and Tyr residues contribute to the intrinsic fluorescence of BHb, it primarily originates from β-37 Trp that plays a key role in the quaternary state change upon ligand binding [21]. Changes in the intrinsic fluorescence of BHb can provide considerable information about its structure and dynamics [20].