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Investigation on the interaction of the toxicant, gentian violet, with bovine hemoglobin

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ABSTRACT

Gentian violet (GV) is a well-known triarylmethane dye that is used in aquacultural, industrial and medicinal fields. But concerns in growing number have been paid to its potential health problems to human beings and its hazardous effects to environment. Herein, the toxic interaction of GV with bovine hemoglobin (BHb) was investigated by a series of spectroscopic methods and molecular modeling method. The fluorescence emission profile exhibited a remarkable quenching upon addition of GV to the buffered aqueous solution of BHb and the analysis of results revealed the dominant role of static quenching mechanism in GV–BHb interaction. The negative ΔH and positive ΔS values demonstrated that the electrostatic interactions mainly stabilized this toxicantprotein complex. Synchronous fluorescence, UV–Vis absorption and CD spectroscopic studies proved that the conformational change of BHb was induced by GV's combination. Molecular modeling studies exhibited the binding mode of GV–BHb complex and the detailed information of related driving forces. During the ^{1}H nuclear magnetic resonance spectra (^{1}H NMR) study, the chemical shift perturbation and spin–lattice relaxation times of different protons were further used to investigate the interaction of GV with BHb and the results indicated that GV bound orientationally to BHb.

1. Introduction

Gentian violet (GV, structure shown in Fig. 1) is a well-known triarylmethane dye to give deep violet color. It has been widely used around the world not only as dye products, such as biological stain and pH indicator, but also as drugs, such as microbicide and anthelmintic, due to its antibacterial, antifungal, anthelmintic and antiseptic properties (Budavari, 1989; Docampo and Moreno, 1990; Harris, 1991; Schuetze et al., 2008; Thompson et al., 1999). However, many reports have proved that this dye shows potentially mutagenic and carcinogenic effects on humans and animals (Au et al., 1978; Littlefield et al., 1985, 1989; Rosenkra and Carr, 1971). GV has been determined to be mutagenic to multiple microorganisms, cytotoxic to mammalian cells, and carcinogenic to mice and other animals (Aidoo et al., 1990; Alvarez, 1924; Au et al., 1979; Littlefield et al., 1985; McDonald et al., 1984). GV has also been related to the increased risk of human bladder cancer and mouth cancer (Case and Hosker, 1954; Drinkwater, 1990; Rushing and Bowman, 1980). When GV is absorbed by human body, the major part can be reduced rapidly to its non-chromophorous metabolite leuco-gentian violet (LGV) (Mcdonald and Cerniglia, 1984), which has also been reported to cause human carcinogenesis and mutagenesis (Safarik and Safarikova, 2002). Since GV is generally considered to be safe for a long history, it has been produced worldwide and released directly to the environment in wastewater (Mittal et al., 2010; Mohanty et al., 2006; Omata and Disraely, 1956; Rushing and Bowman, 1980). Furthermore, although the use of GV in aquaculture has been banned in some countries, this harmful dye is still used in many parts of the world due to its low cost and high efficacy. Therefore, the ecological influence of the release of GV into the environment is getting more and more concerns.

Hemoglobin (Hb), the major protein responsible for oxygen carrying in the vascular system of vertebrates, is composed of two α and two β subunits (Scheller et al., 2005; Yuan et al., 2002). Each α-chain has 141 amino acids, and each β-chain has 146 amino acids. Each subunit has a polypeptide chain attached to an iron-containing component called heme which exhibits uniquely bright red color (Yonetani and Laberge, 2008). Most of the amino acids in hemoglobin form alpha helices which are connected by short non-helical segments. Hemoglobin's quaternary structure comes from its four subunits in a tetrahedral arrangement. Except for oxygen carrying, Hb can also aid the transport of carbon dioxide and regulate the pH of blood (Scheller et al., 2005). Thus, as an important protein in red blood cells, Hb can affect the biodistribution, metabolism and elimination of many exogenous molecules by ligand–protein

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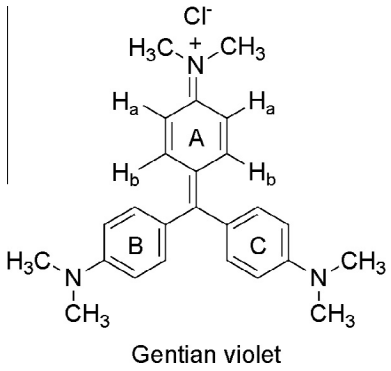

Fig. 1. Molecular structure of gentian violet (GV).

interaction. On the other hand, when exogenous molecules enter organisms, they may penetrate erythrocytes and interact with Hb (Fang et al., 2011) to affect the normal physiological functions and conformation of Hb. Due to the fact that the structural and functional alteration of Hb is closely related to many clinical diseases such as leukemia, anemia, heart disease, and excessive loss of blood (Bao et al., 2001), the research on the toxic effects of exogenous pollutants on Hb through ligand–protein interaction is very important for elucidating the related pathogenic mechanisms.

Although GV has shown toxic effects at the levels of organism and cell and exhibited potential health and environmental hazards, the binding activity between GV and Hb at molecular level has not been reported. In the present study, the interaction between GV and bovine hemoglobin (BHb), which shares high amino acid sequence homology (~90%) with human hemoglobin (HHb), was thoroughly investigated by fluorescence, UV–Vis absorption, synchronous fluorescence, circular dichroism (CD), ^{1}H NMR spectra and molecular modeling techniques under similar physiological conditions. The GV–BHb binding parameters, such as binding constants, the number of binding sites, binding modes and intermolecular distances, were estimated. The effects of GV on the microenvironment and conformation of BHb were also studied comprehensively. The research on the toxicological behavior of GV to BHb at molecular level through appropriate measurements is not only helpful for understanding the metabolism and distribution of this toxicant in vivo, but also can further elaborate the toxic effects of GV on protein function during the blood transportation process.

2. Materials and methods

2.1. Materials

Bovine hemoglobin (BHb) was purchased from Sinopharm Chemical Reagent Beijing Company. Gentian violet (GV) was purchased from Acros Organics with >99.0% purity. All other reagents of analytical grade were used. Double-distilled water was used throughout experiments. BHb was dissolved in 0.05 M phosphate buffered saline solution (PBS) to form a 2.0×10^{-6} M solution, and then preserved at 4 °C for later use.

2.2. Equipments and spectral measurements

Absorption spectra were recorded on a Lambda-35 spectrophotometer (Perkin-Elmer corporate, America) equipped with 1.0 cm quartz cells at room temperature. Fluorescence data were obtained on a Cary Eclipse Spectrofluorimeter (Varian corporate, America) equipped with 1.0 cm quartz cells. Circular dichroism (CD) spectra were measured on a MOS-450/AF-CD Spectropolarimeter (Bio-Logic corporate, France) with a 1 mm quartz cell at room temperature under constant nitrogen flush. NMR studies were carried out on an Avance AV 400 MHz spectrometer (Bruker). All pH measurements were conducted with a PHS-3C acidity meter.

2.3. Methods

For UV–Vis absorption spectra, the sample solution with fixed concentration was added to a 10 mL volumetric flask, and then diluted with PBS solution to the mark. The equilibrated solution was poured into quartz cells (4 cm × 1 cm × 1 cm) and scanned in the ultraviolet range of 190–350 nm using PBS solutions (pH 7.40) as references.

For the fluorescence measurement, GV solution was added into the protein solutions in sequence. Both GV and BHb concentrations were calculated by weight for consistency. The systems were excited at 280 nm, and the emission wavelength was adjusted from 300 to 500 nm with a scanning speed of 600 nm min^{-1}. The excitation and emission slit widths were both set at 5.0 nm. All the experiments were conducted in triplicate and further analyzed using Origin 8.0.

Circular dichroism (CD) spectra were measured on a MOS-450/AF-CD Spectropolarimeter at room temperature under constant nitrogen flush over a wavelength range of 260–200 nm. The path length and volume of quartz cells were 0.1 cm and 400 μl, respectively. The scanning speed was set at 100 nm min^{-1}. Each spectrum was measured three times in succession and the PBS buffer solutions treated under the same conditions were taken as blank. The concentration of BHb was fixed at 2.0×10^{-6} mol L^{-1} and the molar ratios of BHb to GV were varied from 1:0, 1:2 to 1:5. The contents of α-helix structure of BHb were further calculated.

Synchronous fluorescence spectra of solutions prepared as described above were measured on the Cary Eclipse fluorescence spectrophotometer. The initial excitation wavelength (λ_{ex}) was set at 280 nm. The excitation and emission slit widths were both set at 5 nm. The D-value ($\Delta\lambda$) between the excitation and emission wavelengths was set at 15 or 60 nm, at which the spectrum only showed the spectroscopic behavior of Tyr and Trp residues of BHb, respectively.

For the molecular modeling study, the compound structure of ligand molecule (GV) was built using Gaussian 03 software for energy minimization. The molecular geometry of this model was optimized employing DFT method with the B3LYP functional and the 6-31+G* basis set. The ligand root of GV was detected and rotatable bonds were defined before docking. Docking calculations were carried out on BHb protein model with an ICM (v3.4-8) software. Molsoft ICM is a flexible docking program that predicts favorable ligand–protein complex structures with reasonable accuracy and speed. Local search was used to search for the optimum binding site of small molecules to the protein. A grid size of 90 × 90 × 90 points with a grid spacing of 0.4 Å was applied. The protein structure was taken from the protein data bank (1G09) with a crystalline resolution at 2.04 Å.

The ^{1}H NMR spectra experiments were collected with 32,000 data points, 1200 Hz spectral width, 3.4 s acquisition, and 3 s relaxation delay. The spin–lattice relaxation times (T_1) were measured with an inversion recovery pulse sequence. The molar ratios of GV to BHb measured were set at 1:0, 200:1, 500:1, 800:1, 1000:1, 1500:1 and 2000:1. All the NMR experiments were carried out at 298 K.

3. Results and discussion

3.1. Effect of GV on the absorption spectra of BHb

The UV–Vis absorption spectroscopy technique can be used to explore the structural changes of proteins and to study the binding activity of ligand to protein (Demas and Addingto, 1974; Lakowicz, 1999). The absorption spectra of BHb from 195 to 450 nm in the presence of different amounts of GV in buffer solution are shown in Fig. 2. There are three absorption peaks at 204 nm, 280 nm and 406 nm in BHb absorption spectra, which reflect the conformations of the peptide bonds, the aromatic amino acids (the phenyl group of Trp and Tyr residues) and the porphyrin-Soret band (π–π^* electronic transition) of BHb, respectively (Nassar et al., 1996). It can be seen that the strong absorbance peak intensity at 204 nm increases in the presence of GV, and there is a slight shift of GV–BHb spectra towards longer wavelength (Fig. 2A). The comparison is also made towards the absorption peak values between the GV–BHb complex and GV + BHb (the sum values of free GV and free BHb) at 250 nm and 300 nm, respectively. The calculated results show that the sum values (GV + BHb) are lower than the measured ones (GV–BHb complex) by ~32.46% and ~36.76%, respectively. It indicates that GV affects BHb molar absorbance rather than a simple absorption spectral overlapping. Moreover, as shown in Fig. 2B, the Soret band intensity of BHb is gradually decreased with the increased concentration of GV and GV has no absorption interference in this wavelength range, which means that GV can directly disturb the structures of BHb and change the microenvironment around the heme group. The alteration in