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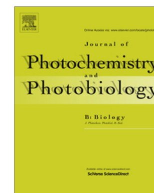

Exploring the biophysical aspects and binding mechanism of thionine with bovine hemoglobin by optical spectroscopic and molecular docking methods

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ABSTRACT

In the present investigation, we have elucidated the interaction between thionine (TH) and bovine hemoglobin (BHb) under physiological conditions by using absorption, emission, time resolved fluorescence, synchronous fluorescence, circular dichroism (CD) and three dimensional emission (3D) spectral studies. Molecular docking experiment was also carried out to establish the possible binding site of TH on BHb. The emission spectral studies revealed that, TH have the ability to bind with BHb and form a ground state complex via static quenching process. The calculated binding constant and the number of binding sites was found to be $3.65 \times 10^4\ dm^3\ mol^{-1}$ and 1.04, respectively. Förster Resonance Energy Transfer (FRET) theory was employed to calculate the distance (r) between donor (BHb) and acceptor (TH) as 3.64 nm. Furthermore, the conformational changes of BHb induced by TH complexation showed some degree of structural unfolding. In addition, molecular docking study confirmed that the most probable binding site of TH was located within the active cavity constituted by α1 and α2 subunits of BHb.
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1. Introduction

The studies on protein–ligand interactions are important in biological process and it is regarded as the second step in rational drug design [1]. Proteins are important biomolecules that play different roles in living beings. The functions of most proteins are mainly depends upon precise three dimensional structures, which are affected by a number of noncovalent interactions such as hydrogen bonds, van der Waals interactions, electrostatic interactions, hydrophilic/hydrophobic effects and so on [2]. The various specific and non specific interactions of ions with the residues of the proteins have important consequences to the biological function of the protein [3]. In addition, protein conformational changes may also be associated with particular properties such as function, transportation, assembly, tendency to aggregate and potential cytotoxicity [4]. Therefore, detailed elucidation of secondary and tertiary formation, stability and their structural and dynamic properties has been one of the main topics studied in chemistry and biology.

Hemoglobin (Hb) is a most important representative of hemoprotein and it is a major functional protein responsible for carrying oxygen in the vascular system of animals. It also helps the transport of carbon dioxide and regulates the pH of blood. It is associated with many clinical diseases such as leukemia, anemia, heart disease and excessive loss of blood [5]. The structure of Hb contains four polypeptide chains and four heme prosthetic groups, in which the iron atoms are present in ferrous (Fe^{2+}) state. The protein portion, called globin, consists of two α-chains (141 amino acid residues each) and two β-chains (146 amino acid residues each). Each α-chain is in contact with β-chain [6]. The α-chains contain seven and the β-chains eight helical regions. Every chain carries one heme group in a pocket, to which oxygen and several other ligands can bind reversibly [7].

Hb has long been used as the paradigm for understanding the structure–function relationships of proteins [8–11]. Moreover, the unfolding of this kind of protein is closely associated with physiological abnormalities. For example, under very acidic conditions, the cooperative oxygen binding property of Hb is decreased and the pro-oxidative activity is dramatically increased mainly due to the significant conformational changes in its structure and heme crevice [12,13]. The knowledge of exact mechanism involved in the pharmaceutical interactions with blood proteins will provide a molecular basis for understanding the pharmacokinetic properties of drug like ligands. Moreover, it would pave the way for new approaches for drug design and therapy [14].

Thionine (3,7-diamino-5-phenothiazinium) (Fig. 1), a planar tricyclic heteroatomic derivative with two amine groups at the C3 and C7 sites [15], is monocationic under physiological conditions,

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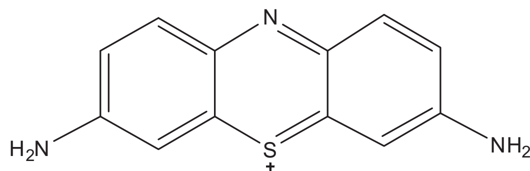

Fig. 1. Structure of thionine dye.

and it has been widely studied because of its intercalative binding with DNA. TH and its derivatives have been used in photodynamic therapy (PDT) due to its ability to generate singlet oxygen [16]. TH has been used to induce photodynamic inactivation of bladder cancer cells, *Escherichia coli*, and *Saccharomyces cerevisiae* [17]. The biological activity of TH has also been employed in graphene-based inmuno sensors [18]. In addition to its favorable applications, TH possess mutagenic activity in *eukaryotic cells*, demonstrating its potential cytotoxic and genotoxic activity in prokaryotic cells and photo induced mutagenic action upon binding to DNA [19–21]. Therefore, it is necessary to understand TH binding to biological systems to exploit their utility on one hand and toxic effect on the other.

The studies on molecular aspects of the interaction of TH with DNA [20,21] and RNA [22] are widely available in literature. In spite of its broad applications, the molecular interaction of TH with BHb protein remains unclear. Thus, in the present work the binding interaction of biological photosensitizer TH with BHb is investigated by spectroscopic methods including absorption, emission, time resolved fluorescence, 3D emission, synchronous fluorescence and CD spectral studies. An attempt is also undertaken to unravel the effect of TH binding on the secondary structure of BHb to rationalize the applicability of TH molecule as an effective photo therapeutic agent. The binding location of TH within BHb has been further confirmed by molecular docking studies.

2. Materials and methods

2.1. Materials

BHb was purchased from Sigma–Aldrich, USA and used without further purification. TH dye was obtained from Himedia chemicals, India and used as received. The stock solution of BHb was prepared by using phosphate buffer solution (PBS) of pH = 7.40. A stock solution of TH was prepared at a concentration of 1.14×10^{-3} mol dm^{-3} and kept protected in dark until further use. All other reagents were of analytical grade and water used in this investigation was doubly distilled over alkaline potassium permanganate using an all glass apparatus.

2.2. Absorption and emission spectral measurements

Absorption spectral measurements were carried out using JASCO V-630 UV–visible spectrophotometer. Quartz cuvettes of path length 1 cm were used to record the absorption spectra. The emission spectral studies were performed with JASCO FP-6600 spectrofluorometer equipped with a 1 cm quartz cuvette. A 10^{-6} mol dm^{-3} solutions of BHb were prepared daily for experiments. For the study of influence of TH, TH solutions were prepared daily from the stock solution. The various concentrations of TH solutions were prepared by pipetting an aliquot of the stock solution into a 5 ml SMF containing 1 ml of BHb solution (10^{-6} mol dm^{-3}), and then the solutions were made up to the mark with PBS. The mixture of BHb and TH solutions are mixed uniformly and allowed to equilibrate for 15 min before recording the spectral data. BHb was excited at 280 nm and the emission was monitored at 338 nm. The emission and excitation slit widths used throughout the experiment were 5 and 6 nm, respectively. The synchronous fluorescence spectra were recorded at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm. The 3D emission spectra were performed under the following conditions; the emission was recorded between the wavelength range of 200–500 nm and the excitation was recorded between the wavelength ranges from 200 nm to 340 nm. The excitation and emission bandwidths for 3D emission spectra were 10 and 6 nm, respectively. All the measurements were carried out at room temperature (25 °C).

2.3. Time resolved fluorescence lifetime measurements

The fluorescence lifetime measurements were done in time-correlated single photon counting (TCSPC) technique using Horiba Jobin Yvon. The samples were excited at 280 nm using a picosecond diode (IBH NanoLED-280) in an IBH fluorocube apparatus. The emission data were collected at a magic angle (54.7°) relatively to the excitation, passed through a monochromator and into a fast detector, using a Hamamatsu MCP photomultiplier (2809U). The repetition rate was 1 MHz. The fluorescence decays were deconvoluted using IBH DAS6 software.

2.4. Circular dichroism measurements

Circular dichroism measurements were performed with a JASCO-810 spectropolarimeter using a 0.1 cm path length quartz cell. The CD spectra were recorded in the range of 200–260 nm with 0.1 nm step resolution and averaged over two scans at a speed of 50 nm min^{-1}. All observed spectra were baseline subtracted for buffer solution and the α-helical content was calculated on the basis of change of molar ellipticity value.

2.5. Molecular docking studies

Molecular docking study was carried out on AutoDock 4.2 program which utilizes Lamarckian Genetic Algorithm (LGA) [23]. The crystallographic coordinates of TH was obtained from the PubChem Database. The native structure of BHb (PDB ID: 1G09) was retrieved from Protein Data Bank. As required in the Lamarckian Genetic Algorithm all water molecules were removed and hydrogen atoms were added followed by the calculation of Gasteiger charges. The grid size along the *x*-, *y*-, *z*- axes was set to 36 Å, 36 Å and 36 Å. The grid spacing was set as 0.375 Å. The AutoDocking parameters used were as follows: GA population size = 150, maximum number of energy evaluations = 250,000 and GA crossover mode of two points. The lowest binding energy conformer was searched out of 50 different conformers for the docking simulation and the resultant one was used for further analysis. The docked conformations were viewed using PyMOL (http://www.pymol.org) software package.

3. Results and discussion

3.1. Emission spectral studies of BHb with TH

It is well established that the emission intensity of a compound can be decreased by a variety of molecular interactions, such as excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching [24]. Such decrease in emission intensity is called fluorescence quenching. The intrinsic emission of proteins is mainly due to the trytophan (Trp) and tyrosine (Tyr) residues, which are highly sensitive to their local environment. BHb is a multi-tryptophan protein, which contains two α-14 Trp, two β-15 Trp and two

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