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Probing the binding interaction of thionine with lysozyme: A spectroscopic and molecular docking investigation

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ABSTRACT

In this article, an attempt is made to explore the binding mechanism of thionine with lysozyme by using multi-spectroscopic and molecular docking methods. The results from emission and time resolved fluorescence studies revealed that the emission quenching of lysozyme with thionine is initiated by static quenching mechanism. The binding constant and number of binding site of lysozyme–thionine complex was evaluated as 4.01×10^5 dm^3 mol^{-1} and ≈ 1, respectively. Furthermore, the results from absorption, constant wavelength synchronous fluorescence, three dimensional emission and circular dichroism spectral studies showed that thionine induced conformational changes in the secondary structure of lysozyme. Molecular docking study confirmed that the probable binding site of thionine is located near trptophan-63 residue of lysozyme and it is further revealed that the existence of hydrogen bonding along with hydrophobic interaction are the primary forces responsible for the complexation of thionine with lysozyme.

1. Introduction

Studies on the interaction between photoactive organic molecules and proteins have attracted great interest in the field of chemistry, life science and clinical medicine for decades. The binding interactions between proteins and low molecular weight molecules are the primary initiators of most of the biochemical reactions observed *in vitro* and *in vivo* [1,2]. Many drug molecules bind reversibly to proteins, and this in turn alters the distribution, free concentration, the metabolism and elimination of the drug and consequently affects the levels of the drug's activity and toxicity [3]. The nature and the magnitude of protein–ligand interactions are known to influence the biosafety, delivery rate, pharmacological response, therapeutic efficacy and the design of drugs. Therefore, detailed investigation of drug–protein interactions assumes significance in understanding the structural features essential for the bio-affinity of drug and pharmacokinetic behavior of the drug in protein environment [4–8].

Lysozyme (Lys), also known as muramidase, was first discovered by Alexander Fleming in 1922 [9]. Lys is a small monomeric low molecular weight (~14 kDa) globular protein that contains α-helix, β-sheet turns and disordered structural elements. It is abundant in a number of secretions such as tears, saliva, human milk, mucus and also present in cytoplasmic granules of the polymorphonuclear neutrophils. The primary structure of Lys consists of 129 amino acid residues including six tryptophan (Trp) and three tyrosine (Tyr) residues. In addition, the amino acid framework in Lys is linked together by four disulfide bonds [10,11]. The six Trp residues are located at the substrate binding sites, out of which two are in the hydrophobic matrix box, while the lone Trp residue is separated from the others [12]. Among the six Trp residues, Trp-62 and Trp-108 are considered to be the most dominant fluorophores in Lys [13]. Lys has many physiological and pharmaceutical functions, such as antibacterial, antiviral activity, etc. An important characteristic function of Lys protein is their ability to carry drugs to the target receptors, whereby, the effectiveness of such drug targeting depends on their binding abilities [14].

Thionine (3,7-diamino-5 phenothiazinium acetate) (TH) (Fig. 1) is a planar cationic phenothazinium dye that contains one heterocyclic nitrogen atom and two amine groups symmetrically distributed on each side of the molecule [15]. TH and its derivatives have the capability of generating singlet oxygen and it is widely used in many areas such as photodynamic therapy (PDT) [16,17], development of biosensors [18,19], as polymerization photoinitiators [20,21], in the decontamination of blood products [22], as nucleic acid probes [23] and against bacteria [24–27], viruses and

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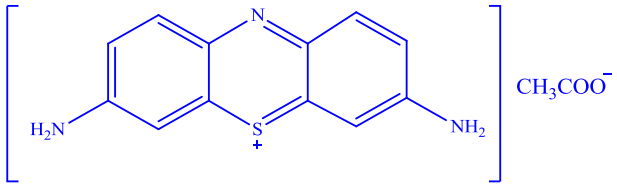

Fig. 1. Structure of thionine dye.

yeasts [28]. TH also used to induce photodynamic inactivation of bladder cancer cells, *Escherichia coli*, and *Saccharomyces cerevisiae* [23]. Previous studies have revealed that TH showed mutagenic activity towards eukaryotic cells [29]. In addition, TH dyes are known to undergo potential cytotoxic and genotoxic activity in prokaryotic cells and photoinduced mutagenic action upon binding to DNA [30]. Therefore, it is necessary to understand the binding mechanism of TH with biological systems to elucidate their potential applications as well as their toxic effects on biological macromolecules.

In recent years, the complexation mechanisms of TH with RNA [31] and bovine hemoglobin [32] have been widely reported by many research groups. However, the binding mechanism of TH with Lys is yet to be studied. The present work aims to explore the binding mechanism of photodynamic therapeutic agent TH with Lys *in vitro* under physiological conditions by means of emission, time resolved fluorescence, absorption, constant wavelength synchronous fluorescence, circular dichroism and three dimensional emission spectral studies. The AutoDock-based 'blind docking' strategy has also been utilized to ascertain the probable binding location of the TH in Lys backbone.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white was acquired from Sigma–Aldrich, USA and used without further purification. TH dye was procured from Himedia chemicals, India and TH was purified by column chromatography on silica gel using ethanol:benzene (7:3 v/v) containing 0.4% glacial acetic acid and then recrystallized from ethanol [33]. 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. The stock solution of Lys was prepared by using phosphate buffer solution (PBS) of pH 7.40 and stored in the dark at 4 °C for further use. The concentration of Lys was determined spectrophotometrically from extinction coefficient (ε_{280} = 37,646 dm^3 mol^{-1} cm^{-1}) [34]. A 10^{-6} mol dm^{-3} of Lys solutions 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 standard measuring flask containing 1 mL of Lys solution (10^{-6} mol dm^{-3}), and then the solutions were made up to the mark with PBS. Lys and TH solutions are mixed uniformly and allowed to equilibrate for 15 min before recording the spectral data.

2.2. Absorption and emission spectral measurements

Absorption spectral measurements were performed on a JASCO V-630 UV–Visible spectrophotometer. Quartz cuvettes of path length 1.0 cm were used to record the absorption spectra. The emission spectra were carried out using JASCO FP-6600 spectrofluorometer equipped with a 1.0 cm path length quartz cuvette. Lys was excited at 295 nm and the emission was monitored at 346 nm. The emission and excitation slit widths used throughout the experiments were 5 and 10 nm, respectively. The constant wavelength 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 wavelength was recorded between 200 nm and 500 nm, the excitation wavelength scan range was recorded between from 200 nm to 340 nm. The excitation and emission bandwidths for 3D emission spectra were 5 and 10 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 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 instrumental response function (IRF) was determined experimentally on the basis of light signal scattered from Ludox (colloidal silica in water) and was used for subsequent deconvolution of the fluorescence signal. The fluorescence decays were deconvoluted using IBH DAS6 software.

2.4. Circular dichroism measurements

Circular dichroism (CD) measurements were recorded with a JASCO-810 spectropolarimeter equipped with a quartz cuvette of 0.1 cm path length. The spectra were recorded in the wavelength range of 200 nm–260 nm with a scan speed of 50 nm min^{-1}. Each spectrum presented was the average of three scans. All observed CD 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 of the Lys and TH system was carried out on AutoDock 4.2 program which utilizes Lamarckian Genetic Algorithm (LGA) [35]. The native structure of Lys (PDB ID: 6LYZ) was retrieved from Brookhaven protein data bank (http://www.rcsb.org/pdb). The crystallographic coordinates of TH was obtained from the PubChem database (http://pubchem.ncbi.nlm.nih.gov). As required in the Lamarckian Genetic Algorithm (LGA) 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 56 Å, 56 Å and 56 Å. The grid spacing was set as 0.375 Å. The grid center along the *x*-, *y*-, *z*-axes was set as 5.788 Å, 23.488 Å and 19.244 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 25 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 Lys with TH

The intrinsic emission property of many proteins arise mainly from the tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe)