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Spectroscopic and theoretical investigation of conformational changes of proteins by synthesized pyrimidine derivative and its sensitivity towards FRET application

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ABSTRACT

Interest in synthesizing and characterizing (IR, NMR and HRMS spectroscopic methods) a pyrimidine based Schiff-base ligand, 2-(2-(Anthracen-9-ylmethylene) hydrazinyl)-4,6-dimethyl pyrimidine (ANHP) has been developed for its application to ascertain the conformational change of protein and sensitivity towards fluorescence resonance energy transfer (FRET) process. Location of ANHP in bovine serum albumin (BSA) and human serum albumin (HSA) proteins environment has been determined using different spectroscopic techniques. Weakly fluorescent ANHP have shown greater protein induced fluorescence enhancement (PIFE) in case of HSA than BSA, though in both cases energy transfer efficiency are almost same but difference in binding constant values encourages us to find the location of ANHP within the complex protein environment. From the FRET parameter and α-helicity change, it has been found that ANHP bound with Trp-214 of HSA and surface Trp-134 of BSA. Conformational changes of proteins have been observed more for HSA than BSA in presence of ANHP, which has confirmed the location of ANHP in both the protein environments. Coupled with experimental studies, molecular docking analysis has also been done to explain the locations and distance dependent FRET process of ANHP in both proteins.

1. Introduction

Numerous studies have been made to show the biological significance of different pyrimidine derivative [1–3]. Pyrimidine moiety is one of the essential classes of N-containing heterocycles and extensively used as key building blocks for pharmaceutical agents. Moreover it exhibits a wide spectrum of pharmacophore such as fungicidal [4], analgesic [5], anti-hypertensive [6] and anti-tumor agents [7]. Pyrimidines and fused pyrimidines, being an integral part of DNA and RNA, play an essential role in several biological processes like antibacterial, antitumor, cardiovascular [8–16] etc. and have considerable chemical and pharmacological importance. Pyrimidine derivative may also interact with serum albumin proteins. Regardless of the size and complexities of human serum albumin (HSA), there is only a single tryptophan residue (Trp-214, in hydrophobic pocket) whereas in bovine serum albumin (BSA), there are two tryptophan residues (Trp 134, in surface and Trp 213, in hydrophobic pocket) [17]. BSA and HSA are frequently used to bind different categories of small molecule, drug and other bioactive molecules because of the availability of hydrophobic pockets inside the protein network and the flexibility of the albumins in adapting their shapes [18,19].

Spectroscopic method has been applied in investigating drug binding with protein under physiological conditions because of its accuracy, sensitivity, rapidity, and ease in handling [20]. Fluorescence resonance energy transfer (FRET) is a distance dependent (the distance between the donor and acceptor transition dipoles generally between 20 and 80 Å) spectroscopic phenomenon during which radiation less transfer of energy has been occurred from donor molecule to suitable acceptor molecule [21, 22–25]. FRET is observed different domains of science [26] whether it is chemistry, molecular biology, cell biology or genetics.

Due to biological significance of pyrimidine moiety, pyrimidine containing Schiff base 2-(2-(Anthracen-9-ylmethylene) hydrazinyl)-4,6-dimethyl pyrimidine (ANHP) has been synthesized and characterized by using different spectroscopic technique. The interaction of ANHP with proteins (BSA and HSA) has been considered in this present work. Proteins induced fluorescence enhancement (PIFE) of ANHP has been exploited to make weakly fluorescent ANHP as a potential candidate for FRET couple. Although it is a challenge to make out ANHP as FRET couple another part open for the present study is to identify the exact location of synthesized ANHP molecule in both complex proteins environment. The strength of binding, energy transfer efficiency, the α-helical changes of proteins and the exact location of ANHP probe in buffer have been determined here by using steady state, time-resolved and CD spectroscopic methods. In conclusion, the experimental outcomes have been supported from molecular docking viewpoint. The importance of ANHP as biologically significant molecule for in vivo application will require more investigation in future.

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2. Experimental Section

2.1. Materials

The spectral grade solvents ethanol (EtOH), methanol (MeOH), conc. HCl, anthracene-9-carboxaldehyde, phosphorousoxy chloride ($POCl_3$), acetyl acetone, hydrazine hydrate and urea were purchased from E. Merck, India. Solvents were purified and dried according to standard method described elsewhere [27] and used only after checking their purity fluorimetrically in the wavelength range of interest. BSA (Sigma,>98%), HSA (Sigma, >96%) and HEPES buffer (N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulphonicacid]), were used as received from Sigma. Millipore water was used throughout the study.

2.2. Synthesis of ANHP

3,5-dimethylpyrimidinehydrazine (compound **3)** was synthesized following a literature method [28]. A methanolic solution of compound **3** (0.5 g, 3.618 mmol) was slowly added to a solution of anthracene-9-carboxaldehyde (0.74 g, 3.618 mmol) in methanol. Then the solution was refluxed for 3 h. After completion of the reaction, the obtained yellow precipitate was filtered and washed several times with cold methanol to give **ANHP** as pure yellowish solid (Scheme 1). Yield: 0.93 g, 77.5%. ^{1}H–NMR (300 MHz, DMSO-d_6), 11.39 (s, 1H, NH), 9.32 (s, 1H, CH=N), 8.75 (d, J = 9 Hz, 2H, Ar-H), 8.62 (s, 1H, Ar-H), 8.10 (d, J = 9 Hz, 2H, Ar-H), 7.54–7.62 (m, 4H, Ar-H), 6.63 (s, 1H, Py-H), 2.06 (s, 6H, Py-CH_3); (Fig. S1); ^{13}C–NMR (300 MHz, DMSO-d_6): δ (ppm), 31.10 (2C, 2 m), 112.26 (1C, 1 L), 125.65, 125.87, 126.81, 127.15, 128.79, 129.31, 129.85, (13C, 2a, 2b, 2c, 2d, 2e, 2f &1 g), 131.49 (1C, 1 h), 140.14 (1C, 1i) 160.33(1C, 1j), 167.88 (2C, 2 k), (Fig. S2); IR (KBr, cm^{-1}) υ: 3217 (N-H), 1597 (C=N) (Fig. S3). ESI-MS: *m/z* calculated for $C_{21}H_{18}N_4$ $[M + H]^+$ 327.15, found 327.31 (Fig. S4); Anal. Calc. for $C_{21}H_{18}N_4$: C, 77.40; H, 5.61; N, 17.18%. Found: C, 77.28; H, 5.56; N, 17.17%.

2.3. Methods

^{1}H and ^{13}C NMR spectra were obtained from a Bruker Advance DPX 300 spectrometer using DMSO-d_6 solution. Infrared spectra (4000–400 cm^{-1}) were taken on KBr pellets using PerkinElmer Spectrum BX-II IR spectrometer. Mass spectroscopic analysis of the ligand was performed in a QTOF Micro YA263 ESI-TOF mass spectrometer using methanol as solvent. Elemental analyses (carbon, hydrogen and nitrogen) were performed with a Perkin-Elmer CHN analyzer 2400. Absorption and fluorescence spectra were recorded using a Shimadzu (model UV1700) UV–vis spectrophotometer and Shimadzu spectrofluorimeter (model RF 5301) respectively. Fluorescence decay curves were obtained from time resolved intensity decay by the method of time-correlated single photon counting (TCSPC) using a nanosecond diode LED at 280 and 370 nm (IBH, nanoLED) as a light source. The data stored in a multichannel analyzer was routinely transferred to IBH DAS-6 decay analysis software. For all the lifetime measurements, the fluorescence decay curves were analyzed by bi and tri exponential iterative fitting program provided by IBH such as in Eq. (1)

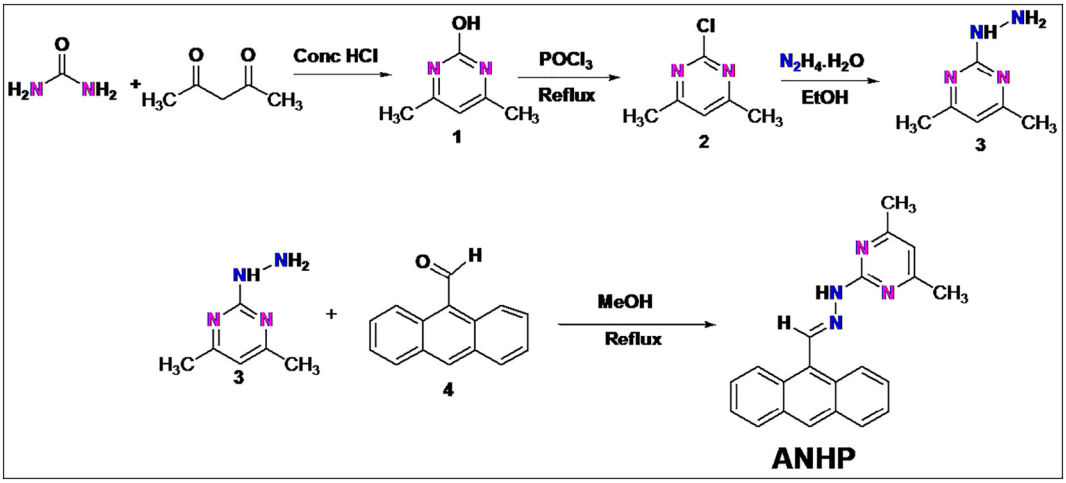
$$F(t) = \sum_i \alpha_i \exp\left(-t/\tau_i\right) \quad (1)$$

Where is the pre exponential factor representing the fractional contribution to the time resolved decay of the component with lifetimeτ_i. Average lifetime ⟨τ⟩for bi exponential decay can be calculated as in Eq. (2)

$$\langle\tau\rangle = \frac{\alpha_1\tau_1 + \alpha_2\tau_2}{\alpha_1 + \alpha_2} \quad (2)\alpha i$$

Throughout the experiment a stock solution of ANHP and proteins (BSA and HSA) were taken at 1.0 mM and 300.0 μM respectively. All the above required measurements were carried out in 10.0 mM HEPES buffer. Circular dichroism (CD) spectra were recorded using a PC controlled spectropolarimeter, JASCO J815 unit (Jasco, Hachioji, Japan) equipped with a temperature programmer (model PFD 425 L/15) at 25 ± 1 °C using a rectangular quartz cuvette of path length 0.1 cm. Far UV-CD spectra were collected with a protein concentration of 5.0 μM in the range of 190–260 nm. For only CD measurement HEPES buffer of 1 mM was used.

Ground state geometries of ANHP was optimized employing density functional theory [29–30] using the B3LYP [31–32] functional with the standard basis set, 6-311G (d,p), for all atoms in the Gaussian 09 program [33] and the resultant geometry was read in Auto Dock 4.2 software in compatible file format [26]. The crystal structure of BSA (PDB entry 4F5S), HSA (PDB entry 1AO6) were downloaded from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). All hydrogen atoms and Gasteiger charges were added to prepare the protein molecules for docking analysis. Ligand docking was carried out applying the Lamarckian genetic algorithm (LGA) implemented in Auto Dock 4.2 [34]. For docking of ANHP with protein, the required file for ANHP was produced through combined use of Gaussian 09 program and Auto Dock 4.2 software packages [33]. The lowest binding energy conformer was searched out of ten different conformers for each docking simulation and the resultant one was used for further analysis. The output from Auto Dock is rendered with PyMOL [35] which has also been used to calculate the distances between the Trp residue of both protein and ANHP.



Scheme 1. Synthesis of the probe ANHP.