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Interaction of phenazinium-based photosensitizers with the 'N' and 'B' isoforms of human serum albumin: Effect of methyl substitution

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ABSTRACT

The present work is focused on exploring the interaction of two phenazinium-based biological photosensitizers, phenosafranin (PSF) and safranin-O (SO), with human serum albumin (HSA), with particular emphasis on the physiologically significant N−B conformational transition of the protein on the dye:HSA interaction. In addition, the presence of methyl substitution on the planar phenazinium ring in SO paves way for looking into the effect of simple chemical manipulation (that is, methyl substitution on the dye nucleus) on the dye:protein interaction behavior as a function of various (pH-induced) isoforms of HSA. Our results reveal a significantly stronger binding interaction of SO with the B isoform of HSA (at pH 9.0) compared to that with the N isoform (at pH 7.4). On the contrary, the PSF:HSA interaction is found to be reasonably insensitive to the aforesaid conformational transition of HSA. However, the probable binding location of both the dye molecules (PSF and SO) is found to be within the protein scaffolds (domain IB). This is further quantified from the modulation of fluorescence decay behavior of the dyes within the protein scaffolds. It is important to note that the rotational relaxation behavior of the protein-bound dyes reveals an unusual 'dip-rise-dip', an observation not reported earlier. Such unusual anisotropy decay is meticulously analyzed by an associated (or multicomponent) exponential decay model which emphasizes on the fractional contributions from differential classes of fluorophore populations characterized by the fast (due to unbound or solvent exposed part of the fluorophore) and slow (due to embedded or bound part) motions, in combination with their different local mobilities. Furthermore, the translational diffusion of the dye molecules in the presence of the protein in different isoforms (N-form or B-form) at a single molecule level is also measured by Fluorescence Correlation Spectroscopy (FCS).

1. Introduction

Human serum albumin (HSA) is the major transport protein of circulatory system comprised of 585 amino acids [1]. HSA is asymmetric in structure composed of three domains (domain I, II and III) with the principal dye binding sites located in hydrophobic subdomains IIA and IIIB [1]. HSA undergoes different pH-dependent conformational transitions, e.g., the N−F transition between pH 5.0 and 3.5, the F-E transition (or acid expansion) between pH 3.5 and 1.2, and the N−B transition between pH 7.0 and 9.0 [1–4]. The N−B transition is argued to have particular physiological importance with a view to the predominance of the B-isoform in blood plasma under enhanced Ca^{2+} ion concentration [5–7]. Besides, this transition (or analogous mechanism) is believed to play crucial regulatory role underlying the transport function of HSA [6–10], which in turn underscores the enormous physiological importance associated with the N−B structural transition of HSA [11]. Thus, any modulation of the binding affinity of a given dye with various isoforms of HSA may critically influence the dye distribution profile within the body which in consequence may alter the dose-response relationship as well as the rate of excretion from the body [12–14]. Naturally, quantitative knowledge of the differential interaction behaviors of a dye with various isoforms of HSA (particularly accompanying the N−B transition) may be critical to complement the optimization of ADME (adsorption-distribution-metabolism-excretion) profile of a given dye. Though in general the blood pH is stable, differences in blood pH are known in comparison to cerebral blood flow, and extracellular and intracellular milieus which offer prospective avenues for the occurrence of dye(s):HSA interaction [15,16].

To this end, the present work is designed to focus on deciphering the interaction behaviors of Phenosafranin (PSF, 3,7-diamino-5-phenyl phenazinium chloride) and Safranin-O (SO, 3,7-diamino-2,8-dimethyl-5-phenyl phenazinium chloride) with the two isoforms of HSA, namely,

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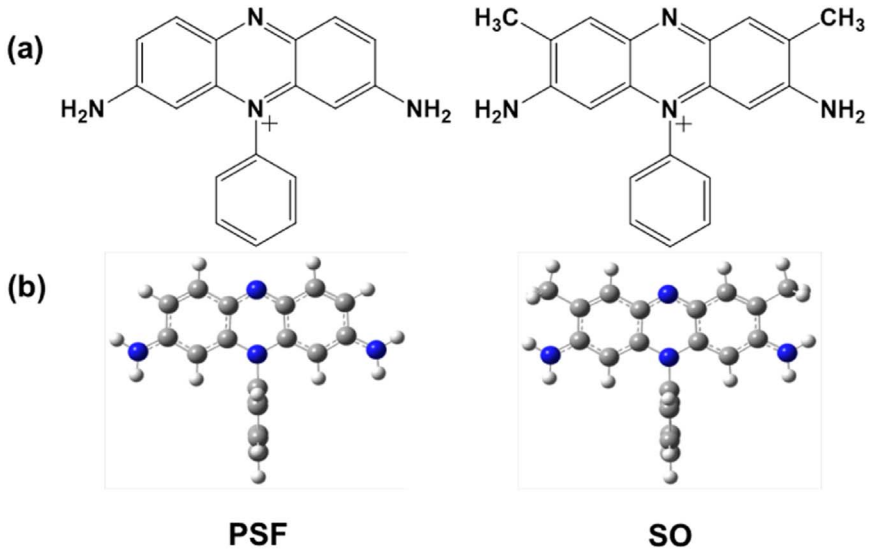

Scheme 1. (a) Schematic and (b) optimized (B3LYP/6–31 + + G(d,p)) structures of PSF and SO.

the N-form (at pH 7.4) and B-form (at pH 9.0). These phenazinium-based dye molecules (PSF and SO) find extensive applications as photosensitizers [17,18], biological probes for estimation of hyaluronic acid, study of DNA intercalation, protein immobilization, membrane organization, inhibition of human ribonuclease reductase and so on [19–23]. The dye molecules (PSF and SO) are structurally analogous barring the degree of methyl substitution on the planar phenazinium nucleus (Scheme 1), which in turn paves way for looking into the effect of simple chemical manipulation (that is, methyl substitution on the dye nucleus) on the dye:protein interaction behavior as a function of various (pH-induced) isoforms of the transport protein, HSA.

Although the interaction of PSF with HSA has been reported in the literature [24,25], the present work emphasizes on various aspects of the overall interaction scenario which remain *hitherto* unexplored, e.g., the effect of protein conformational transition (N—B transition) on the binding affinity of the dyes with HSA. Concurrently, the study also reveals the effect of methyl substitution (PSF and SO differs in the degree of methyl substitution on the planar phenazinium ring, Scheme 1) on the binding affinity of the dyes leading to differential association constants of PSF and SO with various (N-form and B-form) isoforms of HSA. This is further corroborated from the differential fluorescence relaxation behaviors of the dyes with various isoforms of the protein and translational diffusional behaviors at a single molecule level as studied by Fluorescence Correlation Spectroscopy (FCS).

2. Experimental

2.1. Materials

The dyes (PSF and SO, Scheme 1), and HSA (fatty acid free) were used as procured from Sigma-Aldrich Chemical Co., USA. The solvent 1,4-dioxane was obtained from Spectrochem, India (UV spectroscopy grade) and stored in the dark over molecular sieves (5.0 Å, E-Merck Ltd.). 10.0 mM phosphate buffer solution of desired pH (that is, pH 7.4 and 9.0) was prepared from the stock solution of phosphate buffer obtained from Sigma-Aldrich Chemical Co., USA by appropriate dilution in triply distilled deionized Milli pore water.

2.2. Instrumentation and Methods

The absorption and fluorescence spectra were acquired on a Hitachi U-3501 UV–Vis spectrophotometer and Jasco FP-8500 fluorometer, respectively. All the spectroscopic measurements were performed using a low dye concentration (ca. 2.0 μM) in order to minimize inner-filter effects [26–28].

The fluorescence lifetime and depolarization decay profiles were obtained by Time-Correlated Single Photon Counting technique following excitation of the samples at λ_{ex} = 450 nm. The circular dichroic (CD) spectra were obtained on a Jasco J-815 spectropolarimeter using a cylindrical cuvette (path-length = 0.1 cm) at 25 °C. The dynamic light scattering (DLS) measurements were carried out on a Malvern Nano-ZS instrument equipped with a 4 mW He—Ne laser having λ = 632.8 nm. The FCS measurements were carried out on a Confocal Laser Scanning Microscope system from Becker & Hickl DCS-120 equipped with an inverted optical microscope of Zeiss (Carl Zeiss, Germany) [29,30]. For docking simulation the native structure of HSA was obtained from the Protein Data Bank, PDB ID: 1AO6 [31]. The docking simulation was performed on the AutoDock 4.2 software package [32,33]. The three-dimensional structures of the dye molecules (PSF and SO) were prepared on AutoDock 4.2 [32] software utilizing the optimized geometries of PSF and SO (DFT//B3LYP/6–31 + + G(d,p)) as obtained from calculation on Gaussian 03 W [34] suite of programs (Scheme 1). The PyMOL software package [35] was used for visualization of the docked conformations. A detailed description of the experimental methods and protocols is given in the Supporting Information.

3. Results and Discussion

3.1. Characterization of Various Conformational States of HSA

The characterization of various conformational states of HSA is studied by (i) circular dichroism (CD) spectroscopy [1–4,16,24,27,28,36], (ii) intrinsic fluorescence profile of native HSA (at pH 7.4) [2–4,36], and (iii) dynamic light scattering (DLS) measurements. The results are presented in the Supporting Information.

3.2. Interaction of PSF and SO with HSA

3.2.1. Absorption Spectroscopic Studies

The absorption profile of the dyes (PSF and SO) is characterized by a broad unstructured band at ~520 nm in aqueous buffer (pH 7.4 and 9.0), which undergoes a subsequent decrease of absorbance coupled with a red-shift following interaction with the protein, Fig. 1

The red-shift in the absorption profile of the dyes with added HSA is suggestive of the lowering of polarity at the interaction site of the dyes within the protein scaffolds in comparison to that in bulk aqueous buffer [22–24,37–39]. Here, it could be interesting to note that the extent of the red-shift for PSF (from ~520 nm in aqueous buffer to