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Non-enzymatic glycation enhances human serum albumin binding capacity to sodium fluorescein at room temperature: A spectroscopic analysis

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ABSTRACT

Background: Sodium fluorescein (SF) is a fluorescent tracer dye used extensively in diagnostic tools in the field of Ophthalmology, particularly in intravenous fluorescein angiography (IVFA). The binding of SF to human serum albumin (HSA) has been predicted by molecular docking and investigated by circular dichroism (CD) and fluorescence spectroscopy with or without glycation at temperatures 296, 301, and 310 K.

Methods: The binding parameters were calculated by quenching of emission spectrum of a constant concentration of SF (2 μmol/l) at 513 nm against increasing concentrations of glycated or unmodified HSA as quencher starting from stoichiometry ratio of 1:1.

Results: The HSA-SF interaction found to be a static binding. The Stern-Volmer constants (Ksv) were in the range of $\sim 10^4\ M^{-1}$ and other thermodynamic parameters like enthalpy (ΔH°), free energy (ΔG°) and entropy (ΔS°) are similar to albumin ligand bindings reported by previous workers.

Conclusions: The interactions were found to be spontaneous, irrespective of temperature or glycation. Glycated HSA is clinically used to monitor unstable glycemic controls in diabetic patients. A 39% increase in binding affinity (log K) and free energy (ΔG°) is reported on glycation at 310 K (room temperature), which may be important in the SF based angiographies. On glycation HSA-SF binding appears to change from an enthalpy-driven to an entropy-driven reaction. SF shows best binding to FA binding site III of HSA, which also overlaps with drug binding site II of subdomain IIIA. Leu430 seems to play a pivotal role in the interaction.

1. Introduction

Serum albumin, the most abundant protein in blood plasma, account for nearly 60% of the total protein corresponding to a concentration of 42 g/l. Albumin contribute about 80% of the osmotic pressure of blood [1] and is partially responsible for the maintenance of blood pH [2]. Human serum albumin (HSA) is used as a model for studying ligand–protein interactions because it is a major ligand binding and transport protein of circulatory system [3]. The flexibility of the albumin structure adapts it readily to endogenous and exogenous ligands like dyes and drugs, and its three-domain design provides a variety of binding sites. Albumin interacts with a broad range of compounds [4]. Most strong bonds are hydrophobic organic anions of 100–600 Da; like long chain fatty acids, hematin, bilirubin, etc. smaller and less hydrophobic compounds such as ascorbic acid and tryptophan are held weak, but their binding can still be highly accurate [5]. Albumin provides a depot for many endogenous or exogenous compounds so they will be available in quantities well beyond their solubility in plasma. The binding of ligands with albumin is a major determinant of the ligands' distribution in the body. Binding to albumin has a significant effect on ligand dynamics since only unbound ligands are free to interact with other molecules or receptors [6].

The glycation in HSA can occur on several residues that are or around Sudlow's site I and II. warfarin and L-tryptophan have been used as site-selective probes for Sudlow's site I and II respectively. On glycation of HSA, a 20% decreased in affinity of warfarin was observed in vivo, while 30–60% decrease is reported in vitro [7]. But in the case of L-tryptophan, the binding affinity increases on glycation of HSA [8]. In case of the dyes bromocresol purple and bilirubin and drugs: salicylate, phenylbutazone, ibuprofen, ketoprofen and flufenamic acid,

Abbreviations: SF, sodium fluorescein; HSA, human serum albumin; GlyHSA, glycated human serum albumin; Ksv, Stern-Volmer constant; Kq, quenching constant; K, binding constant; n, number of binding sites; ΔG°, standard free energy change; ΔH°, standard change in enthalpy; ΔS°, standard change in entropy

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binding affinity with HSA decreases on glycation [8–10]. The clinical relevance of HSA-ligand binding studies depend on the chemical and physical characteristics of the ligands, the typical therapeutic or physiological range, the domains of HSA that are involved in the interaction and the degree of appropriate modifications, like glycation of HSA, etc. with moderate changes in affinity (due to modification) might be affected to a large extent, if they have a narrow range of concentrations that have undesirable effect [11].

Many of the long-term effects of diabetes can be related to the process of protein glycation. It involves the non-enzymatic addition of reducing sugars and/or reactive degradation products to primary or secondary amine groups on proteins that contributes to retinopathy, nephropathy, and neuropathy [12]. The study of protein containing either early stage glycation products or advanced glycation end-products (AGEs) has become very important due to the suspected effects of glycation on protein tissue damage and function during diabetes [13]. SF is used extensively in intravenous fluorescein angiography (IVFA). IVFA has been widely used in evaluating diabetic retinopathies [14] and for the diagnosis of corneal abrasion, corneal ulcer, herpetic corneal infections, vascular disorders including retinal macular degeneration, etc. [15]. SF is being used during surgery of brain tumor and open heart surgery [16]. SF is a fluorophore (molecular formula: $C_{20}H_{10}Na_2O_5$) having molecular weight 376.3. Its excitation wavelength is 475–490 nm, and the emission wavelength is 510–520 nm. SF is the most frequently used fluorophore in assessing tear turnover and corneal permeability [17]. It is also used to investigate water flow in river and streams to track illegal dumping of waste in waterways and for quantitative studies of ground water contamination of wells [18]. The rate of penetration of fluorescein into the eye following systemic administration can be influenced by many factors, one of which is the binding of the fluorophore to human plasma [19].

The binding mechanism of SF and HSA and the effect of glycation on binding are still not known in details. The binding of ligands to plasma proteins is a major determinant of its distribution and kinetics in the physiological systems. Binding has a substantial effect on the ligand dynamics since only free ligands interact with its receptors. This is the first report on the effect of glycation on the HSA and SF binding evaluated by in-silico docking, circular dichroism and spectroscopic quenching.

2. Materials and methods

2.1. Materials

Human serum albumin (A3782); essentially fatty acid-free and globulin free, and SF were from Sigma Chemical Co. All other reagents were of analytical grade.

2.2. Methods

All the experiments were performed in Sodium phosphate buffer of pH 7.0 (10 mmol/l) at temperature 298 °K, except where specified. The concentration of protein was determined using $E^{1\%}$ of 5.3 (for HSA) at 279 nm [20], while that of SF using solution E^{mM} = 39.8 at 238 nm [21], on a Hitachi spectrophotometer model U-1500.

2.3. CD measurements

CD measurements were carried out with a Jasco spectropolarimeter, model J-720, equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. All the CD measurements were performed at 298 K with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ± 0.1 °C. Far-UV CD spectra were measured at a protein concentration of 7.5 μM. The path length was 0.1 cm. The results are expressed as mean residue ellipticity [MRE] in deg $cm^2 \cdot dmol^{-1}$ defined as

$$MRE = \theta_{obs}\,(mdeg)/10 \times n \times Cp \times l \quad (1)$$

where, θ_{obs} is the CD in millidegree, n = 585 is the number of amino acid residues, l is the path length of the cell in cm and Cp is the molar fraction [22]. Secondary structures like α helix and β sheet were calculated by K2D3 [23].

2.4. Fluorescence measurements

Fluorescence measurements were performed on Shimadzu spectro-fluorophotometer, model RF–5301 PC. The fluorescence spectra were measured at 298 ± 0.1 K with a 1 cm pathlength cell. The excitation and emission slits were set at 3 nm each. Intrinsic fluorescence was measured by exciting the solution at 480 nm, and emission spectra were recorded in the range of 500–600 nm [24]. SF concentration was 2 μmol/l throughout the study.

2.5. Glycation studies

Six hundred micromoles of HSA was incubated with 3000 fold excess glucose (1.8 mol/l) at 310 K for 30 days. A sodium azide solution of 105 mg/ml was added to avoid bacterial contamination. A modified commercial fructosamine assay kit was used to determine the level of glycation [25]. The level of glycation was checked after every 5 days on the basis of the difference in molecular weight by 12% SDS-PAGE (data not shown). Turbidity in samples was monitored by absorbance at 350 nm in every 24 h to check bacterial contamination.

2.6. Ligand binding studies

Binding of SF to HSA and GlyHSA was studied by fluorescence quenching titration method using SF's fluorescence as a probe. To a fixed volume of (3.0 ml) 2 μM SF solution, increasing volume (2–60 μl) of HSA/glyHSA was added.

2.7. Docking studies

The protein was prepared using the Protein Preparation Wizard by removal of crystallographic water molecules and the addition of hydrogen atoms, followed by minimization and optimization using OPLS2005 force field in Schrodinger Glide [26]. The ligand was prepared by using the Ligprep utility of Glide with default parameters. Three-dimensional structure of HSA consists of seven prominent binding sites categorized as I to VII. We have docked SF in each site separately using the Schrodinger Glide software with default settings [26]. Visualization and analysis of protein–ligand complexes were performed using LIGPLOT [27] COOT and PyMOL software [28].

3. Results

3.1. Measurement of the extent of glycation

Fig. 1 shows the extent of glycation of HSA, as measured by fructosamine assay [25]. Most of the increase in glycation occurred during 20 days. The rate of glycation slowed down next 10 days. Similar trends have been observed and reported for HSA [25,29] as well as BSA [30]. The optimum level of glycation was found to be 1.15 ± 0.6 mol hexose/mol HSA which were also similar to previous works mentioned above.

3.2. Secondary structure studies

Typical far-UV CD spectra in the range 200–250 nm for unmodified HSA and glyHSA of 7.5×10^{-6} mol/l in the absence and presence of 187.5×10^{-6} mol/l SF are shown in the Fig. 2. The spectrum of HSA at

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