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Physicochemical aspects of the energetics of binding of sulphanilic acid with bovine serum albumin

Tarlok S. Banipal *, Amandeep Kaur, Parampaul K. Banipal

Department of Chemistry, Guru Nanak Dev University, Amritsar 143005, Punjab, India

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ABSTRACT

The thermodynamic study of the binding of sulphanilic acid with model transport protein bovine serum albumin is a promising approach in the area of synthesizing new sulfa drugs with improved therapeutic effect. Thus, such binding studies play an important role in the rational drug design process. The binding between sulphanilic acid and bovine serum albumin has been studied using calorimetry, light scattering in combination with spectroscopic and microscopic techniques. The calorimetric data reveals the presence of two sequential nature of binding sites where the first binding site has stronger affinity ($\sim 10^4\ M^{-1}$) and second binding site has weaker affinity ($\sim 10^3\ M^{-1}$). However, the spectroscopic (absorption and fluorescence) results suggest the presence of single low affinity binding site ($\sim 10^3\ M^{-1}$) on protein. The contribution of polar and non-polar interactions to the binding process has been explored in the presence of various additives. It is found that sulphanilic acid binds with high affinity at Sudlow site II and with low affinity at Sudlow site I of protein. Light scattering and circular dichroism measurements have been used to study the effect on the molecular topology and conformation of protein, respectively. Thus these studies provide important insights into the binding of sulphanilic acid with bovine serum albumin both quantitatively and qualitatively.

1. Introduction

Serum albumin, well known abundant protein in the circulatory system performs vital physiological functions such as maintaining osmotic pressure and pH of the blood, sequestering oxygen free radicals, and inactivating various toxic metabolites [1]. Bovine serum albumin (BSA), a globular protein consists of 583 amino acid residues in a single polypeptide chain with 17 disulfide bridges and one free thiol group [2]. At neutral pH, BSA exists as negatively charged (net charge −18) due to the presence of 100 negative and 82 positive charges. It has random charge distribution and ellipsoidal structure with axes 41 × 141 Å. The net charge at pH 7 decreases progressively from −10 in domain I, which includes the amino terminal, to zero in domain III, which includes carboxyl terminal. In domain II, between the two domains, the net negative charge is −8 [3]. The presence of multiple high affinity binding sites and its structural resemblance with human serum albumin makes it a broadly studied model protein to bind, transport, and metabolize various endogeneous as well as exogeneous substances to their target positions. The importance of protein binding lies in increase in solubility of ligand in plasma, decrease in its toxicity, protection against the oxidation and have significant impact on the absorption, metabolism, distribution, and excretion properties of ligands [4]. The presence of hydrophobic binding pockets on serum-albumin is responsible for the increase in apparent solubility of the hydrophobic ligands in plasma and also modulates their delivery to the cells *in vivo* and *in vitro* [5]. The reversible nature of the binding of ligands with proteins occurs *via* means of weak chemical bonds such as ionic, van der Waals, hydrogen bonding, and hydrophobic bonds due to the presence of hydroxyl, carboxyl or other reversible sites on the amino acid residues that constitute the binding pockets in the protein [6]. The conformational change induced by the various ligands as a result of their binding with proteins may affect their biological function as the carrier protein [7]. In literature, many studies on the binding of drugs [8–10], vitamins [11,12], environmental pollutants [13], polysaccharides [14], fatty acids [15], plant polyphenols [16], and small ions [17] with BSA have been reported. The transfer of ligands (bound to albumin) to various cells is mediated by the direct interactions between cell surface and ligand-serum albumin complex and such process may involve cell surface receptors [13]. Thus from this point of view, the thermodynamic knowledge obtained from albumin-ligand interactions are also valuable. Sulphanilic acid (SA) (Fig. 1) finds a number of applications in the synthesis of sulfa drugs in pharmaceutical industry, azo dyes, pesticides, and perfumery industry [18]. Owing to its importance in sulfa drugs, many of its side effects on the human body have also been observed [19,20]. SA induces negative effects as oxidative stress in the body that causes gastrointestinal toxicity, bone marrow or hepatic injury [19]. The xenobiotic character [21,22], resistant to biodegradation by bacteria [22], and good water solubility of aromatic sulfonate amines [21] are due to the presence of polar sulfonate group in them. These properties of SA make it an

* Corresponding author.
E-mail address: tsbanipal@yahoo.com (T.S. Banipal).

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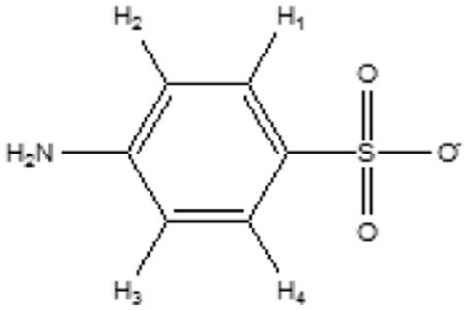

Fig. 1. At pH 7.4 (anionic form). Molecular structure of sulphanilic acid.

environmental pollutant. As an important metabolite of various azo dyes like tartrazine, acid yellow 17 *etc.*, the exposure of SA was found to inhibit both human true and pseudo-cholinesterases activities *in vitro* by non-competitive way [20].

Thus from the point of view of human health concern, it is of interest to study the SA binding with BSA. In the present work, we have studied the detailed energetics of binding, size variation, specific binding site, and effect on the conformation of serum albumin as a result of its binding with SA using various techniques. Isothermal titration calorimetry (ITC) was used to determine the energetics of the binding process. Dynamic light scattering (DLS) measurements were performed to characterize the size variation in BSA as a result of its binding with SA. The various spectroscopic and microscopic techniques like UV–visible, steady-state fluorescence, circular dichroism (CD), proton nuclear magnetic resonance (^{1}H NMR), and scanning electron microscopy (SEM) have also been used to study the binding process as well as change in conformation and morphology of complex formed between BSA and SA.

2. Experimental

2.1. Materials

Bovine serum albumin (Catalog no. B-4287, purity: ≥98%) and sulphanilic acid (Puriss AR, purity: 99%) were obtained from Sigma-Aldrich Chemical Company and Spectrochem, India, respectively. All the solutions were prepared in 0.01 M phosphate buffer at pH 7.4 by using Mettler Toledo AB265-S balance of 0.01 mg readability for mass measurements. Milli-Q water was used to prepare all the solutions. The stock solution of BSA was dialyzed against proper buffer at 4 °C. The reported pH of the dialysate was measured on Systronics μ pH System 362. The protein concentration was determined on Shimadzu-1800 UV–vis spectrophotometer using molar absorption coefficient $^{1\%}A_{1cm} = 6.8$ at 280 nm [23]. All other analytical grade reagents were used as purchased without further purification but dried in vacuum desiccator before use.

2.2. Isothermal titration calorimetry (ITC)

An isothermal titration calorimeter (iTC_{200}, MicroCal, USA) was used to measure various thermodynamic parameters associated with SA-BSA binding. The 0.044 mM BSA solution or 0.01 M phosphate buffer was loaded in sample cell of capacity 200 μl and the reference cell was filled with phosphate buffer for all experiments. The 40 μl syringe of the calorimeter was filled with 4.4 mM SA solution. As the baseline stability had been achieved, the injections of SA into BSA were started. The total 19 injections of 2 μl each of 4.4 mM SA solution were titrated into the sample cell containing 0.044 mM BSA. The contents of the sample cell were stirred at a fixed speed of 500 rpm to ensure the complete mixing of the ligand-protein solution but it did not cause foaming on the protein solution. The time interval between successive injections was 120 s so as to reach the thermal equilibrium during each titration. Raw data peaks were obtained by measuring the enthalpy change at each injection. An integrated heat profile *i.e.* a plot of observed enthalpy change per mole of injectant (kJ mol^{-1}) against molar ratio was obtained by transforming raw data peaks using Origin 7 software of the instrument.

To correct the data for heat of dilution of ligand, protein, and buffer mixing, control experiments included the titration of ligand into buffer, buffer into protein, and buffer into buffer were performed by using same concentration of ligand and BSA and subtracted from the experimental titrations. The experimental data were analyzed with two sequential binding site model by using Origin 7 software provided by MicroCal.

2.3. Dynamic light scattering (DLS) measurements

To monitor the variation in size of BSA upon SA binding, dynamic light scattering measurements were performed at 298.15 K by using Zetasizer NanoZS (Malvern, instruments, U·K) equipped with a He-Ne laser (632.8 nm, 4 mW), a built in temperature controller having an accuracy of ±0.1 K, and at a scattering angle of 173° to the incident beam. All the solutions were filtered before use by Millipore filter with a pore-size of 0.22 μm. The suitable aliquots of SA solution were added to the 2 ml BSA solution (0.02 mM) and the average value of six measurements is reported. The DLS experiments were performed in disposable polystyrene cuvettes of 1 cm path length and the data were analyzed using standard algorithms.

The instrument monitors the speed of the particles at which they diffuses within the solvent due to the Brownian motion, by measuring the rate of fluctuations in the intensity of light scattered from the particles at a fixed scattering angle. The hydrodynamic diameter (d_h) of the particles were calculated using Stoke's-Einstein equation:

$$d_h = k_B T / 3\pi\eta D_0 \quad (1)$$

where k_B is the Boltzmann constant ($= 1.38 \times 10^{-23}$ m^2 kg s^{-2} K^{-1}), T (in K) is the absolute temperature, η (in cP) is the viscosity of the medium measured, and D_0 (in m^2 s^{-1}) is the translational diffusion coefficient.

The value of D_0 has been obtained from distribution decay rates (τ) as

$$D_0 = \tau / q^2 \quad (2)$$

where

$$q = \text{scattering vector} = (4\pi n/\lambda)\sin(\theta/2) \quad (3)$$

here, n is the refractive index of water, λ is the wavelength of the incident light, and θ is the scattering angle.

2.4. UV–visible absorption spectroscopy

The absorption measurements were carried out on Shimadzu-1800 UV–visible spectrophotometer by using the quartz cuvettes having 1 cm path length. By keeping the concentration of BSA as constant (8 μM), the concentration of SA varies from 0 to 18.2 μM during absorbance titrations. The pure buffer was used as reference for each titration. The absorbance of each experiment was recorded at least three times and the average absorbance values obtained after each titration were used to calculate the binding constant.

2.5. Steady-state fluorescence spectroscopy

The steady-state fluorescence measurements were monitored on a Hitachi fluorescence spectrophotometer, F-4600 at 298.15 K. The concentration of BSA was kept at 4 μM in all the experiments. The emission spectrum was recorded by selectively exciting the tryptophan (Trp)