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Binding of glutathione and melatonin to human serum albumin: A comparative study

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ABSTRACT

Binding of glutathione and melatonin to human serum albumin (HSA) has been studied using isothermal titration calorimetry (ITC) in combination with UV–vis absorption spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, and circular dichroism (CD) spectroscopy. Thermodynamic investigations reveal that glutathione/melatonin binds to HSA is driven by favorable enthalpy and unfavorable entropy, and the major driving forces are hydrogen bond and van der Waals force. For glutathione, the interaction is characterized by a high number of binding sites, which suggests that binding occurs by a surface adsorption mechanism that leads to coating of the protein surface. For melatonin, one molecule of melatonin combines with one molecule of HSA and no more melatonin binding to HSA occurs at concentration ranges used in this study. The UV–vis absorption, FT-IR, and CD spectroscopy suggest that glutathione and melatonin may induce conformational and microenvironmental changes of HSA.

1. Introduction

Glutathione is one of the most prominent endogenous low-molecular-weight thiols found in mammals and significantly in demand as a drug for therapeutic purpose because of multiple biological functions in various tissues and its involvement in many diseases and malnutrition [1]. It is a tripeptide composed of glutamic acid, cysteine and glycine, and has two characteristic structural features: a glutamyl linkage and a sulfydryl group [2]. Glutathione is found in cells exists in two forms: one is a reduced form (GSH) and other is in oxidized form as glutathione disulfide (GSSG) [3]. In healthy living cells, more than 90% of glutathione is found as GSH, which can be converted to the oxidized form (GSSG) during oxidative stress, and can be reverted to the reduced form by the action of the enzyme glutathione reductase [4]. Thus, the important physiological function of GSH is an essential endogenous antioxidant that plays a central role in cellular defense against toxins and free radicals [5]. In such case, two GSH molecules form a molecule of oxidized glutathione (GSSG) via the formation of double-sulfur bond [1]. GSH is also an important cofactor in cell metabolism, differentiation, proliferation, and apoptosis [6]. A decreased GSH level is associated with aging and various diseases, including cardiovascular, inflammatory, immune, and neurodegenerative diseases [7]. Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous neurohormone secreted primarily from the pineal gland in mammals [8]. The other organs and tissues including retina, the gastrointestinal tract, lymphocytes, gut, ovary, testes, bone marrow and lens have been reported to produce it as well [9]. Melatonin can also be synthesized in non-mammalian vertebrates, invertebrates and in some organisms including dinoflagellates, algae and bacteria and it even can be found in a variety of plants [10–12]. The presence of melatonin in such a variety of organisms suggests that this substance is phylogenetically highly conserved and plays an important role in the function and survival of organisms. Melatonin plays roles in numerous physiologic activities such as neurogenesis, immunomodulation, improving immune defense, regulating circadian rhythms and sleep, intervening in lipid metabolism, and inhibiting cancer growth [9]. It has also been proposed as a natural antioxidant and potent free radical scavenger [13]. In contrast with usual antioxidants, melatonin is known as a suicidal antioxidant, because it does not contribute in a redox cycling. Once melatonin is oxidized, it cannot be reduced to its initial state, because some irreversible end-products are formed through reaction of melatonin with free radicals [14].

Glutathione and melatonin represent the major antioxidants in plasma and act as a primary defense in the blood against free radical attack. However, to our knowledge, an accurate and full basic data for clarifying the binding mechanisms of glutathione and

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melatonin to plasma proteins remain unclear. Serum albumin is the most abundant protein in blood plasma (~60%) and serves as a depot and transport protein for numerous endogenous and exogenous compounds [15]. Knowledge of interaction mechanisms between these two antioxidants and serum albumin is very important for us to understand the pharmacokinetics and pharmacodynamics of them. First, the drug-serum albumin interaction plays a dominant role in the bioavailability of drugs because the bound fraction of drugs is a depot, whereas the free fraction of drugs shows pharmacological effects [16]. Second, drug distribution is mainly controlled by serum albumin, because most drugs circulate in plasma and reach the target tissues by binding to serum albumin [17,18]. If a drug is metabolized and excreted from the body too fast because of low protein binding, the drug would not be able to provide its therapeutic effect. Alternatively, if a drug has high protein binding and is metabolized and excreted too slowly, it may increase the drug's half-life in vivo and lead to undesired side effects [19]. Furthermore, very high affinity binding of a drug to serum albumin may prevent the drug from reaching the target at all, resulting in insufficient tissue distribution and efficacy. Third, the competition between two drugs for the binding sites on serum albumin may result in a decrease in binding and hence an increase of the concentration of the free biologically active fraction of one or both of the drugs. Co-administration of two drugs increases the free concentration of the drug with the lower affinity to serum albumin [20]. In addition, these hydrophobic binding pockets enable serum albumin to increase the apparent solubility of the hydrophobic drugs in plasma and modulate their delivery to the cells in vivo [21]. In a word, the absorption, distribution, metabolism, and excretion properties of a drug can be significantly affected as a result of its binding to serum albumin.

Isothermal titration calorimetry (ITC), which measures directly the heat evolved during a reaction, is the method of choice for obtaining thermodynamic information. This is because only ITC allows researchers to obtain directly the variations of enthalpy ΔH^0 and of entropy ΔS^0, as well as the association constant K and the stoichiometry of binding n, for an association process [22]. Unlike other methods, ITC does not require chemical modification or immobilization of reactants since heat of binding is a naturally occurring phenomenon [23,24]. This sets the technique apart from fluorescence methods that often require labeling or are specific to proteins that contain a fluorophore that is accessible to a quencher. ITC can also be applied to systems where the complex formed is insoluble. This is a distinct advantage over many solution based techniques, including capillary electrophoresis, where complex insolubility can be problematic [25].

The present study examines the thermodynamics of the binding of these two antioxidants to HSA using isothermal titration calorimetry (ITC), and the consequent conformational changes have been monitored using UV–vis absorption spectroscopy, FT-IR, and CD. The cooperativity displayed between glutathione and melatonin has long been thought to be key to the activity of both compounds in vivo. Glutathione is a hydrophilic antioxidant and melatonin is a hydrophobic antioxidant, thus, the binding mechanism of these two antioxidants interact with HSA may be different. In the study, the calorimetric results are then coupled with spectroscopic observations to understand these mechanisms underlying these interactions.

2. Materials and methods

2.1. Materials

HSA, glutathione and melatonin were purchased from Sigma–Aldrich Chemicals Company (USA). Glutathione was directly dissolved in phosphate buffer solution of pH 7.40 (0.01 mol L^{-1} PBS), and melatonin was dissolved in 99.5% ethanol and then diluted with phosphate buffer solution of pH 7.40 (0.01 mol L^{-1} PBS). The stock solutions of glutathione and melatonin were prepared and used immediately because of oxidation under light and air. Double distilled water was used to prepare solutions. The HSA was dissolved in a phosphate buffer solution of pH 7.40 (0.01 mol L^{-1} PBS). The HSA stock solution was prepared by extensive overnight dialysis at 4 °C against the buffer. The concentration of the HSA was determined on a TU-1810 spectrophotometer (Puxi Analytic Instrument Ltd., Beijing, China) using the extinction coefficient ε_{280} = 36,600 mol^{-1} L cm^{-1} [26]. The pH was determined on a pHS-2 C pH-meter (Shanghai DaPu Instruments Co., Ltd, Shanghai, China) at ambient temperature. Sample masses were accurately weighed on a microbalance (Sartorius, BP211D) with a resolution of 0.01 mg. All other reagents were all of analytical reagent grade and were used as purchased without further purification.

2.2. Isothermal titration calorimetry

Titration of HSA with glutathione/melatonin was performed using a Model Nano-ITC 2G biocalorimetry instrument (TA, USA) at 298 K. All these solutions were thoroughly degassed prior to the titrations to avoid the formation of bubbles in the calorimeter cell. The sample cell was loaded with the phosphate buffer (PBS, 0.01 mol L^{-1}) or protein solution and the reference cell contained double distilled water. In a typical experiment, buffered HSA solution was placed in the 950 μL sample cell of the calorimeter and glutathione/melatonin solution was loaded into the injection syringe. Injections were started after baseline stability had been achieved. Glutathione/melatonin was titrated into the sample cell by means of syringes via 25 individual injections, the amount of each injection was 10 μL. The first injection of 10 μL was ignored in the final data analysis. The contents of the sample cell were stirred throughout the experiment at 200 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat (μJ) against injection number and featured a series of peaks for each injection. These raw data peaks were transformed using the instrument's software to obtain a plot of enthalpy change per mole of injectant (ΔH^0, kJ mol^{-1}) against molar ratio. Control experiments included the titration of glutathione/melatonin solution into buffer, buffer into HSA, and buffer into buffer, controls were repeated for the same HSA concentration used. The last two controls resulted in small and equal enthalpy changes for each successive injection of buffer and, therefore, were not further considered in the data analysis [27]. Corrected data refer to experimental data after subtraction of the glutathione/melatonin into buffer control data.

Estimated binding parameters were obtained from ITC data using NanoAnalyze software provided by the manufacturer. Data fits were obtained using either the independent binding sites (single site) model or the multiple binding sites (two sites) model. For the independent binding sites model the analytical solution for the total heat measured (Q) is determined by the formula:

$$Q = V\Delta H^0 \left\{ [L] + \frac{1+[M]nK - \sqrt{(1+[M]nK - [L]K)^2 + 4K[L]}}{2K} \right\} \quad (1)$$

where V is the volume of the calorimeter cell, ΔH^0 is enthalpy, $[L]$ is ligand concentration, $[M]$ is macromolecule concentration, n is the molar ratio of interacting species, and K is the equilibrium binding constant [28]. The analytical solution for Q in the multiple binding sites model is determined by the formula

$$Q = V[M] \left\{ \frac{n_1 \Delta H_1^0 K_1 [L]}{1 + K_1[L]} + \frac{n_2 \Delta H_2^0 K_2 [L]}{1 + K_2[L]} \right\} \quad (2)$$