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Full Length Article

Experimental and theoretical investigation of bezafibrate binding to serum albumins

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ABSTRACT

The purpose of this investigation was to provide insight into the possible mechanism of the intermolecular interactions between antilipemic agent bezafibrate and serum albumins (SAs) including human (HSA) and bovine (BSA). The aim was to indicate the most probable sight of these interactions. Both experimental (spectroscopic) and theoretical methods were applied. It was determined that bezafibrate binds to SAs in one specific binding site, the fatty acid binding site 6. The results obtained from the steady-state and time-resolved fluorescence experiments suggested that existing two distinct stable conformations of the proteins with different exposure to the quencher. The dominate conformer of HSA and BSA characterized by the Stern–Volmer quenching constant (from ratio F_0/F) equal to $1.24 \cdot 10^4$ and $8.48 \cdot 10^3\ M^{-1}$ at 298 K, respectively. The docking results and calculated thermodynamics parameters may be suggested that the binding process is spontaneous and might involve van der Waals and hydrogen bonding forces.

1. Introduction

Bezafibrate (2-(4-{2-[(4-chlorophenyl)formamido]ethyl}phenoxy)-2-methylpropanoic acid) is an antilipemic agent belonging to the phenoxyacetic acid derivatives. These are compounds containing an anisole where the methane group is linked to an acetic acid or a derivative (Fig. 1).

Bezafibrate is clinically used as a hypolipidemic agent, a drug that lowers the levels of lipids in the blood [1]. Up to 94–96% of bezafibrate is bound to protein in human serum. Treatment with this drug has been shown to lower serum triglyceride and cholesterol levels by 15–25% in patients with hyperlipidemia [2]. The molecular mechanism underlying the effect of bezafibrate is due to activation of peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor superfamily [3,4]. This receptor is highly expressed in the liver, kidneys and heart functioning as a critical regulator of the metabolism of fatty acids [4].

SAs are relatively large (66 kDa) and negatively charged proteins [5] that play a vital role in the transportation of many substances through the circulatory system to target cells, including the transport of drugs and fatty acids [6]. This group of proteins contribute to an estimated 52–60% of the total proteins in the blood of mammals [6]. These proteins have been extensively studied due to their multifunctional properties and extraordinary importance on the delivery and pharmacokinetics of many drugs.

For this study, two different SAs were used: HSA, BSA. These albumins are initially expressed as preproalbumins, meaning that they have a signal sequence and a propeptide [7]. BSA and HSA preproalbumins are 607 amino acids long. The corresponding mature proteins are comprised of 583 (BSA) and 585 (HSA) amino acids. The overall sequence identities of mature BSA compared to HSA is 75.6% [5].

SAs are composed of three structurally similar helical domains (I, II, and III) arranged in a heart-shaped molecule. Each domain can be divided into two subdomains (A and B), with six and four helices, respectively. Several binding sites exist in the structure of SA, which allow them to bind a variety of compounds. There are two main drug-binding sites in HSA known as Sudlow site 1 and Sudlow site 2. Sudlow site 1 is located in subdomain IIA and

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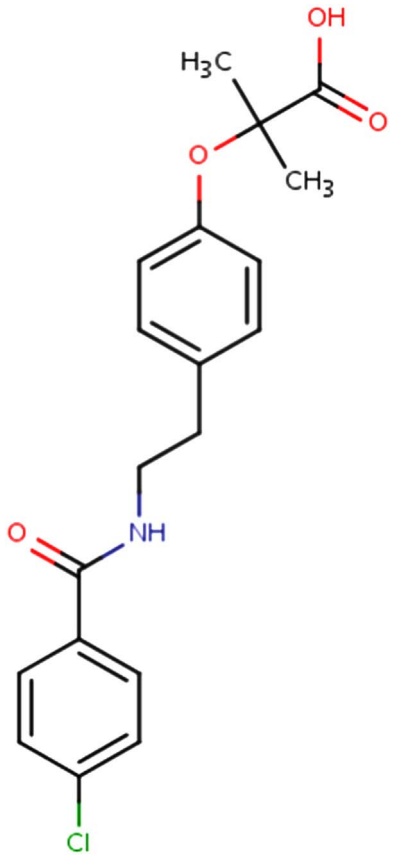

Fig. 1. The structure of bezafibrate.

prefers large heterocyclic and negatively charged compounds, while Sudlow site 2 in subdomain III is the preferred site for small aromatic carboxylic acids [8]. In addition, there are a number of minor binding sites that allow multiple drug molecules to be bound simultaneously onto HSA and lead to higher drug binding capacity of HSA.

The distribution of binding sites differs in individual SAs. Another fundamental difference between these proteins is in the content of tryptophan residues. HSA contains one TRP (TRP214), while BSA has two, TRP212 and TRP135 [9]. Due to the presence of TRP residue(s) in SA, it is possible to apply spectroscopic methods to study ligand binding to these proteins.

The interaction between bezafibrate and HSA has been previously studied [10], and it was observed that bezafibrate interacts with the TRP residue of the subdomain IIA. Bezafibrate is attracted electrostatically to HSA and then binds to the first drug site of HSA. Non-covalent interactions lead to tight complexing and the interaction occurs through a hydrophobic mechanism. The binding is exothermic whereby both enthalpic and free energy values are negative [11].

In this study several spectroscopic methods and molecular modeling tools were used to provide a more detailed description of the possible mechanism of the interaction between bezafibrate and SAs and their pharmacokinetics.

2. Materials and methods

2.1. Materials

SAs fatty acid free and bezafibrate were of the highest purity grade available from commercial sources (Sigma-Aldrich) and were used without further purification. The proteins (pH 7, phosphate buffer 0.005 M) were freshly prepared using high-purity water (18.2 MΩ) from Milli-Q system by mixing stock solutions (10 μM) in aqueous medium. Since the solubility of bezafibrate in water is very low, the stock solution of this drug (1 mM) was prepared using ethanol.

2.2. Methods

2.2.1. Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed with a FluoroMax2 (JobinYvonSpex) spectrofluorimeter, using an excitation wavelength of 295 nm. All measurements were performed in a standard quartz cuvette at 25 °C. Fluorometric titrations of bezafibrate quenching of SAs fluorescence were carried out at 25 °C using 2 μM SAs and drug ranging from 0.01–13 μM. The dosage of the ligand was carried out in a continuous manner by a stepper motor with the selected rate. The excitation and emission wavelengths were set to 295 nm and 320–350 nm, respectively. The emission spectrum was integrated over.

2.2.2. Fluorescence emission spectral fitting

Fluorescence emission spectra were normalized and fit by application of Franck–Condon analysis of emission bands profiles, as described previously [12–14] using the following equations:

$$I(v)_i = \sum_{\upsilon=0}^{4} \left\{ \left(\frac{E_0 - \upsilon\hbar\omega}{E_0}\right)^3 \left(\frac{S^{\upsilon}}{\upsilon!}\right) \exp\left[-4\ln(2)\left(\frac{v - E_0 + \upsilon\hbar\omega}{\Delta v_{1/2}}\right)^2\right] \right\} \quad (1)$$

$$I(v) = f \cdot I(v)_1 + (1-f) I(v)_2 \quad (2)$$

where: $I(v)_i$ is the emission intensity at the energy v in wavenumbers (cm^{-1}) of i-th component, relative to the intensity of the $0 \rightarrow 0$ transition, E_0 is the energy gap between the zero vibrational levels of the ground and excited states, $\hbar\omega$ and S are the quantum spacing and the Huang–Rhys factor [15] reflecting the degree of distortion in the single, average mode as the difference in equilibrium displacements, $\Delta v_{1/2}$ is the full width at half maximum for individual vibronic lines [14], f is the fractional contribution of the $I(\overline{v})_1$. The results of the fits were identical when the summation was carried out over 4 and 6 ground-state vibrational levels ($\upsilon = 0 \rightarrow 4$, $\upsilon = 0 \rightarrow 6$).

The parameters in the Franck–Condon analysis were the 0–0 energy gap (E_0), the bandwidth at half height $\Delta v_{1/2}$, which includes the classical reorganization energy, the quantum spacing for the averaged acceptor mode ($\hbar\omega$) and the electron-vibrational coupling constant or Huang–Rhys factor (S). Emission intensities were fit by optimizing the parameters E_0, $\hbar\omega$, $\Delta v_{1/2}$ and S with a least squares minimization routine.

2.2.3. Time-resolved fluorescence measurements

Fluorescence lifetime measurements were carried out at 25 °C with a FL900CDT time-correlated single photon counting fluorimeter from Edinburgh Analytical Instruments with 295 nm excitation source (nanoLED-295) having pulse FWHM ~1.18 ns. Data acquisition and analysis were performed using the software provided by Edinburgh Analytical Instrumentation. The goodness of fit was estimated by using reduced χ^2 values.

2.2.4. Docking

The binding conformation of SAs and bezafibrate was predicted using the Autodock 4.2 software package [16,17], which uses an empirical free energy force field with a Lamarckian Genetic Algorithm to predict the binding conformation and the free energies of association [16]. The structures of HSA, BSA and bezafibrate were