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Study on the thermodynamic behavior of betaxolol–bovine serum albumin interacting system

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ABSTRACT

The binding reaction of betaxolol (BET) with bovine serum albumin (BSA) in aqueous buffer solution has been investigated using isothermal titration calorimetry (ITC) and circular dichroism (CD) spectroscopy. The thermodynamic results indicate that there were two classes of binding sites on each BSA molecule for BET molecules. The changes of standard Gibbs free energy (ΔG_1° and ΔG_2°) are almost the same when the drug molecules bind to the first and the second classes of sites. However, the changes of standard enthalpy (ΔH_1° and ΔH_2°) are -38.35 ± 0.50 and 18.06 ± 0.03 kJ mol^{-1}, respectively. The first class of binding is an enthalpy driven process while the second class of binding is an entropy driven one. The results of spectroscopic experiment were applied to investigate the structure of the BSA–BET complex and to understand the thermodynamic data.

1. Introduction

The interactions between small molecules and bio-macromolecules have attracted increasing research interest in the fields of chemistry, life science and clinical medicine in recent years [1–5]. The binding ability of drug to protein in blood stream may have a significant impact on its distribution, free concentration and metabolism. Serum albumin (SA) is the most abundant protein in the circulatory system. It has ability to transport multiple ligands such as fatty acids, amino acids, steroids, metal ions and drugs [6]. Besides the function of participating in the mass transportation of biological system, the protein provides the osmotic pressure of blood in body of human or animal. Bovine serum albumin (BSA) has been applied extensively in the areas of molecular biology and cell biology, because it can be easily obtained from the blood and milk of cows and its physicochemical properties are well characterized. Its molecule is in a relatively large globular shape and often causes increase in the apparent solubility of hydrophobic drug in plasma [7,8].

Investigation on interactions of drugs with BSA can elucidate the properties of drug–protein complex, as it can provide useful information of the structural features that determine the therapeutic effectiveness of drugs, and also be of critical importance for understanding the drug toxicity as well as its distribution in the organism. Therefore, studies on binding of drugs to serum albumin are of great significance not only to the improvement of bio-macromolecules detection, but also to finding out the essence of interactions between small molecules and bio-macromolecules at molecular level [9,10]. Our group investigated the interaction of oxymatrine, an anti-tumor drug, with BSA using isothermal titration calorimetry and circular dichroism (CD) spectrometry in earlier work [11].

Among the several classes of effective cardiovascular medications, β-blockers are widely used for treatment of a variety of cardiovascular disorders such as hypertension, coronary artery disease, heart failure and arrhythmias [12]. Betaxolol (BET) is an approved clinically used β-blocker [13], which produces vasodilatation and is effective for the treatment of arterial hypertension, intraocular hypertension [14,15], coronary artery disease [16], and chronic cardiovascular diseases [17]. Studies on the interaction of BET with BSA can help to understand the pharmacological effect of the drug. However, few attempts had been made so far to investigate the binding mechanism of BET with BSA.

In the present work, isothermal titration calorimetry (ITC) combining with circular dichroism (CD) spectroscopy was used to investigate the interaction of BET with BSA. Thermodynamic parameters including the number of binding sites (N), binding equilibrium constants (K), changes of standard enthalpy (ΔH°), Gibbs free energy (ΔG°) and entropy (ΔS°) were determined. The corresponding conformational changes in the protein molecule as the result of binding process have been probed by CD spectra.

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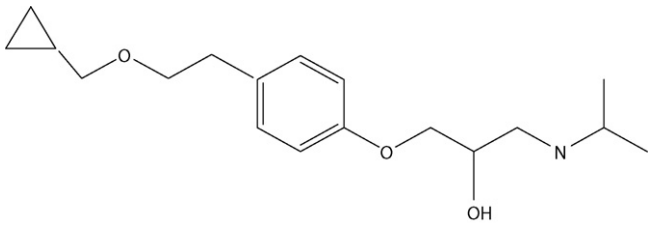

Scheme 1. The molecular structure of betaxolol.

2. Experiment

2.1. Materials

Bovine serum albumin (BSA, purity 98%) was purchased from Sigma Company, whose average molar mass is 66.0 kg mol^{-1} [18], and its solutions were prepared by the weight method. Betaxolol hydrochloride (purity >98%) was purchased from J&K Chemical Company and its molecular structure was shown in Scheme 1. Tri-(hydroxymethyl) aminomethane (Tris), sodium chloride and hydrochloric acid were all of analytical grade. The double distilled water used in the experiment was prepared in the presence of basic potassium permanganate. All other reagents were of analytical purity and solutions used in calorimetric and spectroscopic experiments were prepared in 0.01 M Tris–HCl buffer solution (pH 7.40).

2.2. Isothermal titration calorimetry (ITC)

The isothermal titration micro-calorimeter was provided by 2277 Thermal Activity Monitor (Thermometric, Sweden). The reaction and reference cells were initially loaded with 500 μL titrand, the Tris–HCl buffer solution or BSA solution at the concentration 1.25×10^{-4} M, and 750 μL Tris–HCl buffer solution, respectively. A 25.0 mM BET solution was sequentially injected into the stirred reaction cell (15 μL injections) using a 500 μL Hamilton syringe controlled by a 612 Lund Pump. The representative titration curves are given in Figs. 1 and 2, which respectively demonstrate the dilution heat of BET and the summation of this dilution heat plus the reaction heat of BSA with BET. During the calculating process to be described in Section 3, the dilution heat of BET has been excluded, i.e., the data used for fitting calculation are the differences got by subtracting the corresponding values shown in Fig. 1 from those shown in Fig. 2. The capability and precision of this instrument, and the calorimetric experiments to determine the heat effects of the interaction and dilution processes were the same as described in our earlier work [19].

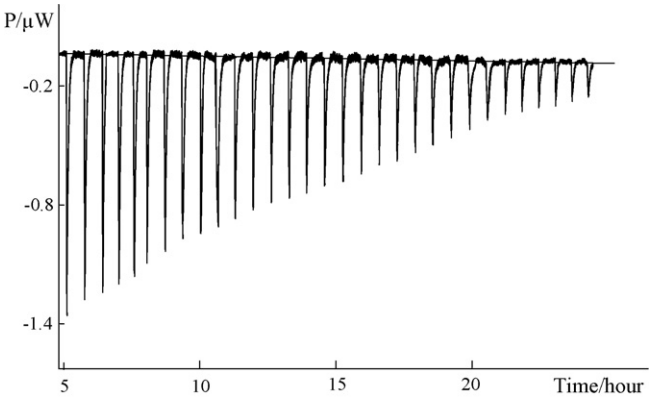

Fig. 1. Variation of heat-flow/electrical power as a function of time, titrant: BET (25.0 mM); titrand: Tris–HCl.

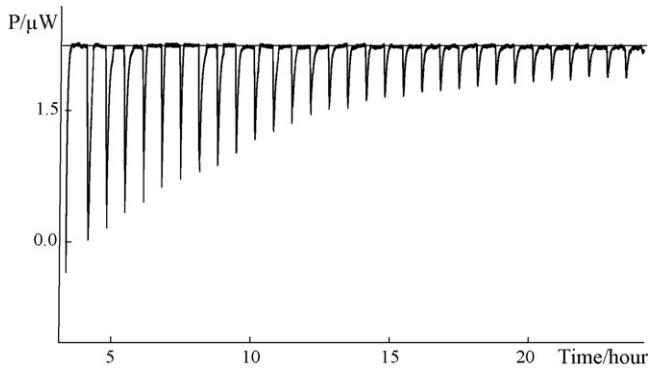

Fig. 2. Variation of heat-flow/electrical power as a function of time, titrant: BET (25.0 mM); titrand: BSA (1.25×10^{-4} M).

2.3. Circular dichroism (CD) spectroscopy

The alterations in the secondary structure of the protein molecule in the presence of the drug were detected on a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan), which was attached to a Jasco temperature controller and thermal programmer and circular quartz cells of 1 cm path length. The lamp–house system was in protection of nitrogen atmosphere during the experiment. The spectra were measured as the average of two scans from 250 to 200 nm at a scan rate of 200 nm/min and the obtained results expressed as millidegrees (mdeg). The concentration of BSA was kept at 1.00 μM and that of BET in the solution was varied from 4.90×10^{-5} to 6.80×10^{-5} M.

3. Results and discussion

3.1. Thermodynamic assumptions of the binding system

The basic assumptions on binding process of BSA with BET which can be regarded as a kind of ligand in the system may be expressed as reported in literature [20,21]. (a) One protein molecule may have several (i) classes of binding sites, which can bind with the same ligand. (b) All sites within one class of binding are thermodynamically identical. (c) The i classes of binding sites are assumed to be mutually independent, so that the binding ratio on one class of sites does not depend on that of another class of ones. (d) Each site can only bind with one ligand molecule. On the basis of the above assumptions and the supposition that the binding of drug to protein can be regarded as adsorption according with the Langmuir's model [22,23], the following equations can be obtained.

$$\theta_i = \frac{K_i c_L}{1 + K_i c_L} \tag{1}$$

$$c_{L,0} = c_L + c_{P,0} \sum_{i=1}^{m} N_i \theta_i \tag{2}$$

where $c_{L,0}$ and $c_{P,0}$ are respectively the total concentrations of BET and BSA within the mixture of the titrant and the titrand. c_L is the concentration of dissociative drug after the binding process having reached equilibrium. θ_i, K_i and N_i are the binding ratio, equilibrium constant and site number of ith class of binding sites, respectively. The following equation can be obtained by combining Eq. (1) with Eq. (2).

$$c_{L,0} = c_L + c_{P,0} \sum_{i=1}^{m} \frac{N_i K_i c_L}{1 + K_i c_L} \tag{3}$$

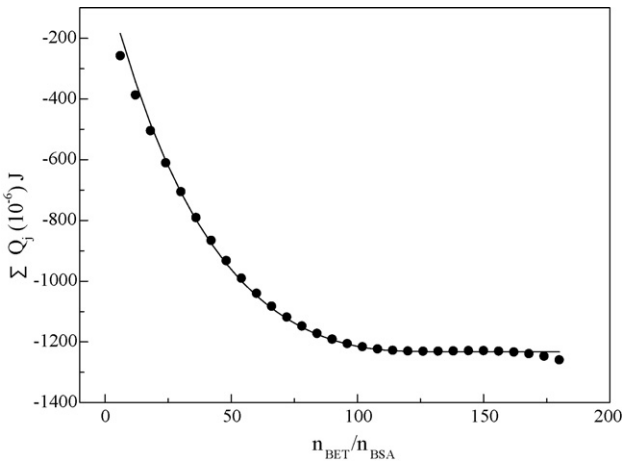

Fig. 3. The nonlinear fitting curve of the reaction heat (●) versus the molar ratio of BET to BSA.

The heat of the jth injection (Q_j) in this experiment may be expressed as:

$$Q_j = c_{P,0} V_{cell} \sum_{i=1}^{m} N_i \ \Delta\theta_i \, \Delta H_i^\circ \tag{4}$$

where V_{cell} is the volume of the titrand in the reaction cell, $\Delta\theta_i$ is the increment of binding ratio from $(j-1)$th to jth injection, and ΔH_i° is the standard change of binding enthalpy. The concentrations $c_{L,0}$ and $c_{P,0}$ are known quantities and Q_j can be obtained from calorimetric experiment. In fact, Q_j is a function of $c_{L,0}$ and $c_{P,0}$ with N_i, K_i and ΔH_i° as undetermined parameters while c_L and θ_i as eliminated variables. The fitting curve of accumulated binding heat, $\sum Q_j$, versus the molar ratio of the drug to the BSA, which is obtained from nonlinear least variance fitting principle and by using of MATLAB 7.01 software, is shown in Fig. 3. By analyzing the deviation between the calculated curve and the experimental data points, it can be reasonably determined that there may be two classes of binding sites on the protein molecule. So such thermodynamic parameters as N_1, N_2, ΔH_1°, ΔH_2°, K_1° and K_2° can be obtained from Eqs. (1) through (4). The changes of standard Gibbs free energy (ΔG°) and entropy (ΔS°) can be calculated using the thermodynamic formulas, Eqs. (5) and (6), and the results were gathered in Table 1.

$$\Delta G^\circ = -RT \ln K^\circ \tag{5}$$

$$\Delta G^\circ = \Delta H^\circ - T \ \Delta S^\circ \tag{6}$$

3.2. Thermodynamic parameters

It can be seen from the data in Table 1 that K_1° is almost equal to K_2°, which means that the affinity of the drug molecules is nearly the same to the first and the second classes of binding sites, while the number of the second class of binding sites (N_2) is evidently larger than that of the first class of ones (N_1). Because the equilibrium constants and the numbers of the different classes of binding sites are got from the calorimetric data which directly relate to the binding enthalpies, the difference between changes of standard enthalpy (ΔH°) corresponding to different classes of binding process may distinguish the classes clearly. Furthermore, the values of ΔH° can be understood by considering the interacting model and the interacting forces of the drug and the protein molecules.A drug molecule may (partly) insert itself into the interior of the protein molecule at a site of the first class, while another one may only interact with the amino acid residues on the surface of the macromolecule at a site of the second class [24,25]. The interaction forces between protein and ligands may comprise hydrophobic, hydrogen bonds and electrostatic interactions [26]. For the first binding site of this system, the negative enthalpic change (ΔH_1°) and entropic change (ΔS_1°) values indicate that this class of binding is an exothermic and entropy decreasing process. The reason may be that the hydrophobic interaction can result in an exothermic process when the drug molecules (partly) enter into the hydrophobic part at the binding sites of the protein molecules. Meanwhile the rigid ordered structure of BSA formed by water is destroyed when drug molecules insert into the interior of BSA molecule. This leads to some solvent molecules transferred into bulk medium from the hydrophobic core at the binding sites, which is an exothermic process. On the other hand, the drug molecules loose some water molecules in their hydration layers when they are approaching to the binding sites, which is an endothermic one. But the absorbed heat is less than the released heat in the above mentioned processes. BET molecules should lose some freedom degrees of movement when they are approaching to the BSA molecules from the bulk medium. This direct interaction leads to an entropy decrease which surpasses the entropy increase caused by the dehydration effect. So this binding is an exothermic and entropy decreasing process. As $\Delta H_1^\circ < 0$ and $\Delta S_1^\circ < 0$, the heat effect makes a contribution to the negative change of standard Gibbs free energy (ΔG_1°), that is, this class of binding is an enthalpy driven process in the viewpoint of thermodynamics.

The positive enthalpy and entropy values indicate that the second class of binding is an endothermic and entropy increasing process, which is completely different from the first class of binding by thermodynamic behavior. It may be explained by the following reasons: the BET molecules lose some water molecules when they are approaching to the binding sites, and simultaneously, the hydration layer of hydrophilic groups at the binding sites of BSA molecule is partly destroyed, too. Both the dehydration processes are endothermic and make a major favorable contribution to the entropy increase. This endoergic and entropy increase effect surpass the opposite effect caused by the direct interactions between the hydrophilic groups and the drug molecules, which are hydrogen bonds and electrostatic force according to literature [27]. Because $T\,\Delta S_2^\circ > \Delta H_2^\circ$ and the entropy change result in $\Delta G_2^\circ < 0$, the second class of binding is entropy driven process.

Overall speaking, the negative changes of standard Gibbs free energy (ΔG_1° and ΔG_2°) show that the both classes of binding processes are spontaneous and the BSA–BET supramolecular complex can form in the aqueous buffer solution. Nevertheless, by comparing the changes of standard enthalpy and entropy, evident difference between the binding forces at the two classes of sites can be discovered. This conclusion is not only obtained from the thermodynamic demonstration made above but also in accordance with the general consensus in literature [28], i.e., there are two principal binding areas for small heterocyclic or aromatic carboxylic acids; there are at least two to three dominant long-chain fatty acid-binding sites unique and separate from the binding sites for small anionic compounds on the albumin molecule. Since the drug

Table 1
Thermodynamic parameters of the binding of BET to BSA at 298.15 K.

Class of sites	N	$10^{-4}(K^\circ)$	ΔH° (kJ mol^{-1})	ΔG° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
First	24.0 ± 0.2	9.69 ± 0.31	−38.35 ± 0.50	−28.46 ± 0.40	−33.16 ± 0.50
Second	50.0 ± 0.3	9.48 ± 0.32	18.06 ± 0.03	−28.41 ± 0.04	155.84 ± 0.40

Table 2
Conformational changes of the secondary structure of BSA (1.00×10^{-6} mol/L) with and without BET at 298.15 K.

Number	[BET] (μM)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
1	0.0	46.2 ± 0.3	22.5 ± 0.2	4.8 ± 0.3	26.5 ± 0.2
2	49.0	45.8 ± 0.2	24.2 ± 0.3	4.2 ± 0.3	25.8 ± 0.2
3	59.0	44.6 ± 0.1	26.1 ± 0.3	3.8 ± 0.4	25.5 ± 0.2
4	68.0	43.4 ± 0.2	28.9 ± 0.1	3.1 ± 0.4	24.6 ± 0.3

(BET) molecule is quite long (Scheme 1) and amphiphilic, the binding force might be mainly caused by hydrophobic interaction when it associate to the site in such location as subdomain II [28], while that might be mainly caused by hydrophilic interaction if the ligand molecule is bound by a site at the surface of the protein molecule. The subtle differences between the equilibrium constants, changes in standard enthalpy and entropy of the two classes of binding process may be the result from synergy of hydrophobic and hydrophilic interactions as well as solvent effect, which is similar to the phenomena found in BSA–oxymatrine system. Probably, the evident difference between the two binding equilibrium constants of oxymatrine binding to BSA is mainly decided by the shorter length and less flexible hydrophobic part of oxymatrine molecule [11].

3.3. CD spectra of BSA–BET binding system

To further investigate whether any conformational changes of BSA molecule occurred in the binding process, circular dichroism (CD) experiment at room temperature and pH 7.40 was carried out. The CD spectrum of BSA exhibits two negative bands in the UV region at 208 and 220 nm, characteristic of α-helix structure units of protein [29]. Fig. 4 displays the CD spectra of BSA at 1.0 μM in the presence of different relative amounts of BET. The data in Table 2 show the variation of the contents of BSA with increase of BET concentration. It can be observed that there is a reduction in both of the bands, clearly indicating the decrease of the α-helix structures of protein. The loss of the α-helix indicates that BET molecules associate with the amino acid residues of the main polypeptide chain of protein and partly destroy their hydrogen bonding networks [30]. However, the CD spectra of BSA in the absence and presence of BET are quite similar in shape, which means that the structure of BSA after addition of BET is still predominantly α-helix. From the above analysis, we can conclude that the BSA–BET supramolecular complex can form in the aqueous buffer solution and the binding process induces some secondary structure change in the bio-macromolecule.

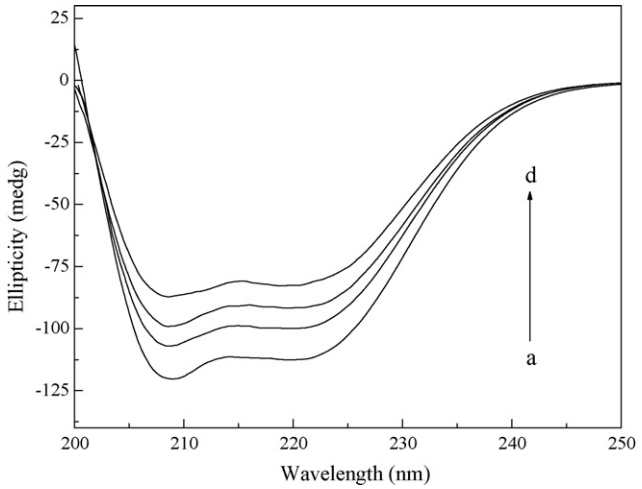

Fig. 4. CD spectra of BSA–BET system at 298.15 K, (a)–(d) the concentration of BSA was always 1.00 μM and that of BET ($\times 10^{-5}$ M) from (a) to (d): 0, 4.90, 5.90 and 6.80.

4. Conclusion

The interaction of BET with BSA was investigated by ITC combined with CD spectroscopy. The thermodynamic parameters, binding constants, changes of standard enthalpy, Gibbs free energy and entropy are obtained, and these data reveal that there are two classes of binding sites for the ligand molecules. The first class of binding is an enthalpy driven process while the second one is entropy driven one. The CD data indicate that the conformation of BSA molecule is slightly changed upon addition of BET. The calorimetric and spectroscopic results jointly show that supramolecular complex of BSA with BET can form in aqueous solution.

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