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Interaction of bovine serum albumin with two alkylimidazolium-based ionic liquids investigated by microcalorimetry and circular dichroism

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ABSTRACT

The interactions of bovine serum albumin (BSA) with two alkylimidazolium-based ionic liquids, 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF_4) and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6), in buffer solutions at pH 7.0 were investigated by isothermal titration calorimetry (ITC) and circular dichroism (CD). CD spectra showed that the two ionic liquids changed the secondary structure of BSA. Data process was based on the supposition that there were several independent types of binding sites on each BSA molecule for the two ligand molecules. The results obtained by using this supposition combined with Langmuir adsorption model showed that there were two types of such binding sites. One was the high affinity binding site, and the other was the low affinity binding site. The binding constants, changes in enthalpy, entropy and Gibbs free energy for the two types of binding were obtained, which showed that the two types of binding were driven by a favorable entropy increase. Furthermore, for either the ionic liquids, the number of the high affinity binding sites is much smaller than that of the low affinity ones. These results were interpreted with the molecular structure of BSA and the different substituent groups on imidazole ring of the two ionic liquid molecules.

Keywords: Isothermal Titration Calorimetry; Circular Dichroism Spectra; Alkylimidazolium-Based Ionic Liquids; Bovine Serum Albumin

1. INTRODUCTION

Protein pharmaceuticals are subjected to a number of stresses during production, storage, and shipping, resulting in loss of the protein concentration and activities or formation of soluble and insoluble aggregates. The general method for stabilizing liquid protein pharmaceuticals is the use of formulation excipients. Surfactants are indispensable as solubilizing agents in the isolation and purification of proteins. To use them correctly, it is necessary to have an idea of how and in which amounts they interact with proteins. Although surfactant-protein interactions have been widely studied for half a century [1-5], the mechanism of interaction is not well understood. Knowledge of the interactions is not only fundamental in theoretics, but also practical in industrial applications. In the cosmetic and food fields, protein function is largely influenced by an added surfactant [6].

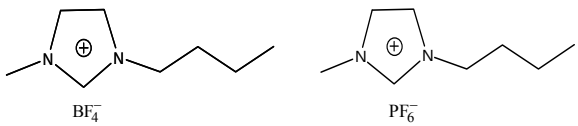
In the studies of surfactant-protein interactions, the serum albumin, e.g., human serum albumin (HSA) or bovine serum albumin (BSA), is commonly used as model protein due to its well-established primary structure, stability, water solubility and versatile binding capacity [7-9]. Ionic liquids (ILs) are a class of organic molten electrolytes at or near ambient temperature [10]. Their physical and chemical properties can be tailored by judicious selection of cation, anion, and substituent. They have no significant vapor pressures, outstanding catalytic properties, high ion-conductivity, non-flammability, and are relatively inexpensive to manufacture [11]. Thus ILs have attracted much attention as electrolytes and solvent media for reactions and extractions [12-14]. So study on binding of ionic liquid-type surfactants to bovine serum albumin would be very necessary to further understand the structural and functional information of surfactant-protein interactions. Among various ILs, the alkylimidazolium salts which belong to ionic liquid-type surfactants have been extensively studied in the field of colloid and interface science [15-17]. In this paper, 1-butyl-3-methyl imidazolium tetrafluoroborate

([bmim]BF_4) and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) (see Scheme 1) were selected as ligands to the protein. Isothermal titration calorimeter (ITC) was used to determine the thermodynamic parameters (enthalpy, binding site number and binding constant, etc) of the interactions of the both alkylimidazolium-based ionic liquids, [bmim]BF_4 and [bmim]PF_6, with BSA at the temperature of 298.15 K. CD spectroscopy was also employed to determine the dependence of α-helical content in the protein molecules on ionic liquid concentration.

2. MATERIALS AND METHODS

2.1. Materials

Bovine serum albumin was purchased from Acros, which was used without further purification. The concentration of BSA was determined by using the extinction coefficient at 280 nm of 44720 $M^{-1}cm^{-1}$ at pH 7.0 [18]. 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF_4) and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) were both obtained from Aldrich (mass fraction > 99%). All solutions were prepared with thrice distilled water in calorimetric experiment. Tris (hydroxymethyl) aminomethane (Tris-HCl) used in the preparation of the buffer was of analytical grade.



(a) (b)

Scheme 1. Molecular structure of two ionic liquids (a) 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF_4); (b) 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6).

2.2. Circular Dichroism (CD) Measurements

CD measurements of BSA in tris-HCl buffer solutions of pH 7.0 were performed using a Jasco J-810 spectropolarimeter (Japan) at the temperature 298.2 K. The light source system was protected by nitrogen (flow rate: 5 L min^{-1}). The spectra of protein solutions (2 μM) were determined in 1 mm cells. The wavelength region of scanning was 190 - 250 nm. The solutions were scanned at 100 nm min^{-1} using a 1 s time constant with step resolution of 0.1 nm. The average of three scans was recorded.

2.3. Microcalorimetric Measurements

Titration microcalorimetry was performed on a nanowatt-scale isothermal titration micorcalorimeter supported by Thermal Activity Monitor TAM 2277 (Thermometric, Sweden), which was controlled by Digitam 4.1 software. This instrument has an electrical calibration with a precision better than ±1% that can be determined by measuring the dilution enthalpy of a concentrated sucrose solution [19]. Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. For the measurement of the protein (BSA)—ligand ([bmim]BF_4 or [bmim]PF_6) solutions, the 1 mL sample cell of the calorimeter made from stainless steel were initially loaded with 800 μL BSA solution whose concentration was 100 μM. 30.00 mM [bmim]BF_4 or [bmim]PF_6 solution was injected into the stirred sample cell in 30 portions of 12 μL using a 500 μL Hamilton syringe controlled by a Thermometric 612 Lund Pump. The interval between two injections was 35 min., which was sufficiently long for the signal to return to the baseline. The system was stirred at 30 rpm with a gold propeller. All experiments were performed at a fixed temperature of (298.15 ± 0.01) K and repeated thrice. To deduct the dilution heats of ionic liquid and BSA solutions, we performed titration experiments of ionic liquid solution into buffer solution and buffer solution into BSA solution, respectively.

3. RESULTS AND DISCUSSION

3.1. Circular Dichroism Studies

Figure 1 shows the CD spectra of BSA under the coexistence of one of the two ionic liquids at different concentrations. [bmim]BF_4 and [bmim]PF_6 do not present any CD signal in the spectral range 190 - 250 nm. This indicates that the observed CD signal is only produced by BSA. The CD spectrum of BSA exhibits two negative bands in the ultraviolet region at 208 ($\pi \rightarrow \pi^*$ transition) and 222 nm ($n \rightarrow \pi^*$ transition), which is characteristic of the α-helical structure of a protein [20]. It can be seen from **Figure 1** that the interaction of [bmim]BF_4 or [bmim]PF_6 with BSA caused a slight decrease in band intensity at all wavelengths of the far UV CD without any significant shift in the peak position. This clearly indicates the minor changes in the protein secondary structure, namely the decrease in the α-helical content in protein. This maybe caused by the interaction between the ionic liquid and BSA which leads to a swelling of the biomacromolecule and exposing of the hydrophobic residues [21]. Thus some of the original α-helices are broken to give a more open disordered structure. However, it can be seen from **Figure 1** that the change of secondary structure is quite small in

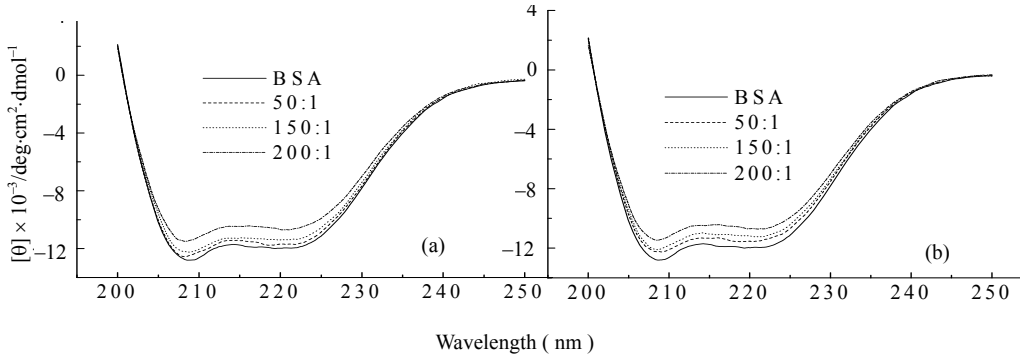


Figure 1. Circular dichroism spectra of BSA in Tris-HCl buffer (10 mM) at pH 7.0 as a function of ionic liquid concentration: (a) BSA + [bmim]BF_4; (b) BSA + [bmim]PF_6. (The ratio in this figure is the analytic concentration ratio of ionic liquid to BSA).

the molar ratio range of titration experiment (ionic-BSA: 135 : 1). So we can conclude that the heat effect caused by conformational change of BSA can be neglected. That is, the measured enthalpy changes are mainly caused by the binding of the ionic liquid molecules to BSA.

3.2. Thermodynamic Data Analysis

3.2.1. Titration Calorimetric Model for the Binding of Ionic Liquid to BSA

A multiple-site model has been proposed for the binding of ligand molecules to protein, which can be regarded as adsorption of the ligand particles on the sites belonging to different classes and abiding Langmuir isotherm [4,22-23]. For the *i*-th class of binding sites, there are:

$$\theta_i = K_i c_L / (1 + K_i c_L) \quad (1)$$

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

where θ_i is the degree of occupancy of the *i*-th types of sites. $c_{L,0}$ and $c_{P,0}$ indicate total concentration of ligand and protein, respectively. c_L is concentration of free ligand molecule at equilibrium state and *m* is the number of binding classes.

The net heat of interaction between the ligand molecule and the biomacromolecule evolved from the *j*-th injection in an ITC experiment can be expressed

$$Q_j = c_{P,0} V_{P,0} \sum_{i=1}^{m} N_i \Delta\theta_i \Delta H_i^{o} \quad (3)$$

where $V_{P,0}$ is the volume of the protein solution as titrand in the calorimeter cell and $\Delta\theta_i$ is the change in occupancy from the $(j-1)$th injection to the *j*th one.

Eq.1 and Eq.2 indicate that c_L is the function of N_i and K_i when $c_{L,0}$ and $c_{P,0}$ are known. So there are 3 *m* unknown parameters in (3), which are K_i, ΔH_i^{o} and N_i. These parameters for single-class ($m = 1$), two-class ($m = 2$) and three-class ($m = 3$) binding model were computed from the actual calorimetric data with an iterative non-linear least-square regression program for minimizing the value of $\Sigma(Q_{exp} - Q_{calc})^2$ by using of software MATLAB 7.01. The coincidence degree between calculated curve and experimental integrate heat indicates that existence of two types of binding sites is most reasonable when either [bmim]BF_4 or [bmim]PF_6 binds to BSA. The nonlinear fitting curves of integrated heat versus the mixed ligand/BSA molar ratio are shown in **Figure 2**. According to the thermodynamic formula:

$$\Delta G^{o} = -RT \ln K^{o} \quad \Delta G^{o} = \Delta H^{o} - T\Delta S^{o} \quad (4)$$

Standard changes of Gibbs free energy (ΔG^{o}) and entropy effect ($T\Delta S^{o}$) for the binding process of ionic liquid to BSA can be derived. The thermodynamic results for the two ligand molecules + BSA complexes are listed in **Table 1**.

3.2.2. Binding Site Number and Binding Constants

Nonlinear fitting curves of integrated heat versus the mixed ionic liquid/BSA molar ratio are shown in Figure **2**. It can be seen that the binding of the two ionic liquids to BSA is exothermic in the range of selected ionic liquid concentration. Crystal structure analyses have revealed that the binding sites on BSA molecules are located in subdomains ⅡA and ⅢA. A large hydrophobic cavity is present in the ⅡA subdomain [24]. Moreover, due to the presence of amino acid residues on the surface of HSA, most authors agree on the existence of a limited number of binding sites on the surface of protein molecules [25]. The optimum simulated results show that there are two types of binding sites on BSA molecules for the binding of the two ionic liquids to BSA: 1) binding to ionic sites on BSA surface driven by hydrogen bonding interaction of anions of [bmim]BF_4 or

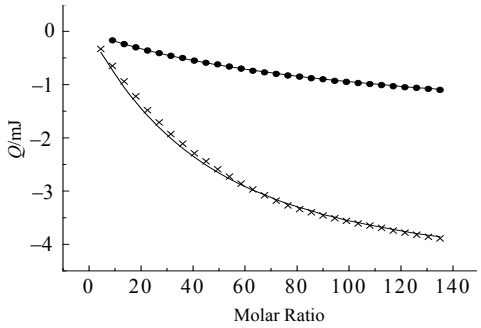


Figure 2. Non-linear fitting curve of the binding heat versus the molar ratio of the two ionic liquid to BSA, where points are gotten from experiments and the solid line is the result of simulation, ×: [bmim]BF_4; •: [bmim]PF_6.

[bmim]PF_6 with amino acid residues on BSA surface, which corresponds to the high affinity sites; 2) hydrophobic interaction of imidazole ring and the hydrophobic chain on either of the two ionic liquids with the hydrophobic cavities of BSA molecules, which corresponds to the low affinity sites. The work of Swati De *et al.* indicates that high affinity sites corresponding to the ionic sites on protein surface, and low affinity sites corresponding to the hydrophobic cavities of protein molecules [26]. From the data in **Table 1**, we can reach two conclusions, which are: when the same ionic liquid binds to BSA, the high affinity site number (N_1) is smaller than the low affinity site number (N_2), while the binding constant for the high affinity sites is evidently larger than that for the low affinity sites. This difference may be explained as follows. The isoelectric point of serum album is at pH 4.70 [27] and BSA molecule is negatively charged at pH 7.0. The actually ligand particles are $[bmim]^+$ cations, for which the mainly binding force to the first type of sites is electrostatic interaction. This can also explain the fact that the two values of the equilibrium constant K_1 respectively for [bmim]BF_4 and [bmim]PF_6 are almost the same. As to the difference between the two values of N_1 respectively corresponding to [bmim]BF_4 and [bmim]PF_6 might be interpreted from the structures of the two anions, BF_4^- and PF_6^-. The size of the former is smaller than the later, and so there is more opportunity for the former to approach the biomacromolecule, which obstructs the ligand cation i.e. decrease the binding site. The relative differences between values of K_2, N_2, ΔH_2^o, $T\Delta S_2^o$ respectively corresponding to [bmim]BF_4 are all not evident. This phenomenon indicate that the actually ligand is also the cation in the second type of binding process driven by hydrophobic interaction.

3.2.3. Enthalpy and Entropy Effects

The standard enthalpy effects, standard entropy effects and changes in standard Gibbs free energies for the binding of [bmim]BF_4 and [bmim]PF_6 to BSA are shown in **Figure 3**. It is necessary to know the main existing form of the two ionic liquids in sample cell in order to interpret the above experimental results. The critical micelle concentrations (CMC) of BF_4 and [bmim]PF_6 are about 0.8 M [28] and 0.031 M [29] respectively, which exceed the initial concentration (30.00 mM) of the two ionic liquids in the syringe. Therefore, the two ionic liquids in the sample cell are both existent as monomers instead of micelles. In other words, there is no demicellization of the ionic-liquid-type surfactant in the experimental concentration ranges. Furthermore, as described above, the heat effect caused by conformational change of BSA can be neglected. It can be seen from **Figure 3** that the standard enthalpies of formation for the binding of the two ionic liquids to the both types of binding sites of BSA are negative, which indicates that the binding process are both exothermic. The reasons for the negative values of ΔH_1^o are manifold. Firstly, the electrostatic attraction of the cation, $[bmim]^+$, with the negatively charged protein molecule is an exothermic process. Secondly, the repulsion force between the anions of the ionic liquid and the negatively charged BSA surface makes positive contribution to ΔH_1^o. Thirdly, destroy of iceberg structure surrounding hydrophobic chains of the ionic liquid is an endothermic process. The negative values of ΔH_1^o indicate that the hydrogen bonding interaction is predominant over the electrostatic repulsion interaction and the destroy effects of iceberg structure. As for ΔH_2^o, there are also several types of weak interactions simultaneously contribute to its negative value, including the expulsion of high energy water molecule from hydrophobic cavity of the protein molecule to bulk solution (exothermic process), destroy of iceberg structure surrounding hydrophobic groups of the ionic liquid

Table 1. Thermodynamic parameters for the binding of [bmim]BF_4 and [bmim]PF_6 to BSA at 298.15 K[a].

Parameter	ionic liquid	
	[bmim]BF_4	[bmim]PF_6
High affinity sites		
N_1	1.0 ± 0.2	3.1 ± 0.1
$K_1/(M^{-1})$	(1.12 ± 0.04) × 10^3	(1.48 ± 0.07) × 10^3
$\Delta H_1°/(kJ\cdot mol^{-1})$	−2.14 ± 0.08	−2.46 ± 0.09
$\Delta G_1°/kJ\cdot mol^{-1})$	−17.40 ± 0.19	−18.09 ± 0.28
$T\Delta S_1°/(kJ\cdot mol^{-1})$	15.26 ± 0.27	15.63 ± 0.37
Low affinity sites		
N_2	25.0 ± 0.9	27.0 ± 1.0
$K_2/(M^{-1})$	65.20 ± 2.60	98.50 ± 3.50
$\Delta H_2°/(kJ\cdot mol^{-1})$	−1.38 ± 0.05	−1.52 ± 0.06
$\Delta G_2°/(kJ\cdot mol^{-1})$	−10.36 ± 0.12	−11.38 ± 0.18
$T\Delta S_2°/(kJ\cdot mol^{-1})$	8.98 ± 0.17	9.86 ± 0.24

[a]Data are expressed as mean ± S. D. (N = 3).

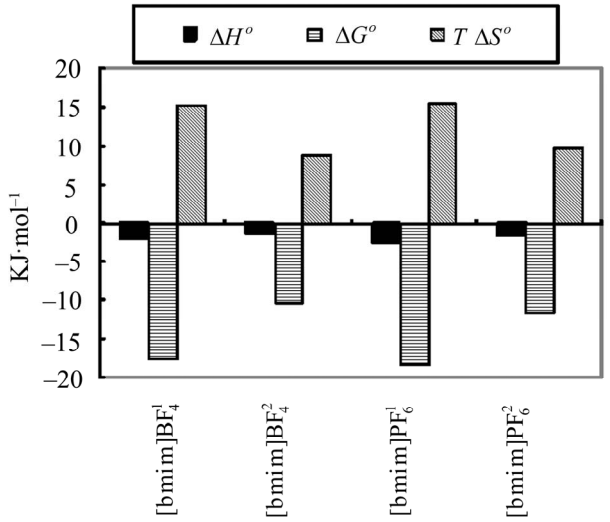


Figure 3. Proportion of the thermodynamic parameters for the binding of bmim]BF_4 and [bmim]PF_6 to BSA (Superscript 1 and 2 represent high affinity sites and low affinity sites, respectively.).

(endothermic process), and the hydrophobic interaction of imidazole ring and the hydrophobic chain on it with the hydrophobic cavities of BSA molecules (exothermic process) [30]. Among them, the hydrophobic interaction should be the main driving force, evidenced by the experimental results that the difference between the ΔH_2^o for [bmim]BF_4 – BSA (–1.38 ± 0.05 kJ·mol^{-1}) and that for [bmim]PF_6 – BSA (–1.52 ± 0.06 kJ·mol^{-1}) is subtle because the two ionic liquids contain the same hydrophobic chain.

It can be seen from **Figure 3** that the entropy effects for the binding of [bmim]BF_4 and [bmim]PF_6 to BSA are all positive, which is beneficial for this interacting process. This may be due to the total result of the binding of the two ligand ions to BSA molecule (negative contribution to entropy) and the releasing of water molecules from cavity (positive contribution to entropy) as well as the disruption of hydration layer structure on the surface of BSA molecule (positive contribution to entropy). In addition, we can infer from **Figure 3** that the binding process is predominantly entropy driven.

4. CONCLUSIONS

ITC experiments demonstrate that there are two types of binding sites on BSA molecules for the Alkylimidazolium cation in the both ionic liquids, [bmim]BF_4 and [bmim]PF_6. One type of binding with high affinity binding is caused electrostatic interaction of the cation with negatively charged sites on BSA molecules, and the other one is low affinity binding due to the hydrophobic interaction of imidazole ring and the hydrophobic chain on it of the both ionic liquids with the hydrophobic cavities of the protein molecules. The thermodynamic results obtained from the calorimetric data with an iterative non-linear least-square regression program show that when the same ionic liquid cations bind to BSA molecules, the number of high affinity sites (N_1) is smaller than that of the low affinity sites (N_2). On the contrary, the binding constant for the high affinity sites (K_1) is evidently larger than that for the low affinity sites (K_2). The entropy effects for the two binding sites are both negative while the entropy effects for the two binding sites are both positive. Circular dichroism (CD) spectra show that the two ionic liquids change the secondary structure of BSA. These results can be understood by considering several weak interactions of the biomacromolecule with the cation, the anion as well as the solvent effect of water on the protein-ligand interaction system. The data and message obtained in this study may be important for understanding the influence of protein-surfactant interactions on the functionality of globular proteins in the biochemical systems.

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