Binding of Halide Ions to Bovine Serum Albumin and Hemoglobin: Studied with Ion-Selective Electrodes

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The interactions of F^-, Br^- and I^- with bovine serum albumin (BSA) and with hemoglobin (Hb) were studied in acetate buffers of pH 5.68, at different temperatures, by using ion-selective electrodes. The data for the anion-protein systems were treated according to Klotz equation, and the number of binding sites and the binding constants were determined. It is shown that the binding sites of F^- on BSA and on Hb molecules are more than those of Br^- and I^-, and that the number of the binding sites for F^-, Br^- and I^- on BSA and on Hb molecules increases with increasing temperature. This study also indicates that the binding constants for the interactions of F^-, Br^- and I^- with BSA and with Hb gradually decrease as the size of halide ions increases. In addition, as temperature increases, the binding constants gradually decrease. These were reasonably interpreted with the structural and thermodynamic viewpoints. The thermodynamic studies indicate that the interactions of halide ions with proteins are mainly electrostatic interaction.

1. INTRODUCTION

It is very important that the interactions of small anions with protein in their transportation and distribution processes in biological systems, and it is helpful to study these interactions to understand the nature of the transportation and distribution processes. The capacity of a protein binding anions is mainly dependent on the chemical and structural properties of protein. In addition, the pH value of the solution, the ionic size, the ionic charge and the ionic concentration are also the influencing factors [1, 2]. Bovine serum albumin (BSA) and hemoglobin (Hb) have high affinities for various ligands. There have been relatively few studies on Hb [3], but more on BSA [4-9]. Although the amino acid

composition and sequence of BSA which are important in its conformational analysis are now known [10], the nature of the binding of anions to proteins is not yet well interpreted. Equilibrium dialysis and UV-visible spectrophotometry have been used to study the binding of fluoride ion to BSA and obtained significant binding [11-15]. Peters [16] and Carter [17] have reviewed the studies on the binding of various ligands to serum albumin, respectively, expounding the binding sites of small organic molecules, fatty acid compounds with long side chain and metal ions on serum albumin molecule.

Ion-selective electrodes are useful tools to study the binding of ions to protein in aqueous and non-aqueous solutions, which can obtain accurate data for the binding of ions at low concentrations. Luehrs and Johnson studied the binding of F^- to ovalbumin (OVA) by using ion-selective electrode and obtained the thermodynamic binding constant [18]. Sideris et al. studied the interaction of diflunisal ion and cyclodextrins with ion-selective electrode and discussed the cooperative binding between them [19]. Ayranci and Duman studied the binding of F^-, Br^- and I^- to BSA with ion-selective electrodes. They calculated the binding sites and stepwise constants and interpreted the influence of charge on the binding sites [20,21]. Investigating the effect of temperature on the interactions between ions and proteins is also very important, which can help people to understand the nature of the interactions. However, few studies in this aspect have been reported. The objective of this work is to investigate the binding of F^-, Br^- and I^- to BSA and Hb by using ion-selective electrodes. The emphasis is to study the effects of temperature on the interactions of halide ions with proteins, and discuss the nature of the interactions with thermodynamic and structural viewpoints according to hofmaister series [22].

2. MATERIALS AND METHODS

2.1 Materials and Reagents

BSA and Hb (from bovine blood) were purchased from Sigma Chemical Co. without further purified before use. The standard regents of NaF, KBr and KI were from the First Chemical Co. of Shanghai, and $NaNO_3$ was from the Hongxing Chemical Co of Beijing. All of the chemical reagents were kept over P_2O_5 in a desiccator prior to use, and weighed on an electronic balance (Sartorius, Germany) with a sensitivity of 10^{-5} g. All solutions were prepared in acetate buffers of pH 5.68 and ionic strength 0.10 M. Doubly distilled water was used throughout.

A model of 201 fluoride ion-selective electrode, 302 Bromide ion-selective electrode and 303 iodide ion-selective electrode, which were purchased from Jiangsu Jiangfen Electroanalytical Instrument Co. LTD, were used for the studies of F^-, Br^- and I^-, respectively. A model of 802 single-junction calomel electrode and a model of 217 double-junction calomel electrode were used as the reference electrodes for F^- and for Br^- and I^- studies. The two reference electrodes were purchased from Shanghai Ruosull Technology Co. LTD. The measurements of the potentials were made by using a PXSJ-216 Ion Analyzer which was from Shanghai Leici Instrument Co., with a readability of ± 0.1 mV.

Titrations for binding experiments were carried out with a Proline Mechanical Single-Channel Pipettors (Shanghai, China). All measurements were carried out in a 50 ml double-walled glass cell which was immersed in a model of DC-2006 Circulating Thermostat (Shanghai, China) with the precision of ± 0.01 K. The sample solution was continuously stirred using a magnetic stirrer.

2.2 Construction of Calibration Equations of the Ion-Selective Electrodes

The pair of electrodes was immersed in 20 ml NaF solutions of different concentrations. The potential values (mV) were recorded and measured after stabilization (± 0.1 mV). The potential values were plotted against logarithm of F^- concentration according to the Nernst equation, and the calibration curve was determined by least-squares fitting of the Nernst equation to the experimental data. The same procedure was followed for the construction of calibration equations of Br^- and I^- selective electrodes.

2.3 Studies on the Binding of Halide Ions to BSA and Hb by the Ion-Selective Electrode

The pair of electrodes were immersed in 25 ml 2×10^{-4} mol·L^{-1} protein solution. A 6×10^{-3} mol·L^{-1} standard NaF solution was used as a titrant. After the potential was stabilized, small volumes (about 2 ml) of the titrant were added. After each addition, the potential values were recorded by waiting sufficient time for equilibration. The potential values were converted into concentrations of free F^- by use of the previously obtained calibration equation of F^-. The total amount of bound F^- was calculated as the difference between the amount of total F^- added and the amount of free F^- measured at equilibrium. Then the average number of bound anions of each protein molecules, *r*, can be obtained with the total amount of bound F^- and the concentration of protein. The same procedure was followed for the binding experiments of the other two ions, Br^- and I^-.

3. RESULTS AND DISCUSSION

*3.1 Binding Sites of Halide Ions on Protein Molecules*

Klotz has given the relation of the average number of bound anions of each protein molecules (*r*) and the concentration of free anion in equilibrium ([A]) as follows [8]

$$\frac{1}{r} = \left(\frac{1}{nk}\right)\frac{1}{[A]} + \frac{1}{n} \qquad (1)$$

where *n* is the number of binding sites and *k* is the binding constant. We plotted $1/r$ against $1/[A]$ for the systems studied in this paper and represented these plots in Figs. 1-6.
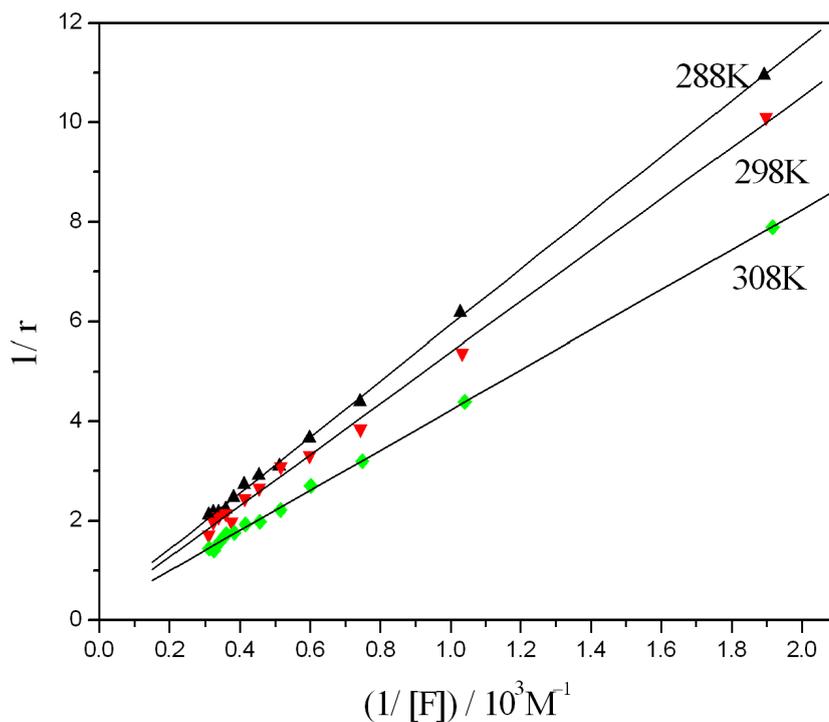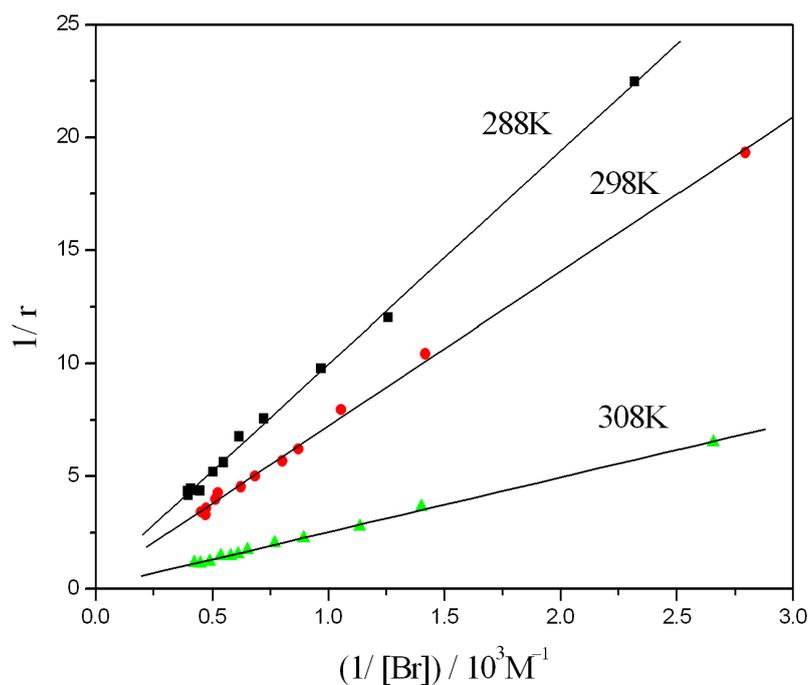
Figure 1. The Klotz plot for the binding of F$^-$ to BSA at different temperature in acetate buffers of pH 5.68
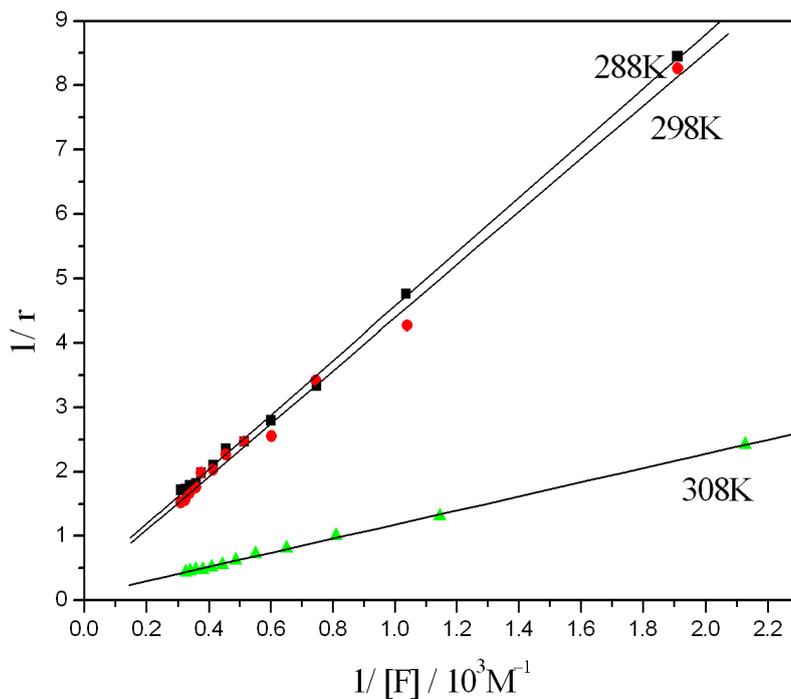
Figure 2. The Klotz plot for the binding of F$^-$ to Hb at different temperature in acetate buffers of pH 5.68
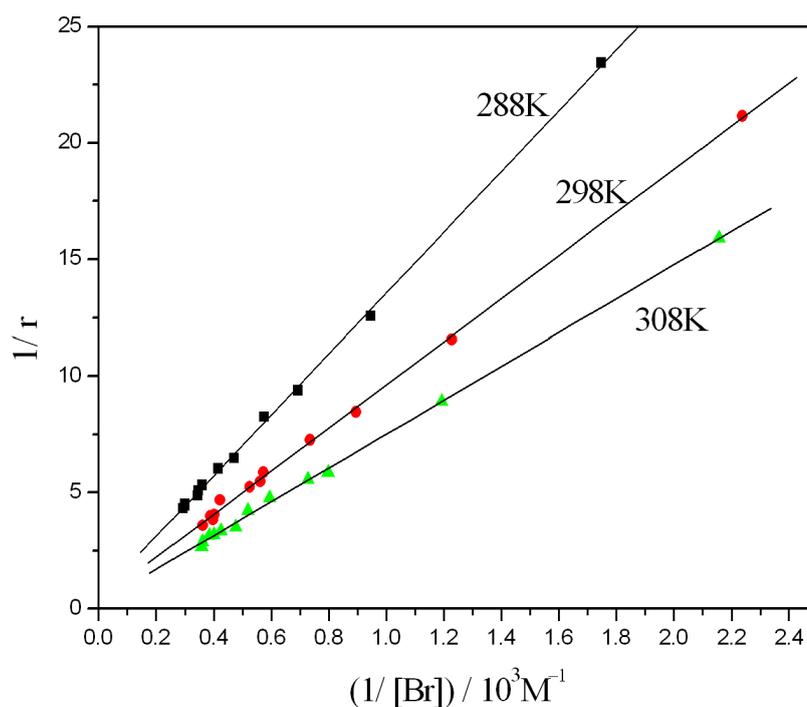
Figure 3. The Klotz plot for the binding of Br⁻ to BSA at different temperature in acetate buffers of pH 5.68
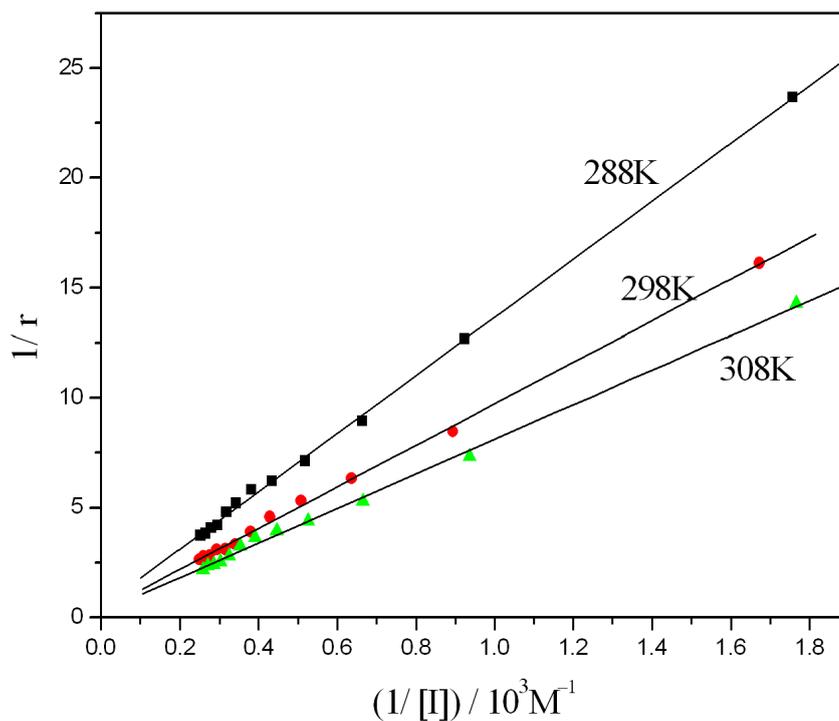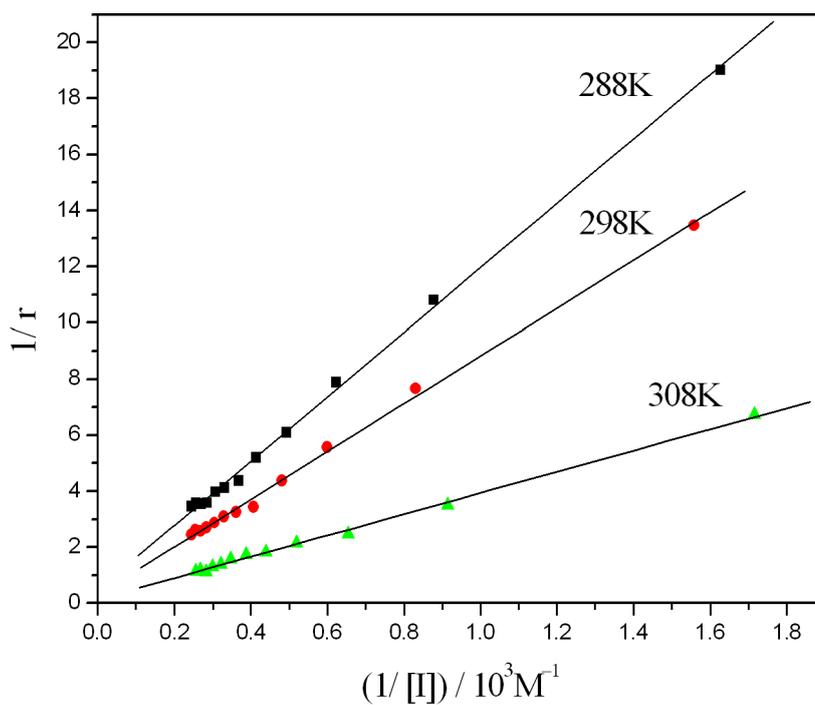

Figure 4. The Klotz plot for the binding of Br⁻ to Hb at different temperature in acetate buffers of pH 5.68

Figure 5. The Klotz plot for the binding of Γ to BSA at different temperature in acetate buffers of pH 5.68

Figure 6. The Klotz plot for the binding of Γ to Hb at different temperature in acetate buffers of pH 5.68

There are obvious linear relations in these figures, which indicate that Eq. (1) is in good agreement with the binding model of the interactions between halide ions and proteins. Fitting the data of $1/r$ and $1/[A]$ according to Eq. (1) by using a least-square method, we obtained the regressing equations and the binding parameters (n and k) which are presented in Tables 1-3.

Table 1. Regression equations, binding parameters and the corresponding correlation coefficients for F^- and proteins systems measured by ion-selective electrode method

T/K	Regression equations	n	k	R^a
BSA				
288.15	$1/r = 0.313 + 5.622 \times 10^{-3}(1/[F^-])$	3	59.2	0.9997
298.15	$1/r = 0.246 + 5.137 \times 10^{-3} (1/[F^-])$	4	48.8	0.9982
308.15	$1/r = 0.197 + 4.026 \times 10^{-3} (1/[F^-])$	5	49.8	0.9995
Hb				
288.15	$1/r = 0.342 + 4.226 \times 10^{-3} (1/[F^-])$	3	78.7	0.9993
298.15	$1/r = 0.279 + 4.111 \times 10^{-3} (1/[F^-])$	4	61.0	0.9975
308.15	$1/r = 0.0822 + 1.098 \times 10^{-3} (1/[F^-])$	12	75.8	0.9993

[a] The correlation coefficient.

Table 2. Regression equations, binding parameters and the corresponding correlation coefficients for Br^- and proteins systems measured by ion-selective electrode method

T/K	Regression equations	n	k	R^a
BSA				
288.15	$1/r = 0.513 + 13.039 \times 10^{-3}(1/[Br^-])$	2	38.2	0.9996
298.15	$1/r = 0.368 + 9.244 \times 10^{-3} (1/[Br^-])$	3	36.0	0.9993
308.15	$1/r = 0.255 + 7.249 \times 10^{-3} (1/[Br^-])$	4	34.5	0.9993
Hb				
288.15	$1/r = 0.505 + 9.447 \times 10^{-3} (1/[Br^-])$	2	52.8	0.9991
298.15	$1/r = 0.371 + 6.847 \times 10^{-3} (1/[Br^-])$	3	49.0	0.9988
308.15	$1/r = 0.097 + 2.427 \times 10^{-3} (1/[Br^-])$	9	46.5	0.9989

[a] The correlation coefficient.

Table 3. Regression equations, binding parameters and the corresponding correlation coefficients for I^- and proteins systems measured by ion-selective electrode method

T/K	Regression equations	n	k	R^a
BSA				
288.15	$1/r = 0.475 + 13.181 \times 10^{-3}(1/[I^-])$	2	37.9	0.9995
298.15	$1/r = 0.301 + 9.433 \times 10^{-3} (1/[I^-])$	3	35.2	0.9993
308.15	$1/r = 0.258 + 7.863 \times 10^{-3} (1/[I^-])$	4	31.6	0.9986
Hb				
288.15	$1/r = 0.469 + 11.492 \times 10^{-3} (1/[I^-])$	2	43.7	0.9992
298.15	$1/r = 0.303 + 8.517 \times 10^{-3} (1/[I^-])$	3	39.5	0.9988
308.15	$1/r = 0.146 + 3.787 \times 10^{-3} (1/[I^-])$	7	37.3	0.9984

[a] The correlation coefficient

It should be pointed out that the values of n calculated from Eq. (1), are not generally integer. However, they are rounded to whole numbers in Tables 1-3, because the number of bound anion can not be fractional. Two factors are responsible for this difference. One is the deviations of the theoretical model and the reality, and the other is the experimental errors.

From Tables 1-3, we can see that the numbers of binding sites for Br^- and I^- on BSA and Hb molecules are, in all case, almost the same value, while that of F^- are greater. Since these ions all have single negative charge, their difference is only in the ionic size. F^- has the least size and the largest charge density among these ions, so its ability of biding with the weaker polar groups on protein is stronger than that of Br^- and I^-. For the systems studied here, it is found that the number of the binding sites for F^-, Br^- and I^- increases with increasing temperature. The secondary structure for the two proteins should be responsible for this kind of behavior. It has been reported that there is 65% α-helix content for BSA, and 70% for Hb [16]. Ikedo et al. have indicated that the α-helix content of BSA was gradually lowered with increasing temperature [23]. The decrease of α-helix content will make the peptidic chains unfolded, so that the latent binding sites in the α-helix structure are accessible by halide ions. Therefore, the number of the binding sites will be greater with increasing temperature. Additionally, it is found that the numbers of the binding sites for F^-, Br^- and I^- on Hb molecules are much lager than that of BSA molecules at 308.15 K. This indicates that the secondary structure of Hb is more unstable than that of BSA at higher temperature. It is equivalent to say that the decrease of α-helix content is quicker and the unfolding of the peptidic chains is greater with increasing temperature. As the temperature increases, however, the thermal motion of molecules and ions will be more rapid, which will make that the binding of halide ions to proteins is more difficulty. Although there is the unfavorable influence on the interactions between ions and proteins, the numbers of binding sites still increase with increasing temperature. This indicates that the change of secondary structure for the proteins is the main effect on the number of binding sites of halide ions to proteins as temperature increases.

3.2 Intrinsic Binding Constant

The intrinsic binding constants of the interactions of F^-, Br^- and I^- with BSA and Hb at 288.15 K, 298.15 K and 308.15 K were presented in Tables 1-3. It can be summed up in two rules: (a) the binding constants for the interactions of halide ions with the two proteins gradually decrease as the size of halide ions increases, (b) the binding constants gradually decrease with increasing temperature (except the systems containing F^-). It is known that the binding of halide ions to proteins is mainly with the electrostatic interaction as described above. Protein molecules and halide ions are highly hydrated, and the hydration structures will be partially destroyed by the electrostatic interactions between them. It is the incompletely hydrated halide ions that can bind with the polar groups on protein molecules. As the size of ions increases, the charge density will decrease, and the electrostatic interactions of ions with the polar groups on protein molecules will also be relatively weak, which leads to the binding constants decreasing. At the same time, the thermal motion of halide ions and protein molecules will be more rapid with increasing temperature, which will make the stability of the

complex of anion-protein lowered. Therefore, the binding constants will be decreased as temperature increase. This is in accord with what Chengnong Yan and co-workers have reported [24].

It is worth mentioning that the binding constants of the interactions of F⁻ with BSA and with Hb first decrease and then increase when the temperature increases from 298.15 K to 308.15 K, which is not consistent with the above rules. The major cause of the behavior is its own characteristics of F⁻. In fact, the ionic hydration structure includes two layers. The first layer which is close to the ions has the compact structure, is also called chemical hydration layer. The second layer next to the first has the unfirm structure, which is also called physical hydration layer. The size of F⁻ is the least among the three halide ions, so its charge density is the largest, and its chemical hydration structure is the thickest. When the temperature is lower, the chemical hydration layer of the ions is difficult to be destroyed completely by the interactions of ions with proteins. However, as the temperature increases gradually, the thermal motion of ions and molecules not only result in the instability of the binding of ions to proteins, but also the instability of the ionic hydration structure. The latter effect will make the water molecules depart from the ions easily. Therefore, this effect will impel the binding of ions to proteins stronger and the binding constants increase. The complexity of the change of the binding constants for the interaction of F⁻ with the two proteins is caused by the competition of the above two effects.

3.3 Thermodynamic Studies

Since the interactions between anions and proteins are rather complicated, the nature of the binding of anions is not yet clearly known [21]. According to the study of the interactions of ions with amino acids [25], the interactions of ions and small molecules with proteins can also be considered to be composed of three effects: (a) electrostatic interaction, (b) partial desolvation of solutes, (c) solvent reorganization effect. The electrostatic interaction is mainly occurring between ions and the polar groups of proteins. This kind of interaction is exothermic and will give negative contribution to the enthalpic function (ΔH^θ). Since protein molecules and anions are hydrated in aqueous solutions, the binding of anions to proteins should be accompanied with the partial desolvation of solutes caused by the cosphere overlap of solvation layer of solutes. The partial desolvation of solutes is endothermic, and will give positive contribution to ΔH^θ. The binding of anions to proteins will, of course, give negative contribution to entropic function (ΔS^θ). However, the partial desolvation of solutes accompanied with the binding, which makes the structure of water molecules of solvation layer destructive to a certain extent, also changes the structural water molecules into free water molecules. Therefore, the partial desolvation effect will give positive contribution to ΔS^θ. As for the contribution of the solvent reorganization effect to ΔH^θ and ΔS^θ, it should be quite complex for different systems. In general, the solvent reorganization effect gives positive contribution if it destroys the solvent structure. On the contrary, it will give negative contribution if it enhances the solvent structure. The values of ΔH^θ and ΔS^θ for the whole process should be mainly determined by the relative contribution of the three effects.

The intrinsic binding constants for the interactions of F^-, Br^- and I^- with BSA and with Hb at different temperatures were obtained as presented in Tables 1-3. With the thermodynamic relation

$$\Delta G^{\theta} = -RT\ln k \qquad (2)$$

Gibbs free energy (ΔG^{θ}) can be calculated. ΔH^{θ} may be regarded as a constant when the temperature changes little, and can be determined by Van't Hoff equation

$$\Delta H^{\theta} = \frac{-R(d \ln k)}{d(1/T)} \qquad (3)$$

ΔS^{θ} of the binding will be given as the following relation

$$\Delta S^{\theta} = \frac{1}{T}(\Delta H^{\theta} - \Delta G^{\theta}) \qquad (4)$$

The thermodynamic functions for the interactions of F^-, Br^- and I^- with BSA and with Hb at 288.15 K, 298.15 K and 308.15 K, which were calculated with Eqs. (2)-(4), are presented in Tables 4-6.

Table 4. The thermodynamic functions for the interactions of F^- with BSA and Hb in acetate buffers of pH 5.68 measured by ion-selective electrode method at 288.15 *K*, 298.15 *K* and 308.15 *K*

T/*K*		288.15	298.15	308.15
	ΔG^{θ} /(kJ·mol^{-1})	-9.77	-9.63	-10.00
BSA	ΔH^{θ} /(kJ·mol^{-1})	-6.20	-6.20	-6.20
	ΔS^{θ} /(J·mol^{-1}·K^{-1})	12.40	11.51	12.34
	ΔG^{θ} /(kJ·mol^{-1})	-10.45	-10.18	-11.08
Hb	ΔH^{θ} /(kJ·mol^{-1})	-0.99	-0.99	-0.99
	ΔS^{θ}/(J·mol^{-1}·K^{-1})	32.85	30.84	32.76

Table 5. The thermodynamic functions for the interactions of Br^- with BSA and Hb in acetate buffers of pH 5.68 measured by ion-selective electrode method at 288.15 *K*, 298.15 *K* and 308.15 *K*

T/*K*		288.15	298.15	308.15
	ΔG^{θ} /(kJ·mol^{-1})	-8.72	-8.88	-9.07
BSA	ΔH^{θ} /(kJ·mol^{-1})	-3.75	-3.75	-3.75
	ΔS^{θ} /(J·mol^{-1}·K^{-1})	17.26	17.21	17.27
	ΔG^{θ} /(kJ·mol^{-1})	-9.50	-9.64	-9.83
Hb	ΔH^{θ} /(kJ·mol^{-1})	-4.67	-4.67	-4.67
	ΔS^{θ} /(J·mol^{-1}·K^{-1})	16.77	16.68	16.75

Tables 4-6 show that the values of ΔG^{θ} for the interactions of F^-, Br^- and I^- with the two proteins are negative at the three temperatures, which shows that this kind of binding is spontaneous. The negative value of ΔG^{θ} for the binding of F^- to the two proteins is largest, while for the binding of Br^-

and I^-, the value of ΔG^θ is smaller. This is corresponded to the values of binding constants. In addition, the values of ΔH^θ presented in Tables 4-6 are very small negative, even close to zero. This shows that the electrostatic interaction between ion and protein should be accompanied with strong partial desolvation of them.

Table 6. The thermodynamic functions for the interactions of I^- with BSA and Hb in acetate buffers of pH 5.68 measured by ion-selective electrode method at 288.15 K, 298.15 K and 308.15 K

T/K	288.15	298.15	308.15
ΔG^θ /(kJ·mol^{-1})	-8.70	-8.21	-8.84
BSA ΔH^θ /(kJ·mol^{-1})	-6.07	-6.07	-6.07
ΔS^θ /(J·mol^{-1}·K^{-1})	9.13	9.23	8.99
ΔG^θ /(kJ·mol^{-1})	-9.04	-9.11	-9.27
Hb ΔH^θ /(kJ·mol^{-1})	-5.80	-5.80	-5.80
ΔS^θ /(J·mol^{-1}·K^{-1})	11.25	11.11	11.27

The endothermic effect of the partial desolvation of solutes will compensate for the exothermic effect of electrostatic interaction to a great degree. The values of ΔS^θ listed in Tables 4-6 are all positive, which indicates that the entropic effect will be favorable for the binding of halide ions to proteins. In the interactions of halide ions with proteins, the electrostatic interaction will do negative contribution to ΔS^θ, while the partial desolvation accompanied will do positive contribution to ΔS^θ. When the latter effect is predominant, the values of ΔS^θ will be positive. As the temperature increases, the secondary structure of proteins will be unfolded. This will cause the number of binding sites for halide ions increase, and resulting in the increase in the values of ΔS^θ. However, as is shown in Tables 4-6, the change of the values of ΔS^θ is unnoticeable with increasing temperature. This is because that the hydration structure of solutes becomes more unstable as temperature increases, which causes ΔS^θ to be decreased. As a result, the decrease in ΔS^θ will compensate for the increase in ΔS^θ caused by the increase in the number of the binding sites. Therefore, the change of ΔS^θ with temperature increase is complex.

The smaller the ionic size, the larger the charge density. Therefore, the order of the electrostatic interaction for halide ions is $F^- > Br^- > I^-$. According to hofmaister series [22], the partial desolvation effect is in the order $F^- < Br^- < I^-$. The two orders are opposite and the two effects of electrostatic interaction and partial desolvation on the interaction enthalpy are also opposite. Therefore, the order of the interaction enthalpies of halide ions with the proteins studied are $F^- < Br^- < I^-$. The interaction entropy of halide ions with the proteins studied come mainly from the partial desolvation but not electrostatic interaction. Therefore, the order of the interaction entropy of halide ions with the proteins studied should also be $F^- < Br^- < I^-$. However, the data of interaction enthalpy and entropy in Tables 4-6 are not in the order mentioned above. This shows that the solvent reorganization has larger effect on the interaction enthalpy and entropy. If the solvent reorganization effect makes the structure of water

increasing or decreasing, the interaction enthalpy and entropy will accordingly decrease or increase. The data of ΔH^{θ} and ΔS^{θ} in Tables 4-6 are consistent with the above-mentioned analysis.

4. CONCLUSION

For the purpose of this study, the binding of F^-, Br^- and I^- to BSA and to Hb were investigated by use of ion-selective electrodes, at 288.15 K, 298.15 K and 308.15 K. The binding sites and the binding constants were calculated. The effect of the electrostatic interaction on the binding parameters was discussed with the density of ionic charge and the structure of BSA and Hb. Furthermore, the paper also discussed the variation trends for the binding parameters with increasing temperature due to the effects of temperature on the secondary structure of BSA and Hb and on the stability of complex of anion-protein. The thermodynamic functions of the binding were calculated according to thermodynamic relations. They show that the electrostatic interaction is the driven force in the interactions of F^-, Br^- and I^- with BSA and with Hb. On occasion, partial desolvation of solutes and solvent reorganization effect may play a role in determining the thermodynamic functions of the interactions. The results of the present study indicate that the method of ion-selective electrode can be successfully applied to investigate the interactions of some ions with proteins.

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