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Study on the Binding of Fluoride, Bromide and Iodide to Ovalbumin by Using Ion-Selective Electrodes

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The interactions of F^-, Br^- and I^- with ovalbumin (OVA) were studied in acetate buffers of pH 5.68, at 288.15 K, 298.15 K and 308.15 K, using ion-selective electrodes. The data for the ion-protein systems were treated according to the Klotz equation, and the number of binding sites and the binding constants were determined. It is shown that the binding sites of F^- on OVA molecule are more than those of Br^- and I^-, and that the binding sites of F^-, Br^- and I^- on OVA molecule decreases with increasing temperature. At the same time, our studies indicate that the binding constants for the interactions of F^-, Br^- and I^- with OVA show a same trend: They decrease as temperature increases. These were reasonably interpreted with the structural and thermodynamic factors. The thermodynamic functions (ΔG^0, ΔH^0, ΔS^0) at different temperatures were calculated with thermodynamic equations, and the enthalpy change for the interactions were also determined by isothermal titration calorimetry (ITC) at 298.15 K, which indicate that the interactions of F^-, Br^- and I^- with OVA are mainly electrostatic interaction. Simultaneously, there are also partial desolvation of solutes and solvent reorganization effect.

Key words: binding, halide ion, interaction, ion-selective electrode, ovalbumin.

Abbreviations: OVA, ovalbumin; BSA, bovine serum albumin; ITC, isothermal titration calorimetry.

It is important to study the interactions of small anions with proteins in order to understand the nature of transportation and distribution of these species in biological systems because such interactions play a key role in transportation and distribution processes. The binding capacity of a protein is dependent on its chemical and structural properties. In addition, the value of pH for the solutions, the ionic size and charge, and ionic concentration are also influencing factors (*1*,*2*). Bovine serum albumin (BSA), haemoglobin (Hb) and OVA have high affinities for various ligands. There have been more studies of the binding of anions to Hb and BSA (*3*–*9*), but relatively few studies on OVA (*10*). Although the amino acid composition and sequence of BSA which are important in its conformational analysis are now known (*11*), the nature of binding of anions to proteins is not yet well understood. Equilibrium dialysis and UV-visible spectrophotometry have been used to study binding of fluoride ion to BSA, and significant binding was determined (*12*–*14*). The studies on binding of various ligands to serum albumin have been reviewed by Peters (*15*), and further investigated by Carter (*16*) later. A study on the interactions of two proteins, namely, BSA and protamine, with urea aqueous in relation to the denaturation process was reported previously (*17*,*18*).

Through the use of recently developed ion-selective electrode, which have been applied in investigations on the binding of ions to proteins, polymers and some ligands in aqueous and nonaqueous solutions, accurate data on the binding of ions to proteins, polymers and ligands could be obtained at lower concentration of ions. A detailed study of the interaction of diflunisal ion with cyclodextrins using an ion-selective electrode discussed the cooperative binding between them (*19*). Another detailed investigation of the binding of F^-, Br^- and I^- to BSA with ion-selective electrode calculated the binding sites and stepwise constants, and interpreted the influence of charge on the binding sites (*20*,*21*). However, few studies of the effect of temperature on the interactions between anions and proteins have been reported, and no studies of the thermodynamic and structural factors on the binding of F^-, Br^- and I^- to OVA have been performed. Isothermal titration calorimetry has become an effective tool to thermodynamically characterize the binding of small molecules to macromolecules (*22*,*23*). The thermodynamic information is necessary for a thorough understanding of the mechanism of the interactions between ions and proteins. The objective of the present work is to investigate the binding of F^-, Br^- and I^-, to OVA by using ion-selective electrodes. The emphasis is to study the effect of temperature on the interactions of halide ions with OVA, and discuss the nature of the interactions with thermodynamic and structural factors according to hofmaister series (*24*).

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MATERIALS AND METHODS

Materials—OVA was purchased from Sigma Chemical Co. without further purification 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 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 sodium acetate buffers of pH 5.68 and an ionic strength of 0.10 M. Doubly distilled water was used throughout.

A model of PF-1C (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 analysis of F^-, Br^- and I^-, respectively. A model of 802 single-junction electrode was used as the reference electrode for F^- studies. For the studies on Br^- and I^-, a model of 217 double-junction electrode was used as the reference electrode. The two reference electrodes were purchased from Shanghai Ruosull Technology Co, Ltd. The potentials of F^-, Br^- and I^- were measured with a PXSJ-216 Ion Analyzer, which was from Shanghai Leici Instrument Co., with a readability of ±0.1 mV.

Construction of Calibration Curve of the Ion-Selective Electrode Method—Titrations for binding experiments were carried out with Proline Mechanical Single-Channel Pipettors (Shanghai, China). All measurements were carried out in a 50 ml double-walled glass cell at the desired value of ±0.01 K, by a model of DC-2006 Circulating Thermostat (Shanghai, China), and the sample solution was continuously stirred using a magnetic stirrer.

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

Studies on the Binding of Halide Ions to OVA by the Ion-Selective Electrode Method—The pair of electrodes were immersed in 25 ml 2×10^{-4} mol·l^{-1} OVA 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 potentials were converted into concentrations by use of the previously obtained calibration curve of F^-. The 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. The same procedure was followed for the binding experiments of the other two ions, Br^- and I^-.

RESULTS AND DISCUSSION

Binding Studies—Binding Model and Equation

For the binding of multi-anions to a protein molecule, the successive binding equilibria can be expressed in general as

$$\begin{aligned} \mathrm{P} + \mathrm{A} = \mathrm{PA}_1; \mathrm{PA}_1 + \mathrm{A} = \mathrm{PA}_2; \cdots\cdots \mathrm{PA}_{i-1} + \mathrm{A} \\ = \mathrm{PA}_i; \cdots\cdots; \mathrm{PA}_{n-1} + \mathrm{A} = \mathrm{PA}_n \end{aligned} \quad (1)$$

where P indicates a molecule of free potein, A indicates an anion and n the number of bound anions per protein molecule. The stepwise binding constants will be given by the relations

$$\begin{aligned} k_1 = \frac{[\mathrm{PA}_1]}{[\mathrm{P}][\mathrm{A}]};\ k_2 = \frac{[\mathrm{PA}_2]}{[\mathrm{PA}_1][\mathrm{A}]}; \cdots\cdots; \\ k_i = \frac{[\mathrm{PA}_i]}{[\mathrm{PA}_{i-1}][\mathrm{A}]}; \cdots\cdots; k_n = \frac{[\mathrm{PA}_n]}{[\mathrm{PA}_{n-1}][\mathrm{A}]} \end{aligned} \quad (2)$$

It has been pointed out to us by Professor Klotz that if the bound anion exerts no electrostatic influence on the succeeding bindings, then each anion is bound to the same kind of group on the protein (*8*). In such a case, the strength of binding would be the same for each bound anion, and the relative values of the successive binding constants would be determined only by statistical factors. For this situation the binding constants of the ith is given by

$$k_i = \left(\frac{n - i + 1}{i}\right)\frac{1}{K} \quad (3)$$

where $(n - i + 1)/i$ is the statistical factor for the binding equilibrium of the ith, K the intrinsic disassociation constant which depends on the nature of the anion as well as on the character of the protein and must be determined experimentally. $1/K$ is the intrinsic binding constant which can be expressed as k. If we define that the ratio of bound anions to protein molecules is r, the relation of k and r is given as follows

$$k = \left(\frac{r}{[\mathrm{A}]}\right)\frac{1}{n - r} \quad (4)$$

and Klotz equation can be given by Eq. (5) according to Eq. (4)

$$\frac{1}{r} = \left(\frac{1}{nk}\right)\frac{1}{[\mathrm{A}]} + \frac{1}{n} \quad (5)$$

where [A] is the concentration of free anion in equilibrium. Intrinsic binding constant k is independent of [A] and r.

Furthermore, $1/r$ is plotted as a function of 1/[A] according to Eq. (5). If there is a straight line, which indicates that the binding studied here is in good agreement with the binding model described above, the binding parameters n and k are determined by the intercept and slope.

Binding Sites on OVA Molecule—For the systems of F^-, Br^-, I^- and OVA, $1/r$ was plotted against $1/[F^-]$, $1/[Br^-]$ and$1/[I^-]$ according to the experimental data. These plots are shown in Figs. 1–3 and obviously present linearity. In order to find the value of n and the intrinsic

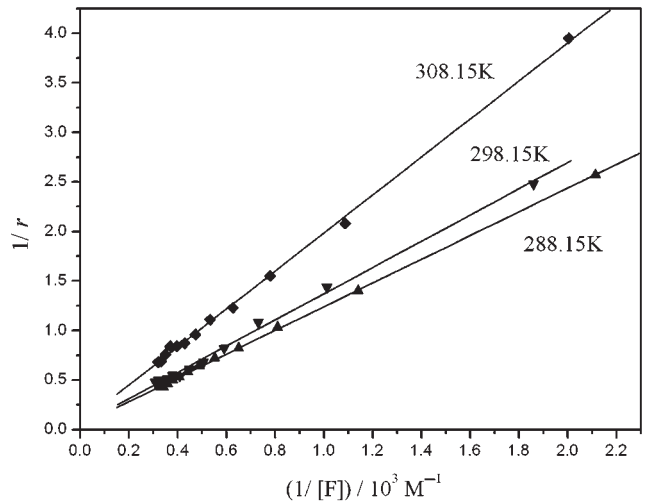

Fig. 1. **The Klotz plot for the binding of fluoride ion to OVA at different temperatures in acetate buffers of pH 5.68.**

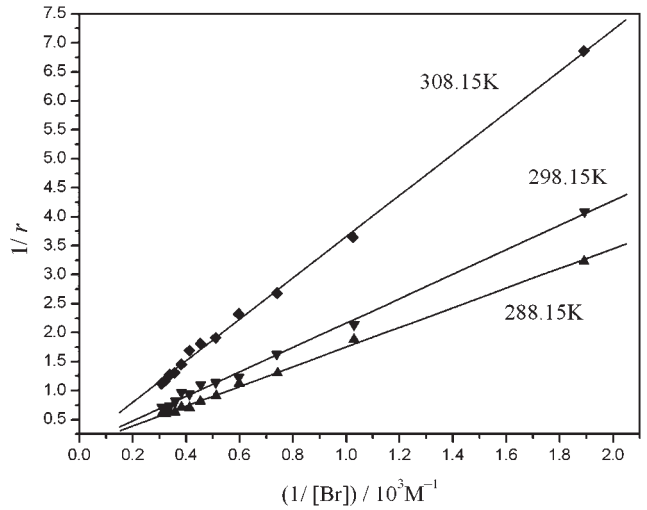

Fig. 2. **The Klotz plot for the binding of bromide ion to OVA at different temperatures in acetate buffers of pH 5.68.**

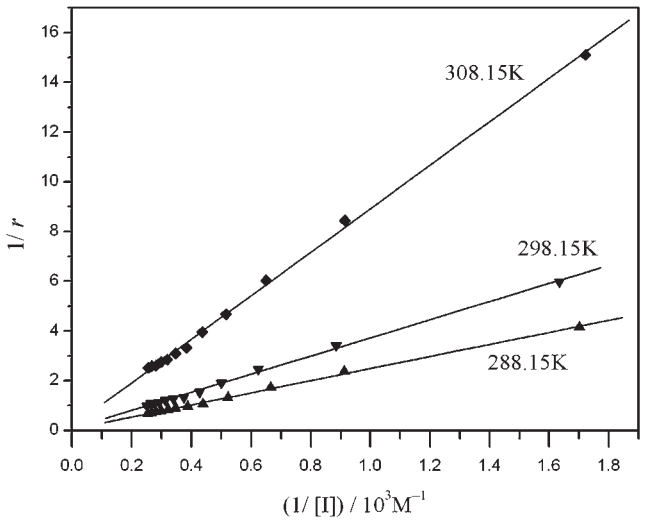

Fig. 3. **The Klotz plot for the binding of iodide ion to OVA at different temperatures in acetate buffers of pH 5.68.**

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

T (K)	Regression equations	n	k	R
F^-				
288.15	$1/r = 0.0438 + 1.197 \times 10^{-3}(1/[F^-])$	23	38.0	0.9998
298.15	$1/r = 0.0488 + 1.323 \times 10^{-3}(1/[F^-])$	21	36.2	0.9982
308.15	$1/r = 0.0684 + 1.917 \times 10^{-3}(1/[F^-])$	15	34.7	0.9992
Br^-				
288.15	$1/r = 0.0513 + 1.699 \times 10^{-3}(1/[Br^-])$	20	30.4	0.9988
298.15	$1/r = 0.0589 + 2.109 \times 10^{-3}(1/[Br^-])$	17	27.6	0.9980
308.15	$1/r = 0.0904 + 3.559 \times 10^{-3}(1/[Br^-])$	11	25.4	0.9989
I^-				
288.15	$1/r = 0.0550 + 2.434 \times 10^{-3}(1/[I^-])$	18	22.8	0.9991
298.15	$1/r = 0.0771 + 3.649 \times 10^{-3}(1/[I^-])$	13	21.0	0.9989
308.15	$1/r = 0.1694 + 8.750 \times 10^{-3}(1/[I^-])$	6	19.8	0.9993

binding constant k, the data of Figs. 1–3 were linearly regressed according to Eq. 5. The regression equations, correlation coefficients and the binding parameters n and k are presented in Table 1.

It should be pointed out that the values of n, calculated from the intercepts, are not generally integer. However, they are rounded to whole numbers in Table 1 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. At the same time, the excellent lines were obtained as indicated by the regression coefficients of the calibration equations. It is shown that the binding of F^-, Br^- and I^- to OVA is consistent with the model of multi-ions successive binding. Therefore, it is obvious that all of the binding sites of F^-, Br^- and I^- are equivalent, respectively, and the strength of binding is same.

Table 1 shows that the number of binding sites for the three halide ions on OVA molecule is found to decrease in the order $F^- > Br^- > I^-$, which can be explained by the increase in ionic size of the three ions in the order $F^- < Br^- < I^-$. These ions have single negative charge. The charge density of these ions increases as their size decreases, which then leads to the electrostatic interactions of halide ions with the weaker polar groups on OVA molecule and make the binding ability of these ions to decrease in the order $F^- > Br^- > I^-$. For the systems studied here, it was found that the number of the binding sites for F^-, Br^- and I^- decreases with increasing temperature. The secondary structure of OVA should be responsible for this kind of behaviour. It has been reported that there is mainly β-sheet and free coil for OVA molecule, and α-helix content is only 30.6% (*25*,*26*). This structural characteristic will make the change of

the secondary structure for OVA molecule less visible, and the binding sites of halide ions on OVA molecule will not increase remarkably with increasing temperature. On the contrary, there is unfavourable influence on the interactions between halide ions and OVA molecule as temperature increases. The thermal motion of molecules and ions will be more rapid with increasing temperature, which will make the binding of halide ions to OVA more difficult. In view of the results, it can be concluded that the secondary structure is the negligible effect on the binding of halide ions to OVA as compared to the thermal motion, as temperature increases.

Intrinsic Binding Constant—The intrinsic binding constants of the interactions of F^-, Br^- and I^- with OVA at 288.15 K, 298.15 K and 308.15 K were presented in Table 1. It can be seen that the binding constants of the interactions of the three ions with OVA gradually decrease in the order $F^- > Br^- > I^-$ at the same temperature, which is also mainly due to the ionic charge density decrease in the same order ($F^- > Br^- > I^-$) as discussed above.

In addition, it was found that the binding constants of the interactions of F^-, Br^- and I^- with OVA decrease with increasing temperature. In the course of the binding of anions to proteins, many factors may effect on the binding constants. However, two aspects may play a key role in the binding. One is the structural factor of proteins, and the other is the thermodynamic factor. As temperature increases, the peptide chains of proteins will unfold; thus, the potential binding sites in the α-helix structure will be exposed. At the same time, the thermal motion of ions will be more rapid with increasing temperature, which leads to the hydration structure for the polar groups of proteins becoming weaker, causing a more feasible binding of halide ions to proteins. In light of the above discussions, it seems that the binding constants should be greater as temperature increases. However, according to the results reported so far, the stability of the complex for anion–protein will be lowered with increasing temperature, so the binding constants will decrease (*27*). This difference is because that the α-helix content of OVA molecule is comparatively limited. As temperature increases, the unfolding of the peptide chains of OVA is also limited, so the increase in the binding constants caused by the structural change of OVA is not clear, which will make the effect of temperature on the stability of the complex of anion–protein being the decisive factor. Therefore, the binding constants for the interactions of F^-, Br^- and I^- with OVA gradually decrease as temperature increases.

THERMODYNAMIC STUDIES

Although it has been active in the study of the interactions between anions and proteins, the interactions are rather complicated. So 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 (*28*), 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 gives negative contribution to the enthalpic function (ΔH^θ). Because protein molecules and anions are hydrated in aqueous solutions, the binding of anions to protein molecules 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 entropic function (ΔS^θ) is lowered for the binding of anions to proteins, which will give negative contribution to ΔS^θ. However, the partial desolvation of solutes accompanied by the binding, which makes the structure of water for solvation layer destructive to a certain extent, also changes the structural water molecules become 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 to 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 electrostatic interaction and the partial desolvation of solutes.

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

$$\Delta G^\theta = -RT \ln k \tag{6}$$

Gibbs free energy (ΔG^θ) can be calculated. ΔH^θ may be regarded as a constant when there is a slight change in temperature and can be determined by Van't Hoff equation

$$\Delta H^\theta = \frac{-R(\mathrm{d} \ln k)}{\mathrm{d}(1/T)} \tag{7}$$

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

$$\Delta S^\theta = \frac{1}{T}(\Delta H^\theta - \Delta G^\theta) \tag{8}$$

The thermodynamic functions for the interactions of F^-, Br^- and I^- with OVA at 288.15 K, 298.15 K and 308.15 K, which are calculated with Eq. (6), (7) and (8), are presented in Table 2.

Table 2 shows that the values of ΔG^θ for the interactions of F^-, Br^- and I^- with OVA are negative at the three temperatures, which in turn shows that this kind of binding is spontaneous. It was found that the binding is exothermic on the basis of values of ΔH^θ; however, the caloric effect is rather weak. The results indicate that there is an adverse effect on the binding with an increase in temperature, which is in accordance with the results mentioned above. As discussed previously, there is a strong exothermic effect in the course of the interactions of anions with proteins. However, ΔH^θ of the systems studied here are calculated as small negative values, which shows that they should be accompanied with strong partial desolvation of solutes in the electrostatic interaction. The endothermic effect of the partial

Table 2. **The thermodynamic functions for the interactions of F^-, Br^- and I^- with OVA 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
F^-	ΔG^θ/(kJ·mol^{-1})	−8.71	−8.89	−9.08
	ΔH^θ/(kJ·mol^{-1})		−3.34	
	ΔS^θ/(J·mol^{-1}·K^{-1})	18.65	18.62	18.64
Br^-	ΔG^θ/(kJ·mol^{-1})	−8.18	−8.22	−8.28
	ΔH^θ/(kJ·mol^{-1})		−6.62	
	ΔS^θ/(J·mol^{-1}·K^{-1})	5.42	5.37	5.39
I^-	ΔG^θ/(kJ·mol^{-1})	−7.49	−7.54	−7.65
	ΔH^θ/(kJ·mol^{-1})		−5.18	
	ΔS^θ/(J·mol^{-1}·K^{-1})	8.03	7.92	8.02

desolvation of solutes will compensate for the exothermic effect of electrostatic interaction to a great degree. At the same time, it is also indicated that the partial desolvation of solutes may play an important role in the interactions of halide ions with OVA by the positive values of ΔS^θ presented in Table 2.

It is also important to note that F^- has the largest charge density in the three halide ions, so there should be stronger electrostatic interaction of F^- with OVA than those of Br^- and I^-, and that the partial desolvation effect of Br^- and I^- is relatively larger than F^- according to hofmaister series (*24*). Therefore, the negative value of ΔH^θ for the interaction between F^- and OVA should be comparatively larger, and the value of ΔS^θ should be comparatively smaller as compared to Br^- and I^-; however, contradictory results are presented in Table 2, possibly caused by the solvent reorganization effect as mentioned above. It is assumed that F^- can destroy the solvent structure to a great degree in the process of the solvent reorganization, but Br^- and I^- have the opposite effect. The destructive solvent reorganization effect caused by F^-gives positive contribution to ΔH^θ and ΔS^θ, whereas the constructive solvent reorganization effect caused by Br^- and I^- gives negative contribution to ΔH^θ and ΔS^θ, which brings forth the favorite values of the thermodynamic functions.

To further investigate the thermodynamic effect of the interactions between halide ions and OVA, the ITC experiments were also performed. ITC experiments were carried out on a Nano-ITC titration calorimeter. To calibrate the heat effect of dilution and mixing of the titrant, a control experiment was performed by injecting the titrant into the buffer alone. The heat released by dilution of OVA was negligible. The values of the enthalpy change for the interactions of F^-, Br^- and I^- with OVA at 298.15 K from ITC measurements (Table 3) indicate that the values are quite similar to the results measured by the ion-selective electrode method (Table 2). In addition, it is found that the values of the enthalpy change for these systems are very small and slightly fluctuate around zero, which indicates that the electrostatic interaction is the driving force behind the interactions of F^-, Br^- and I^- with OVA (*27*,*29*). From Table 3, we can also see the same order of the enthalpy change of these systems $Br^- > I^- > F^-$. The reason has been interpreted above. However, we should note that the values of

Table 3. **The enthalpy change for the interactions of F^-, Br^- and I^- with OVA in acetate buffers of pH 5.68 measured by ITC at 298.15 K.**

Halide ion	F^-	Br^-	I^-
ΔH^θ (kJ·mol^{-1})	0.78	−3.17	−1.96

heat released for the interactions of F^-, Br^- and I^- with OVA measured by ITC are a bit smaller than those measured by ion-selective electrode, which in all likelihood would have originated due to the differences between the two methods.

CONCLUSION

For the purpose of this study, the binding of F^-, Br^- and I^- to OVA were investigated by use of ion-selective electrodes at 288.15 K, 298.15 K and 308.15 K, and the binding sites and the binding constants were calculated. The article discussed the effect of the electrostatic interaction on the binding parameters and the density of ionic charge and the structure of OVA. Furthermore, the article also discussed the variation trends for the binding parameters with increasing temperature due to the effect of temperature on the secondary structure of OVA and the stability of anion–protein complex. The thermodynamic functions of the binding were calculated according to thermodynamic relations. It was shown that the electrostatic interaction is the driving force in the interactions of F^-, Br^- and I^- with OVA. On occasion, partial desolvation of solutes and solvent reorganization effect may play a role in determining the thermodynamic functions of the interactions. Furthermore, the enthalpy change of the interactions of halide ions with OVA measured by ITC is in accord with that measured by the ion-selective electrode method. 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.

SUPPLEMENTARY DATA

Supplementary data are available at *JB Online*.

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CONFLICT OF INTEREST

None declared.

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