# Thermodynamics of the Lysozyme−Salt Interaction from Calorimetric Titrations

**Matjaž Bončina, Jurij Lah,* Jurij Reščič,* and Vojko Vlachy**

*Faculty of Chemistry and Chemical Technology, University of Ljubljana, SI-1000 Ljubljana, Slovenia*

*Received: July 28, 2009; Revised Manuscript Received: February 13, 2010*

It is well-known that the addition of salts influences the properties of proteins in solution. The essential nature of this phenomenon is far from being fully understood, partly due to the absence of the relevant thermodynamic information. To help fill this gap, in this work isothermal titration calorimetry (ITC) was employed to study the ion−lysozyme association in aqueous buffer solutions at pH = 4.0. ITC curves measured for NaCl, NaBr, NaI, $NaNO_3$, NaSCN, KCl, $CaCl_2$, and $BaCl_2$ salts at three different temperatures were described by a model assuming two sets of independent binding sites on the lysozyme. The resulting thermodynamic parameters of binding of anions (counterions) to the first class of sites ($N \approx 7$) indicate that the binding constant ($K \approx 10^2$ $M^{-1}$) increases in the order $Cl^- < Br^- < I^- < NO_3^- < SCN^-$. The anion−lysozyme association is entropy driven, accompanied by a small favorable enthalpy contribution and a positive change in heat capacity. It seems that the entropy and heat capacity increase is due to the water released upon binding, while the net exothermic effect originates from the anion−$NH_3^+$ pair formation. Moreover, the results reveal that the nature of the cation has little effect on the thermodynamics of the anion−lysozyme association under the given experimental conditions. Taken together, it seems that the observed thermodynamics of association is a result of a combination of both electrostatic and short-range interactions. The anion ordering reflects the strength of water mediated interactions between anions and lysozyme.

## 1. Introduction

Understanding the interaction between biologically relevant macromolecules and electrolytes in aqueous solution is of crucial importance for the medical sciences and biology. Addition of salt(s) affects the stability, solubility, and activity of biomacromolecules.[1] For example, the solubility of lysozyme in a water−electrolyte mixture can be strongly affected by simply changing the counterion species.[2−5] These results are supported by the small-angle scattering data obtained by SAXS[6,7] and SANS[8] and also by osmotic pressure measurements[9−11] for this and other proteins. The effect of a salt on the stability of a protein solution, usually called the Hofmeister effect,[12] has been known for over a century, but its nature is still not fully understood. The effect is reflected in the list of ions ranked in terms of their efficiency in precipitating nonpolar solutes.[13] For anions this series reads $F^- > Cl^- > Br^- > NO_3^- > I^- > SCN^-$, that is, the $F^-$ ion is more efficient than the $SCN^-$ ion in precipitation of nonpolar solutes. For cations, the order is $Ca^{2+} > Ba^{2+} > Na^+ > K^+ > Cs^+$. The above series are the so-called direct Hofmeister series. Ions also affect protein stability. It was shown by studying thermal denaturation that some ions increase the thermal stability, while others act in the opposite way, decreasing it.[14−19] Moreover, a direct correlation was found between the protein crystallization polymorphism and salt effects.[20−23]

Many theories have been proposed to explain these findings. It is generally believed that water, through the water−ion interaction, is responsible for many of such effects. Small ions with large surface charge densities order the surrounding water molecules and are therefore called kosmotropes. Such ions (or charged groups on macromolecules) bind their water molecules strongly. On the other hand, large ions or charged groups with small surface charge densities that are more "hydrophobic" may weaken the water structure and are accordingly called chaotropes. These effects, which can be qualitatively explained even by simple models of water,[24,25] influence the properties of ions, their mutual interaction, and interaction with charged groups on the macromolecules. In mixtures of biological macromolecules and electrolytes, the ion-specific effects are the strongest in cases where the anions act as counterions. Collins related these effects to the charge distribution on the water molecule; positively charged hydrogen can approach closer to the anion.[25] Other authors have emphasized the role of dispersion forces in such systems.[26−28] The recent simulations of Lund and Jungwirth[29−32] suggest that small counterions bind to charged groups through electrostatic interactions, while large ions bind to less solvated regions between charged groups by a combination of hydrophobic and electrostatic effects. It has been generally accepted that implicit solvent models cannot reproduce these effects in a satisfactory manner.

The questions we seek to answer in studies of ion−protein interactions are the same as posed about sixty years ago by Scatchard:[33] how many, how tightly, where, and why? Thermodynamic investigations of ion−protein binding are essential to provide answer to these queries. In this regard, a method well suited for determining the basic thermodynamic properties of ion−protein associations is isothermal titration calorimetry (ITC).[34] This method can measure the true, model-independent, enthalpy changes that accompany some binding process. However, ITC studies of ion−protein associations are rather scarce,[35,36] presumably due to problems related to model analysis of calorimetrically determined binding isotherms for relatively weak ligand−receptor associations.[37−42] Namely, most ions interact with proteins rather weakly;[43−45] they simply screen the charge on the protein. In some cases, ions may influence the protein stability and/or activity through binding to specific, well-defined, sites.[36,46−51]

* To whom correspondence should be addressed. E-mail: (J.L.) jurij.lah@fkkt.uni-lj.si; (J.R.) jurij.rescic@fkkt.uni-lj.si.

10.1021/jp9071845 
Published on Web 03/10/2010

In the present study, we employed ITC to measure the heat effects coming from the salt−lysozyme interaction in aqueous buffer solutions (pH = 4.0). The calorimetric binding isotherms obtained for NaCl, NaBr, NaI, $NaNO_3$, NaSCN, KCl, $CaCl_2$, and $BaCl_2$ salts at different temperatures from the salt-to-protein and protein-to-salt titrations were described simultaneously (globally) by a model assuming two sets of independent binding sites for the anions. Since the influence of cations (co-ions) on the anion (counterion) binding was found (as expected) to be weak, the global thermodynamic analysis applied here allowed estimate of the number of binding sites and determination of the complete thermodynamic profile, that is $\Delta G^{\circ}_{1,T_0}$, $\Delta H^{\circ}_{1,T_0}$, $\Delta S^{\circ}_{1,T_0}$, and $\Delta C^{\circ}_{p,1}$ for the anion binding to the first set of binding sites on lysozyme. The observed thermodynamic quantities for this process are discussed in terms of the properties of individual ions, and their position in the Hofmeister series for the salt−protein interaction.

## 2. Experimental Part

**2.1. Materials.** Hen egg white lysozyme ($M$ = 14 388 g $mol^{-1}$)[52] was purchased from Merck (Darmstadt, Germany), product number 105281, lot K35888481 709. The alkali metal and alkaline earth salts (>99%, NaCl, NaI, $NaNO_3$, NaSCN, KCl, $CaCl_2$, and $BaCl_2$) were obtained from Merck as well, while NaBr was obtained from Kemika (>99%; Zagreb, Croatia). The first step was preparation of acetate buffers and various salt and protein solutions. Aqueous buffer solutions were made from water that was demineralized and then distilled twice in a quartz bidistillation apparatus (Destamat Bi 18E, Heraeus). Buffer solutions contained 0.02 M acetate ions ($CH_3COO^-$) and 3.6 mM base; NaOH, KOH, $Ba(OH)_2$ or $Ca(OH)_2$ depending on the cation of the salt used in the ITC experiment. The salts were dried at 130 °C for 2 h in the presence of $P_2O_5$. Aqueous solutions of various salts at concentrations of 0.30 and 0.01 M were prepared gravimetrically using the acetate buffer as solvent. Lysozyme was dissolved in the buffer solution and then extensively dialyzed (three changes of buffer solutions in 24 h) against the same buffer using a dialysis cassette Slide-A-Lyser (Pierce, Rockford, IL $M_w$ cutoff 3500 Da). Since lysozyme was crystallized from hydrochloric acid, the concentration of chloride in the lysozyme solution was checked after dialysis by titration with $AgNO_3$; a chloride electrode was used as an indicator electrode and the antimony electrode served as a reference. No chloride was detected after dialysis. If needed, the protein solutions were concentrated using Vivaspin 4 vessels (Sartorius Stedim, Goettingen, Germany, $M_w$ cutoff 5000 Da). The lysozyme concentration was determined by measuring its absorbance at $\lambda$ = 280 nm and 25 °C using a Cary 100 Bio (Varian) spectrophotometer, which uses a Peltier block for temperature regulation. The extinction coefficient of lysozyme is $\varepsilon$ = 2.635 $dm^3 \cdot g^{-1} \cdot cm^{-1}$.[53] pH was measured by an Iskra pH meter (Ljubljana, Slovenia), model MA5740, using a combined glass microelectrode of type InLab 423 from Mettler Toledo (Schwerzenbach, Switzerland). All experiments were performed at pH = 4.0 in acetate buffer. pH was measured at the beginning of the titration experiment (lysozyme in buffer solution) and at the end (lysozyme in buffer−salt solution). The maximum deviations from the starting pH value were about ±0.2.

**2.2. Isothermal Titration Calorimetry (ITC).** Isothermal titration calorimetry (ITC) experiments were performed between 278.15 and 318.15 K by adding a solution of the titrant ($x$) from the syringe into the solution in the measuring cell containing the solute ($y$) using a VP-ITC isothermal titration calorimeter (Microcal Inc., Northampton, MA; cell volume = 1.3862 mL).

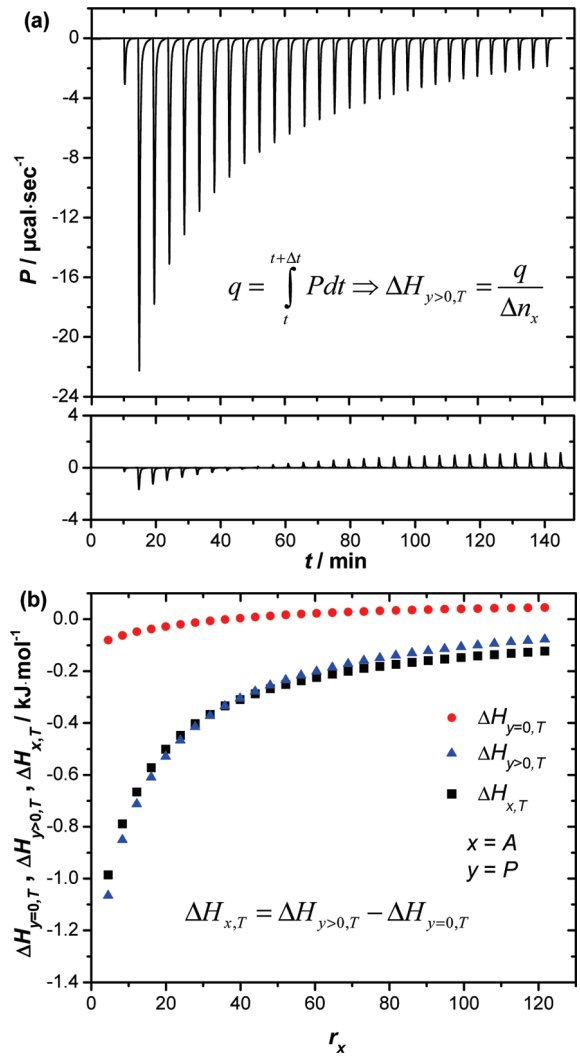


**Figure 1.** Analysis of raw signals to obtain the corresponding calorimetric isotherms. (a) Typical raw calorimetric signal accompanying the anion−lysozyme association. Titration of lysozyme ($[P]_t \approx 0.6$ mM) with NaI ($c_x$ = 0.30 M) in acetate buffer solution at pH = 4.0 and $T_0$ = 298.15 K is shown. The corresponding enthalpy change at a given injection ($\Delta H_{y>0,T}$) is obtained by dividing the integrated signal by the amount of anion ($\Delta n_x$) added to the solution in the measuring cell per injection (inset). Below is the raw signal coming from NaI−buffer solution titration into the buffer solution in the absence of the protein. (b) The corresponding enthalpy of salt−buffer mixing $\Delta H_{y=0,T}$ was subtracted from $\Delta H_{y>0,T}$ to obtain the enthalpy change induced by binding of the anions to lysozyme ($\Delta H_{x,T}$). Note that $r_x$ is defined as anion/protein molar ratio.

In case of the salt-to-protein titrations [$x$ = anion ($A$), $y$ = protein ($P$)] the $M_mA_a$ salt solution with anion concentration $c_A \approx 0.30$ M was added into the lysozyme solution ($[P]_t \approx 0.6$ mM), while in case of the protein-to-salt titrations [$x = P$, $y = A$] the protein solution ($c_P \approx 13$ mM) was titrated into the salt solution with total anion concentration $[A]_t \approx 0.01$ M. The area under the peak following each injection of $x$ obtained by integration of the raw signal (Figure 1a) was expressed per mole of added $x$ per injection, to give the corresponding enthalpy changes at a given temperature $T$ ($\Delta H_{y>0,T}$; Figure 1b). By subtraction of the enthalpy change ($\Delta H_{y=0,T}$) coming from titration of $x$ in the buffer solution in the absence of $y$, one obtains the corresponding enthalpy change induced by the binding of ions (ligands) to the protein ($\Delta H_{x,T}$; Figure 1b).[34,42,54,55] A model analysis of the calorimetric isotherms obtained ($\Delta H_{x,T}$ versus $x/y$ molar ratio, $r_x$) is presented in the next section.

## 3. Thermodynamic Analysis of the Anion−Lysozyme Association

The enthalpy change at a given temperature $T$ induced by the binding of ligand (ion) $A$ to the protein $P$ can be expressed as[42,56]

$$\Delta H_{x,T} = \sum_i \Delta H_{i,T}\left(\frac{\partial n_i}{\partial n_x}\right)_{p,T,n_1} \quad (1)$$

where $\Delta H_{i,T}$ is the enthalpy of formation of the complex $i$ from $P$ and $A$, and $(\partial n_i/\partial n_x)_{p,T,n_1}$ is the corresponding partial derivative at given $p$, $T$ and a constant amount of solvent in which $n_i$ is the amount of the complex $i$ and $n_x$ is the total amount of added $x$. Note that in case of the salt-to-protein titration $x = A$ and $y = P$, while in case of the protein-to-salt titration $x = P$ and $y = A$. The most widely used approach to analyze binding data in biological systems is to treat each set of sites independently.[57,58] By applying this model to describe the anion−lysozyme association and assuming that $\Delta H_{i,T}$ is independent of concentration (and thus equal to its value in the standard state), eq 1 transforms into

$$\Delta H_{x,T} = \sum_i \Delta H^\circ_{i,T}\left(\frac{\partial \bar{\nu}_i}{\partial r_x}\right)_{p,T,n_1} \quad (2)$$

where $\bar{\nu}_i$ represents the average number of occupied sites of each class $i$ per protein $P$, $r_x$ ($r_A = 1/r_P$) the corresponding $x/y$ molar ratio and $\Delta H^\circ_{i,T}$ the standard enthalpy of binding of $A$ to the binding site of class $i$ on the protein $P$. $\bar{\nu}_i$ can be defined as

$$\bar{\nu}_i = \frac{N_i K_{i,T}[A]}{1 + K_{i,T}[A]} \quad (3)$$

where $K_{i,T}$ is the apparent intrinsic equilibrium constant of the binding of $A$ to a binding site of class $i$ and $N_i$ is the total number of sites of class $i$. In eq 3, $[A]$ represents the corresponding equilibrium molar concentration of unbound $A$ that can be expressed as

$$[A] = [A]_t - [P]_t \sum_i \bar{\nu}_i = [A]_t(1 - r_A \sum_i \bar{\nu}_i) \quad (4)$$

where $[A]_t$ and $[P]_t$ represent the total concentrations of anion and protein in the measuring cell. On the other hand, the binding of $A$ to each class of binding site can be described in terms of changes of standard thermodynamic parameters: the Gibbs free energy $\Delta G^\circ_{i,T_0}$ and enthalpy $\Delta H^\circ_{i,T_0}$ at the reference temperature $T_0 = 298.15$ K and the corresponding heat capacity $\Delta C^\circ_{p,i}$ (assumed to be temperature independent). These parameters define $\Delta G^\circ_{i,T}$ and $\Delta H^\circ_{i,T}$ at any $T$ through the Gibbs−Helmholtz relation and Kirchhoff's law

$$\Delta G^\circ_{i,T} = T\left\{\frac{\Delta G^\circ_{i,T_0}}{T_0} + \Delta H^\circ_{i,T_0}\left[\frac{1}{T} - \frac{1}{T_0}\right] + \Delta C^\circ_{p,i}\left[1 - \frac{T_0}{T} - \ln\left(\frac{T}{T_0}\right)\right]\right\} \quad (5)$$

$$\Delta H^\circ_{i,T} = \Delta H^\circ_{i,T_0} + \Delta C^\circ_{p,i}(T - T_0) \quad (6)$$

Thus, a single set of (adjustable) parameters $\Delta G^\circ_{i,T_0}$, $\Delta H^\circ_{i,T_0}$, and $\Delta C^\circ_{p,i}$ defines $K_{i,T} = \exp(-\Delta G^\circ_{i,T}/RT)$ at any $T$. It follows that for known $K_{i,T}$ and $N_i$ values the equilibrium populations of all species in solution and the model function (eq 2) are completely defined at any $T$, $[P]_t$, $[A]_t$, or $r_x$ by eqs 2−6. Global fitting of the model function (assuming two sets of independent binding sites; $i = 1, 2$) to the experimental ITC curves, measured at various $T$, was based on the nonlinear Levenberg−Marquardt $\chi^2$ regression procedure.[59]

## 4. Results and Discussion

**4.1. Anion−Lysozyme Association.** Calorimetric binding isotherms resulting from titration of various sodium salts into the lysozyme solution (Figure 2) show that the ITC data are able to distinguish between the binding behavior of different anions. To describe this behavior quantitatively, we attempted to fit the experiments by the model assuming a single set of independent binding sites. This model was applied simultaneously to all the salt-to-protein and protein-to-salt titration curves for a given salt-lysozyme pair (global fitting). The model failed to describe the ITC curves adequately. Consequently, an attempt was made to describe the same calorimetric data by a model assuming two sets of independent binding sites ($i = 1, 2$). This model gives reasonably good agreement with experimental ITC curves for all the investigated salts (Figure 3a,b and Figure 1-SI in the Supporting Information). Unfortunately, the values of the thermodynamic parameters accompanying binding of anions to the second class of binding sites ($i = 2$) cannot be determined with satisfactory accuracy due to the small (curvature) slopes of binding isotherms ($N_2K_2[P]_t \ll 1$) at higher anion/lysozyme molar ratios ($r_A$) (Figure 2). Nevertheless, these parameters are needed for an appropriate description of ITC curves at higher $r_A$ values. It is important to mention that the $i = 2$ parameters are not highly correlated to the thermodynamic parameters describing binding to the first class of binding sites ($i = 1$). This suggests that the global analysis of the ITC data presented here provides realistic values of parameters $N_1$, $\Delta G^\circ_{1,T_0}$, $\Delta H^\circ_{1,T_0}$, and $\Delta C^\circ_{p,1}$.

The appropriateness of the proposed model has additionally been supported by the following findings: (i) The fitting procedure was repeated many times from different starting values of the adjustable parameters, but they always converged

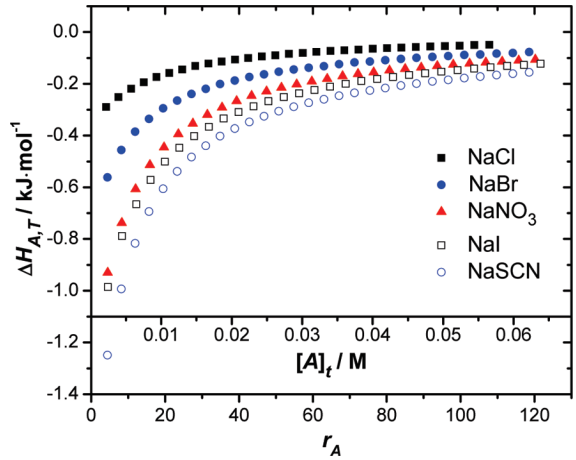


**Figure 2.** Calorimetric binding isotherms for various sodium salt−lysozyme mixtures as a function of the anion concentration $[A]_t$ or anion/lysozyme molar ratio $r_A$ at $T_0 = 298.15$ K and pH = 4.0.

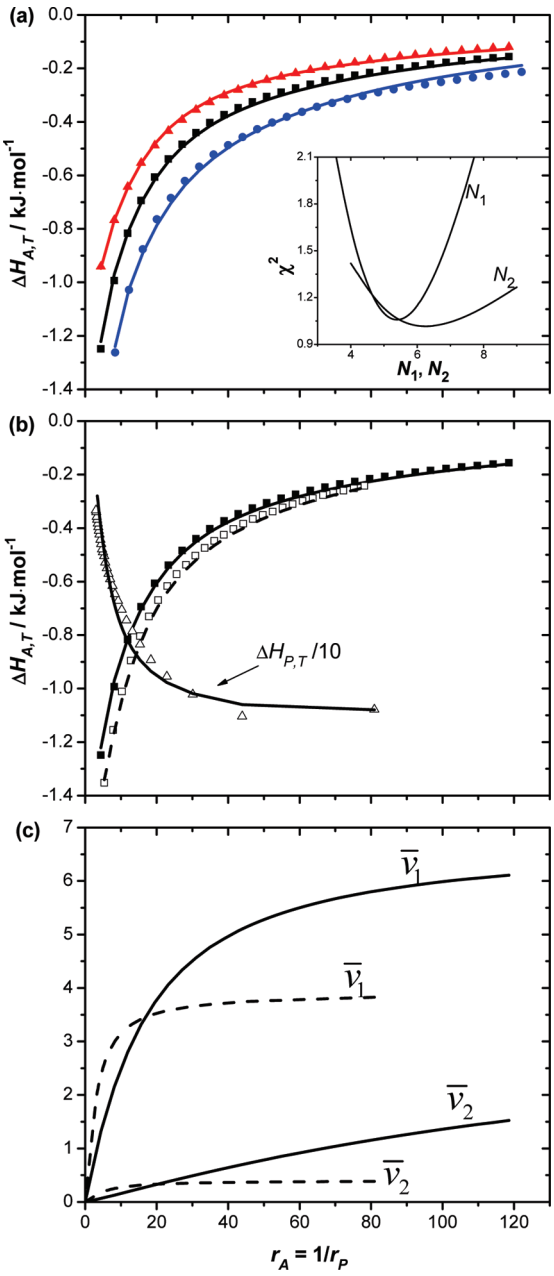


**Figure 3.** Model analysis of salt-to-protein and protein-to-salt calorimetric isotherms. Example: NaSCN−lysozyme titrations at pH = 4.0. (a) Enthalpy of $SCN^-$ association with lysozyme coming from salt-to-protein titration as a function of anion/protein molar ratio $r_A$ at various temperatures is represented by different symbols ($T$ = 278.15 K blue circles, 298.15 K black squares, and 318.15 K red triangles) ($[P]_t \approx 0.6$ mM, $c_A = 0.30$ M), while the corresponding best global fit of the model function (eq 2) obtained using an average value of $N_1$ = 7 as a fixed parameter (see text) is presented by lines. Inset: Dependence of $\chi^2$ as a function of $N_1$ and $N_2$ if they are treated as adjustable parameters. (b) Salt-to-protein isotherms (symbols) measured at two different concentrations of lysozyme ($[P]_t \approx 0.6$ mM, full squares; and $[P]_t \approx 0.9$ mM, open squares) at $T_0$ = 298.15 K. The dashed and full lines represent the model function calculated from the best fit parameters presented in Table 1. Protein-to-salt isotherm $\Delta H_{P,T}$ measured at $[A]_t \approx 0.01$ M, $c_P \approx 13$ mM and $T_0$ = 298.15 K is presented by open triangles while corresponding best global fit of the model function (eq 2) by a solid line. (c) Average number of occupied sites of the first $\bar{\nu}_1$ and the second class $\bar{\nu}_2$ (salt-to-protein titrations, full lines; and protein-to-salt, dashed lines), at $T_0$ = 298.15 K as a function of $r_A = 1/r_P$ obtained from the best fit parameter values (Table 1) using eqs 3−5. Pay attention to slightly increasing $\bar{\nu}_1$ and $\bar{\nu}_2$ accompanying protein-to-salt titration which at high $r_A$ approach to the same limit as the $\bar{\nu}_1$ and $\bar{\nu}_2$ accompanying salt-to-protein titrations ($\lim_{r_A \to \infty} \bar{\nu}_i = N_i$).

**TABLE 1: Standard Thermodynamic Parameters of Anion−Lysozyme Association Obtained by Global Analysis of ITC Data (Equations 1−6)[a]**

| | NaCl | NaBr | NaI | $NaNO_3$ | NaSCN |
|---|---|---|---|---|---|
| $K_{1,T_0}/M^{-1}$ | 63 ± 4 | 76 ± 3 | 105 ± 5 | 122 ± 7 | 128 ± 14 |
| $\Delta G^\circ_{1,T_0}$/kJ·mol$^{-1}$ | −10.3 ± 0.2 | −10.7 ± 0.1 | −11.5 ± 0.1 | −11.9 ± 0.1 | −12.0 ± 0.3 |
| $\Delta H^\circ_{1,T_0}$/kJ·mol$^{-1}$ | −1.2 ± 0.1 | −2.3 ± 0.1 | −3.3 ± 0.1 | −2.7 ± 0.1 | −3.1 ± 0.2 |
| $T\Delta S^\circ_{1,T_0}$/kJ·mol$^{-1}$ | 9.1 ± 0.3 | 8.4 ± 0.2 | 8.3 ± 0.2 | 9.2 ± 0.2 | 9.0 ± 0.5 |
| $\Delta C^\circ_{p,1}$/ J·mol$^{-1}$·K$^{-1}$ | 8.5 ± 0.7 | 22.5 ± 0.9 | 32 ± 1 | 28 ± 1 | 18 ± 2 |

[a] The parameters presented correspond to the anion association with the first set of binding sites ($N_1$ = 7, taken as an average value over all investigated salts; see text in the subsection Anion−Lysozyme Association) on the lysozyme molecule at $T_0$ = 298.15 K and pH = 4.0. They are given together with the corresponding standard deviations, estimated as square roots of diagonal elements of the variance−covariance matrix.

to the same values (global minimum). (ii) The observed high solubility of lysozyme[2] in the presence of $CH_3COO^-$ (present in the buffer solution) at pH = 4.0 and the fact that acetic acid is not fully ionized at this pH suggest that the ability of these ions to screen positive charges on lysozyme $NH_3^+$ groups is rather weak. Therefore, the influence of $CH_3COO^-$ ions on the binding of the other studied anions was neglected. (iii) ITC curves resulting from titrations with NaCl, KCl, $BaCl_2$, and $CaCl_2$ (Figure 5) agree within experimental error. Therefore, the effect of the nature of the cation on the anion−lysozyme association can be neglected (see below). (iv) The successful global fitting of the salt-to-protein and protein-to-salt calorimetric titration curves for a relatively wide $[P]_t$ and $[A]_t$ concentration range indicates, that the resulting thermodynamic parameters are largely independent of $[P]_t$ and $[A]_t$ (Figure 3b).

It should be mentioned that, due to the high correlation between $N_1$ and other adjustable parameters (reflected in a shallow minimum of $\chi^2$ with respect to $N_1$), reliable values of $N_1$ cannot be obtained by fitting the model to salt-to-protein (or protein-to-salt) calorimetric titration curves alone. The problem is a consequence of the nature of the binding process, and concentrations used in the experiments ($N_iK_i[P]_t < 1$).[60] We have successfully solved this problem by simultaneous analysis of the salt-to-protein and protein-to-salt titration curves (Figure 3). The model function (eq 2), namely, depends on the independent variables $[P]_t$ and $r_A = 1/r_P$. Additional experimental data, obtained for more $[P]_t$, $r_A$ combinations (Figure 3), impose additional restrictions on the value of the adjustable parameter $N_1$, compared to salt-to-protein or protein-to-salt titration curves alone. This is resulting in a pronounced minimum of $\chi^2$ as a function of $N_1$ (Figure 3a, see the inset). Moreover, our model analysis shows that $N_1$ does not significantly depend on the salt type $N_1 = 7 \pm 2$. For this reason, we calculated an average value of $N_1$ over all investigated salts. The obtained value of 7 was then used as a fixed parameter in the global fitting of the model to the calorimetric isotherms for each salt−lysozyme pair (Figure 3). Successful model analysis of data, using $N_1$ as salt independent parameter, strongly suggests that positively charged groups on lysozyme can be considered as binding sites to which anions bind with higher affinity.

In summary, the evidence presented strongly suggest that the model assuming the binding of anions to two sets of independent binding sites is capable of a realistic description of the anion−lysozyme association. We believe that the model analysis provides good estimates of the binding parameters $N_1$, $\Delta G^\circ_{1,T_0}$, $\Delta H^\circ_{1,T_0}$, and $\Delta C^\circ_{p,1}$. One important finding is that the binding of anions ($Cl^-$, $Br^-$, $I^-$, $NO_3^-$, $SCN^-$) to about seven binding sites on lysozyme is relatively strong (average $K_{1,T_0} \approx 100$ $M^{-1}$; see

Table 1) compared to monovalent ion−protein associations in which ions are mainly involved in protein surface charge neutralization.[15,17,43,44] For binding of monovalent anions to the second class of binding sites, however, the model can merely provide an estimate; the corresponding association constant $K_{2,T_0}$ is about two orders of magnitude lower than $K_{1,T_0}$. Accordingly, the influence of the second class of binding sites on the properties of salt−lysozyme mixtures is expected to be small (Figure 3c). Nevertheless, our analysis clearly shows that the binding of anions to lysozyme cannot be characterized by a single class of binding sites.

**4.2. The Association is Entropy Driven.** Table 1 reveals that the apparent binding constants for monovalent anions increase in the order $Cl^- < Br^- < I^- < NO_3^- < SCN^-$. The ordering follows the reverse Hofmeister series with the exception that $NO_3^-$ binds with higher affinity than $I^-$. The constant increases from $K_{1,T_0} = 63\ M^{-1}$ for $Cl^-$ to $128\ M^{-1}$ for $SCN^-$. Binding affinities of halides follow the expected order.[2−4,8] The strongest binding of $I^-$ can be explained by its inability to bind its water molecules tightly, which is in close agreement with recent molecular dynamics simulations of lysozyme in explicit water.[31] It can be seen from Table 1 that $NO_3^-$ and $SCN^-$ ions bind to lysozyme more strongly than halide ions. Anions may bind through a combination of electrostatic and short-range interactions; the latter is valid for complex ions ($NO_3^-$, $SCN^-$) as well as for iodine.[29] In general, strongly bound anions decrease the net charge on the protein more efficiently and decrease the range of the repulsive electrostatic interaction between them. Proteins may therefore approach each other to a distance where attractive short-range forces become important.[61]

For all the anions studied here the binding to lysozyme is entropy driven ($T\Delta S^\circ_{1,T} > 0$), accompanied by a small favorable enthalpy contribution ($\Delta H^\circ_{1,T} < 0$). It can be assumed that the entropy increase comes from the water released on ion−protein association, while ($\Delta H^\circ_{1,T} < 0$) probably originates from anion−$NH_3^+$ pair formation. The observed small negative enthalpies of binding are consistent with the results of some previous studies of lysozyme solutions[62] and with the notion that they originate from compensation of two highly negative contributions resulting mainly from the anion−$NH_3^+$ interaction (ions in bound state, see Figure 3-SI), and the ion−dipole (water) interaction (ions in unbound state).

The temperature dependencies of thermodynamic functions accompanying association of $SCN^-$ with lysozyme are shown in Figure 4; the results for other salts are given as Supporting Information (Figure 2-SI). Values for $\Delta H^\circ_{1,T}$ and $\Delta C^\circ_{p,1}$, of the same orders of magnitude for all investigated ions (Table 1), result in similar temperature dependencies of $\Delta G^\circ_{1,T}$, $\Delta H^\circ_{1,T}$, and $T\Delta S^\circ_{1,T}$. Since $\Delta H_{1,T}^\circ < 0$, the anion−lysozyme association is stronger at lower temperatures where $T\Delta S^\circ_{1,T}$ becomes smaller. What makes the association stronger at lower temperature? We believe that water could play an important role here. Interactions between water molecules themselves at low temperatures can be energetically more favorable than the interactions between ions and water.[24]

At this point, it seems worthwhile to compare the thermodynamics of anion−lysozyme binding with anion−cation pairing in simple electrolytes. The $Na^+$-$Cl^-$ pair formation constant in water, for example, is significantly lower ($K < 2\ M^{-1}$)[63,64] than the corresponding constants presented in Table 1. Association of simple electrolytes is entropy driven due to the release of water molecules upon pairing. The enthalpy change in this process is (contrary to the anion−lysozyme association) endothermic and the change in heat capacity is positive.[63] In the case of the anion−lysozyme association, the binding constants are higher than for the alkali halides, most likely due to the higher accumulation of positive charge on the lysozyme. In this context it should be noticed that even for much simpler systems, such as alkali halides in water, the ion−ion association constants obtained from explicit water computer simulations are merely in qualitative agreement with experimental data .[64]

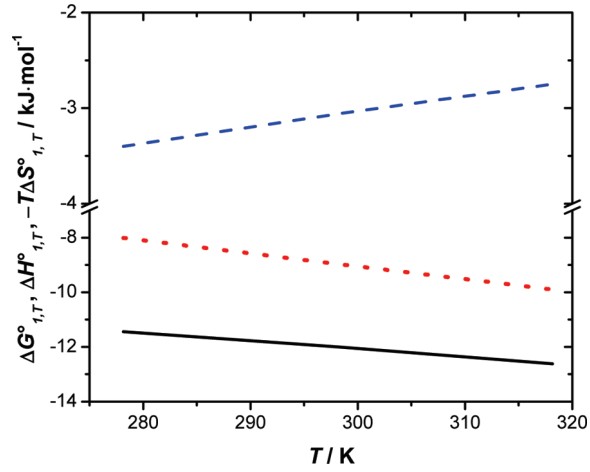


**Figure 4.** Standard thermodynamic parameters of $SCN^-$ binding to lysozyme. $\Delta G^\circ_{1,T}$(black continuous line), $\Delta H^\circ_{1,T}$(blue dashed line), and $-T\Delta S^\circ_{1,T}$(red dotted line) as a function of temperature obtained from analysis of $SCN^-$ binding to the first set of independent sites on lysozyme.

The heat capacity change ($\Delta C^\circ_{p,1}$) is another quantity of interest since it can provide deeper insight into the changes in hydration accompanying the anion−protein association. $\Delta C^\circ_{p,1}$ values are small ($\Delta C^\circ_{p,1} > 0$) but significant (Table 1). We can ascribe this increase to the following process. The water molecules associated with ions cannot easily fluctuate between the energy states. When an ion pair is formed, hydration water is released into the bulk. This bulk water has greater ability to absorb heat from the surroundings due to the higher number of energetically similar microstates accessible to water molecules (higher energy fluctuations).[65]

**4.3. The Effect of Cations (Co-Ions) is Weak.** To obtain a more complete picture on factors that influence the anion−lysozyme association, we examined the effect of the nature of the cation ($Na^+$, $K^+$, $Ba^{2+}$, $Ca^{2+}$) on the $Cl^-$ binding properties. Experiments were performed under identical conditions as for the other salts. The isotherms resulting from calorimetric titrations of lysozyme with NaCl, KCl, $BaCl_2$, and $CaCl_2$ (Figure 5) agree within experimental error, which yields the conclusion that the anion−lysozyme association is, under the given conditions, independent of the nature of the cation. It appears that cations (co-ions) interact with lysozyme very weakly; due to the equal sign of the charge they predominantly occupy the domain distant from the protein molecule.

## 5. Conclusions

In this study we employed isothermal titration calorimetry (ITC) to examine the heat effects accompanying the anion−lysozyme association in aqueous solutions at pH = 4.0. The model assuming two sets of independent binding sites was fitted globally to the calorimetric isotherms resulting from the salt-to-protein and protein-to-salt titrations to determine the thermodynamic parameters for the salt−lysozyme interaction. Aqueous solutions of NaCl, NaBr, NaI, $NaNO_3$, NaSCN, KCl, $CaCl_2$, and $BaCl_2$ salts were examined. The analysis provides

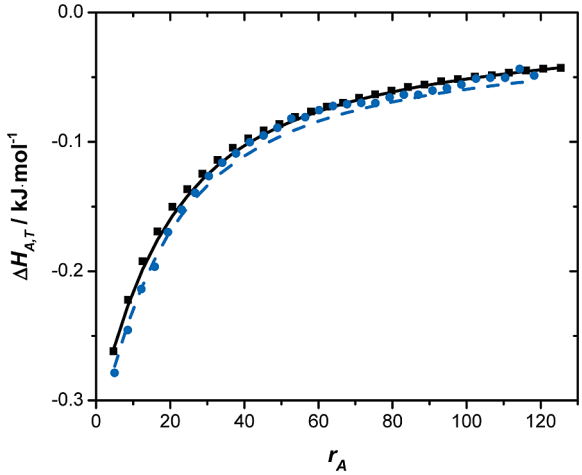


**Figure 5.** Calorimetric isotherms accompanying $Cl^-$ association with lysozyme in the presence of different cations; $K^+$ (KCl, black squares, $[P]_t = 0.56$ mM, $c_A = 0.30$ M) and $Ca^{2+}$ ($CaCl_2$, blue circles, $[P]_t = 0.61$ mM, $c_A = 0.30$ M). Enthalpies were measured by ITC as a function of anion/protein molar ratio $r_A$ at $T_0 = 298.15$ K and pH = 4.0. The corresponding lines ($K^+$, black solid line; $Ca^{2+}$, blue dashed line) are model functions calculated from the best fit parameters obtained from global analysis of the NaCl - lysozyme system (Table 1). Results for $BaCl_2$−lysozyme are not shown for clarity. They are almost identical to those observed with KCl ($CaCl_2$)−lysozyme system. The small differences in experimental enthalpies ($K^+$ versus $Ca^{2+}$) do not reflect the effect of the cations but rather the different lysozyme concentration. Namely, the differences are the same as the differences between the model functions corresponding to the different lysozyme concentrations used.

estimates of $\Delta G^\circ_{1,T_0}$, $\Delta H^\circ_{1,T_0}$, and $\Delta C^\circ_{p,1}$ values, accompanying the binding of anions to the first class of sites ($N_1 \approx 7$) on the lysozyme molecule. This binding is relatively strong, $K_{1,T_0} \approx 10^2$ $M^{-1}$. The corresponding parameters for anion binding to the second class of sites cannot be determined with satisfactory accuracy. Our estimate for the corresponding association constant is $K_{2,T_0} \approx 0.01K_{1,T_0}$. The conclusions emerging from this study are the following: (i) Global fitting of the proposed model to the salt-to-protein and protein-to-salt calorimetric titration curves provide physically sound estimate for the number of binding sites $N_1$. (ii) Model analysis of the ITC curves shows that the sign of standard thermodynamic parameters is the same for all the anions studied here. (iii) Apparent binding constants for the investigated monovalent anions increase in the order $Cl^- < Br^- < I^- < NO_3^- < SCN^-$ ($63 < K_{1,T_0}/M^{-1} < 128$). (iv) The anion−lysozyme association is entropy driven, accompanied by a small negative change in enthalpy and a positive change in heat capacity. It appears that the association is a combination of both electrostatic and short-range interactions. The entropy increases due to water released upon binding, and the net exothermic effect probably originates from anion−$NH_3^+$ pair formation. (v) The results reveal that the cations studied here have no effect on the thermodynamics of the anion−lysozyme association at given experimental conditions. (vi) For anions acting as counterions, the reverse Hofmeister series is observed; the ordering is a consequence of the different strength of water mediated interactions between the anions and lysozyme.

Despite achieving the main goal of this study, that is, to obtain physically sound estimates and qualitative interpretation of thermodynamic parameters of ion binding, the molecular nature of anion−lysozyme association still remains rather elusive and requires additional experimental and theoretical studies.

**Acknowledgment.** Financial support of the Slovenian Research Agency through Grant P1-0201 is gratefully acknowledged.

**Supporting Information Available:** Figure 1-SI: Model analysis of calorimetric isotherms accompanying salt - lysozyme titrations at pH = 4.0. Figure 2-SI: Standard thermodynamic parameters of anion binding to lysozyme for all investigated salts. Figure 3-SI: Calorimetric binding isotherms. This material is available free of charge via the Internet at http://pubs.acs.org.

## References and Notes

(1) Cacace, M. G.; Landau, E. M.; Ramsden, J. J. *Q. Rev. Biophys.* **1997**, *30*, 241–277.
(2) Riès-Kautt, M.; Ducruix, A. F. *J. Biol. Chem.* **1989**, *264*, 745–748.
(3) Retailleau, P.; Ducruix, A.; Riès-Kautt, M. *Acta. Crystallogr., Sect. D* **2002**, *58*, 1576–1581.
(4) Guilloteau, J. P.; Riès-Kautt, M.; Ducruix, A. F. *J. Cryst. Growth* **1992**, *122*, 223–230.
(5) Bončina, M.; Reščič, J.; Vlachy, V. *Biophys. J.* **2008**, *95*, 1285–1294.
(6) Tardieu, A.; Finet, S.; Bonneté, F. *J. Cryst. Growth* **2001**, *232*, 1–9.
(7) Bonneté, F.; Finet, S.; Tardieu, A. *J. Cryst. Growth* **1999**, *196*, 403–414.
(8) Chodankar, S.; Aswal, V. K. *Phys. Rev. E.* **2005**, *72*, 041931.
(9) Curtis, R. A.; Ulrich, J.; Montaser, A.; Prausnitz, J. M.; Blanch, H. W. *Biotechnol. Bioeng.* **2002**, *79*, 367–379.
(10) Curtis, R. A.; Prausnitz, J. M.; Blanch, H. W. *Biotechnol. Bioeng.* **1998**, *57*, 11–21.
(11) Marušič, J.; Reščič, J.; Jamnik, A.; Tomšič, M. *Acta Chim. Slov.* **2009**, *56*, 172–179.
(12) Kunz, W.; Henle, J.; Ninham, B. W. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 19–37.
(13) McDevit, W. F.; Long, F. A. *J. Am. Chem. Soc.* **1952**, *74*, 1773–1777.
(14) Tadeo, X.; Pons, M.; Millet, O. *Biochemistry* **2007**, *46*, 917–923.
(15) Sedlák, E.; Stagg, L.; Wituung-Stafshede, P. *Arch. Biochem. Biophys.* **2008**, *474*, 128–135.
(16) Ahmad, F.; Bigelow, C. C. *J. Protein Chem.* **1986**, *5*, 355–367.
(17) Henzl, M. T.; Larson, J. D.; Agah, S. *Biochemistry* **2000**, *39*, 5859–5867.
(18) Brewer, J. M.; Wampler, J. E. *Int. J. Biol. Macromol* **2001**, *28*, 213–218.
(19) Ramos, C. H. I.; Baldwin, R. L. *Protein Sci.* **2002**, *11*, 1771–1778.
(20) Vaney, M. C.; Broutin, I.; Retailleau, P.; Douanagamath, A.; Lafont, S.; Hamiaux, C.; Prangé, T.; Ducruix, A.; Riès-Kautt, M. *Acta. Crystallogr., Sect. D* **2001**, *57*, 929–940.
(21) Nagem, R. A. P.; Polikarpov, I.; Dauter, Z. *Methods Enzymol.* **2003**, *374*, 120–137.
(22) Lim, K.; Nadarajah, A.; Forsythe, E.; Pusey, M. L. *Acta. Crystallogr., Sect. D* **1998**, *54*, 899–904.
(23) Saludjian, P.; Prange, T.; Navaza, J.; Ménez, R.; Guilloteau, J. P.; Riès-Kautt, M.; Ducruix, A. *Acta Crystallogr.* **1992**, *B48*, 520–531.
(24) Hribar, B.; Southall, N. T.; Vlachy, V.; Dill, K. A. *J. Am. Chem. Soc.* **2002**, *124*, 12302–12311.
(25) Collins, K. D. *Biophys. J.* **1997**, *72*, 65–76.
(26) Boström, M.; Tavares, F. W.; Finet, S.; Skouri-Panet, F.; Tardieu, A.; Ninham, B. W. *Biophys. Chem.* **2005**, *117*, 217–224.
(27) Tavares, F. W.; Bratko, D.; Blanch, H. W.; Prausnitz, J. M. *J. Phys. Chem. B* **2004**, *108*, 9228–9235.
(28) Boström, M.; Tavares, F. W.; Ninham, B. W.; Prausnitz, J. M. *J. Phys. Chem. B* **2006**, *110*, 24757–24760.
(29) Lund, M.; Vácha, R.; Jungwirth, P. *Langmuir* **2008**, *24*, 3387–3391.
(30) Lund, M.; Vrbka, L.; Jungwirth, P. *J. Am. Chem. Soc.* **2008**, *130*, 11582–11583.
(31) Lund, M.; Jungwirth, P. *J. Phys. Condes. Matter.* **2008**, *20*, 1–4.
(32) Lund, M.; Jungwirth, P. *Phys. Rev. Lett.* **2008**, *100*, 258105.
(33) Scatchard, G.; Black, E. S. *J. Phys. Chem.* **1949**, *53*, 88–99.
(34) Ladbury, J. E.; Chowdhry, B. Z. *Biocalorimetry: Applications of Calorimetry in the Biological Sciences*; John Wiley & Sons: Chichester, UK, 1998.
(35) Poznański, J.; Wszelaka-Ryilk, M.; Zielenkiewicz, W. *Biophys. Chem.* **2005**, *113*, 137–144.
(36) Lah, N.; Lah, J.; Zegers, I.; Wyns, L.; Messens, J. *J. Biol. Chem.* **2003**, *278*, 24673–24679.
(37) Turnbull, W. B.; Daranas, A. H. *J. Am. Chem. Soc.* **2003**, *125*, 14859–14866.
(38) Lah, J.; Vesnaver, V. *J. Mol. Biol.* **2004**, *342*, 73–89.

(39) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. N. *Anal. Biochem.* **1989**, *179*, 131–137.
(40) Hallen, D. *Pure Appl. Chem.* **1993**, *65*, 1527–1532.
(41) Lopez, M. M.; Makhatadze, G. I. *Methods Mol. Biol.* **2002**, *173*, 121–126.
(42) Lah, J.; Maier, N. M.; Lindner, W.; Vesnaver, G. *J. Phys. Chem. B* **2001**, *105*, 1670–1678.
(43) Record, M. T.; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, *2*, 103–178.
(44) Makhatadze, G. I.; Lopez, M. M.; Richardson, J. M., III; Thomas, S. T. *Protein Sci.* **1998**, *7*, 689–697.
(45) Scatchard, G.; Yap, W. T. *J. Am. Chem. Soc.* **1964**, *86*, 3434–3438.
(46) Ahmad, A.; Sohail Akhtar, Md.; Bhakuni, V. *Biochemistry* **2001**, *40*, 1945–1955.
(47) Suelter, C. H. *Science* **1970**, *168*, 789–795.
(48) Alexander, P. A.; Ruan, B.; Bryan, P. N. *Biochemistry* **2001**, *40*, 10634–10639.
(49) Hohenester, E.; Keller, J. W.; Jansonius, J. N. *Biochemistry* **1994**, *33*, 13561–13570.
(50) Peracchi, A.; Mozzarelli, A.; Rossi, G. L. *Biochemistry* **1995**, *34*, 9459–9465.
(51) Henzl, M. T.; Larson, J. D.; Agah, S. *Biochemistry* **2000**, *39*, 5859–5867.
(52) http://www.merck-chemicals.si/life-science-research/lysozymechicken-egg-white/EMD_BIO-4403/p_uuid?ProductID=ji.b.s1Oy7AAAAEWMs8W4z8N&PortalCatalogID=merck4biosciences.
(53) Sophianopoulos, A. J.; Van Holde, K. E. *J. Biol. Chem.* **1964**, *239*, 2516–2524.
(54) Lah, J.; Pohar, C.; Vesnaver, V. *J. Phys. Chem. B* **2000**, *104*, 2522–2526.
(55) Lah, J.; Bešter-Rogač, M.; Perger, T. M.; Vesnaver, V. *J. Phys. Chem. B* **2006**, *110*, 23279–23291.
(56) Lah, J.; Drobnak, I.; Dolinar, M.; Vesnaver, V. *Nucleic Acids Res.* **2008**, *36*, 897–904.
(57) van Holde, K. E.; Johnson, W. C.; Ho, P. S. *Principles of Physical Biochemistry*; Prentice Hall: Upper Saddle River, NJ, 1998.
(58) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry*: W. H. Freeman and Company: San Francisco, CA, 1980.
(59) Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical recipes in C++*; Cambridge University Press: Oxford, 1992; pp 650−694.
(60) Turnbull, W. B.; Daranas, A. H. *J. Am. Chem. Soc.* **2003**, *125*, 14859–14866.
(61) McGuffee, S. R.; Elcock, A. H. *J. Am. Chem. Soc.* **2006**, *128*, 12098–12110.
(62) Sibille, L.; Pusey, M., L. *Acta Crystallogr.* **1994**, *D50*, 396–397.
(63) Bešter-Rogač, M.; Neueder, R.; Barthel, J. *J. Solution Chem.* **1999**, *28*, 1071–1086.
(64) Fennell, C. J.; Bizjak, A.; Vlachy, V.; Dill, K. A. *J. Phys. Chem. B* **2008**, *113*, 6782–6791.
(65) Sharp, K. A.; Madan, B. *J. Phys. Chem. B* **1997**, *101*, 4343–4348.

JP9071845