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PREFERENTIAL SOLVATION OF LYSOZYME BY DIMETHYL SULFOXIDE IN BINARY SOLUTIONS OF WATER AND DIMETHYL SULFOXIDE

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To reveal the denaturation mechanism of lysozyme by dimethyl sulfoxide (DMSO), thermal stability of lysozyme and its preferential solvation by DMSO in binary solutions of water and DMSO was studied by differential scanning calorimetry (DSC) and using densities of ternary solutions of water (1), DMSO (2) and lysozyme (3) at 298.15 K. A significant endothermic peak was observed in binary solutions of water and DMSO except for a solution with a mole fraction of DMSO (x_2) of 0.4. As x_2 was increased, the thermal denaturation temperature T_m decreased, but significant increases in changes in enthalpy and heat capacity for denaturation, ΔH_{cal} and ΔC_p, were observed at low x_2 before decreasing. The obtained amount of preferential solvation of lysozyme by DMSO ($\partial g_2/\partial g_3$) was about 0.09 g g^{-1} at low x_2, indicating that DMSO molecules preferentially solvate lysozyme at low x_2. In solutions with high x_2, the amount of preferential solvation ($\partial g_2/\partial g_3$) decreased to negative values when lysozyme was denatured. These results indicated that DMSO molecules do not interact directly with lysozyme as denaturants such as guanidine hydrochloride and urea do. The DMSO molecules interact indirectly with lysozyme leading to denaturation, probably due to a strong interaction between water and DMSO molecules.

Keywords: *denaturation, dimethyl sulfoxide, lysozyme, preferential solvation, water*

Introduction

Dimethyl sulfoxide (DMSO), a typical aprotic solvent having both polar and nonpolar groups, is an important solvent in chemistry, biotechnology and medicine for dissolution of various substances and as an antifreezing agent for living cells [1, 2]. Water is also an important solvent in various fields, especially biology. Water plays an essential role in inter- and intra-molecular interactions such as hydrogen bonding, hydrophobic interactions and ionic bonding that are required in stabilizing protein conformation. The formation of a protein involves a delicate energy balance among these interactions. Therefore, additives such as salts, sugars, denaturants and alcohols influence conformation and stability of proteins via a slight perturbation of water, reducing their activity [3, 4]. Numerous thermodynamic studies have been conducted to determine the properties of binary solutions of water and DMSO indicate a strong interaction between water and DMSO molecules [5–7]. There is a large interest in studying how such a strong interaction can influence protein conformation in this solution.

When a protein dissolves in a binary solution of water and an additive, the three components do not mix equally, and water or the additive preferentially or selectively exist in solvation shell of the protein. Such a difference in the solvent components between bulk solvent and the solvation shell has been described as preferential solvation. A theory of preferential solvation was proposed by Schachman & Lauffer and Casassa & Eisenberg [8, 9]. Timasheff *et al.* determined the preferential solvation of proteins by alcohols and denaturants, and showed that protein unfolding is directly related to the binding of denaturant molecules to particular groups on proteins [10–13]. Through understanding which components influence protein conformation and stability, preferential solvation provides information on the relative affinities to proteins and is, therefore, an effective approach for revealing the mechanism of denaturation.

Lysozyme is a monomeric protein composed of 129 amino acids. Many structural and biophysical studies have been conducted using lysozyme as a model protein [14–16]. In this study, thermal stabilities of lysozyme in binary solutions of water and DMSO were measured by differential scanning calorimetry (DSC). The mechanisms of lysozyme denaturation induced by DMSO were discussed on the basis of preferential solvation of DMSO determined by a new approach.

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Experimental

Materials

Hen egg-white lysozyme was purchased from Sigma (six times recrystallized). DMSO (Kanto Kagaku, spectroscopy grade) was distilled over freshly activated molecular sieves 4 Å under a reduced pressure of 0.3 kPa at 327 K. Water was distilled twice before use.

Sample preparation

In this study, mole fraction of DMSO (x_2) could be regarded as two-component systems containing water (1) and DMSO (2) because the amount of lysozyme (3) was negligible compared to the amounts of DMSO and water. All sample solutions were prepared according to the method described in our previous paper [17]. We did not use buffer in this study because of unclear concentration definition and effect of buffer in the binary solvent and a high stability of lysozyme against pH change. The concentrations of lysozyme were determined using dilution factors obtained from gravimetric and density data for the solvents and solutions. The mole fractions of DMSO (x_2) were determined with an accuracy of 10^{-4}, and the concentrations of lysozyme were calculated with an accuracy of 10^{-5} g cm^{-3}. The differences in x_2 between the solvents and solutions were accurate to within 10^{-4}. The solutions were incubated for at least 6 h at 278 K to allow lysozyme to reach a stable state in the DMSO solution. All sample solutions were clearness and any precipitate was not shown before and after all measurements, supporting that lysozyme existed without insoluble aggregation in these solutions.

Methods

Differential scanning calorimetry

Thermal stability of lysozyme was monitored with a high sensitivity differential scanning calorimeter (MicroCal MCS) with a scanning rate of 1 K min^{-1}. The protein concentrations in the solutions were 1 mg cm^{-3}. All sample solutions and reference solvents were degassed at least 5 min before DSC measurements. A solvent blank was measured before and after each set of experiments. Thermal denaturation temperature, T_m; calorimetric enthalpy change, ΔH_{cal}; van't Hoff enthalpy change, ΔH_{vH}; heat capacity change, ΔC_p; and the half-value width of the denaturation, $T_{1/2}$ were calculated with software supplied with this instrument.

Density measurement

Densities of solutions were measured with a DMA 512/60 (Anton Paar, precision $\pm 10^{-6}$ g cm^{-3}) digital density meter calibrated with dry air and double-distilled water. The gravimetric data were obtained using a Sartorius BP210 with a precision of 10^{-5} g. Temperatures were controlled at 298.15±0.001 K with a TC100 (Tokyo Riko).

Results and discussion

Differential scanning calorimetry

DSC curves of lysozyme in binary solutions of water and DMSO are shown in Fig. 1. x_2 was 0, 0.05, 0.10, 0.20, 0.30 and 0.40 corresponding to the DMSO mass percentages of 0, 18.5, 32, 52, 65 and 74%, respectively. A significant endothermic peak was observed in each binary solution except for the one with x_2=0.4. The endothermic nature of reaction indicates that

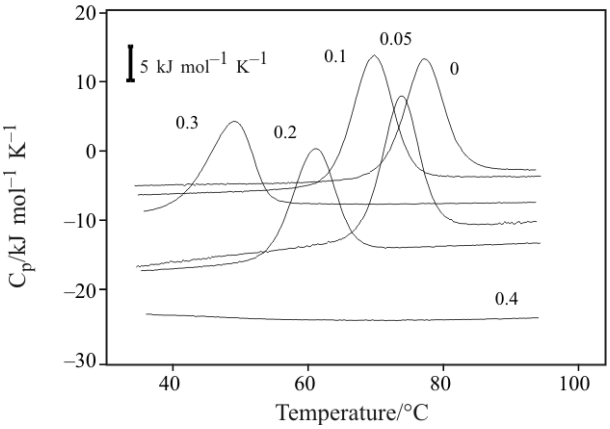

Fig. 1 Heat capacity of lysozyme in binary solutions of water and DMSO. The numbers next to the curves represent the mole fractions of DMSO, x_2

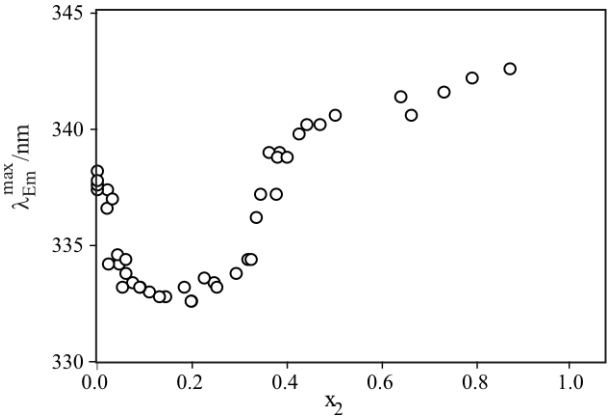

Fig. 2 Maximum emission wavelength of lysozyme, λ_{Em}^{max}, in binary solutions of water and DMSO at 298.15 K [17]

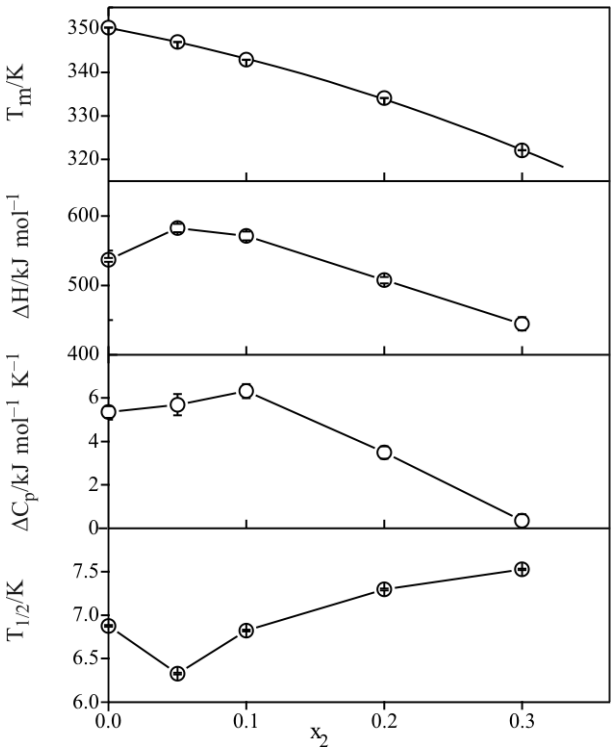

Fig. 3 Dependence of thermodynamic properties for thermal denaturation of lysozyme on the mole fraction of DMSO, x_2

lysozyme unfolds by heating. The fact that no distinct peak could be seen at x_2=0.4 implies that at this mole fraction, lysozyme unfolds due to the addition of DMSO at room temperature. The unfolding was also shown in fluorescence results. Figure 2 shows the dependence of the maximum emission wavelength of lysozyme on x_2 at 298.15 K. As shown in Fig. 2, lysozyme was denatured at x_2>0.4 through an intermediate state at around x_2=0.2. Such conformational changes were also observed in our previous studies focusing on partial specific volume, intrinsic viscosity and free energy of activation for viscous flow of lysozyme [17–19]. These conformational changes were also reflected in our DSC results. The obtained thermodynamic properties – T_m, ΔH_{cal}, ΔC_p and $T_{1/2}$ – are listed in Table 1 and shown in Fig. 3. T_m decreased with an increase in DMSO concentration, according to the equation T_m=350.25–58.89x_2–115.6x_2^2. The estimated x_2 for denaturation at room temperature is about 0.46, consistent with the results of fluorescence measurements shown in Fig. 2. Interestingly, at low x_2, ΔH_{cal} increased with an increase in x_2 before decreasing. A similar trend was observed in ΔC_p, whereas $T_{1/2}$ first decreased and then increased with an increase in x_2. ΔC_p of a protein reflects the difference in the amount of hydration. Therefore, the increase in ΔC_p indicates that dehydration occurs in the native state because of the specific binding of DMSO molecules to lysozyme. Such specific binding of DMSO was observed by NMR and crystal structure analysis with X-ray and neutron diffraction [20–22]. The specific binding of DMSO would increase co-operativity of the denaturation, reflecting the decrease in $T_{1/2}$ at x_2=0.05. The ratio of ΔH_{vh} to ΔH_{cal} reflects the number of states in the thermal transition [23, 24]. The ratios determined in solutions with various x_2 values are listed in Table 1. In water, the ratio was close to 1.0, indicating that the thermal transition consists of two states, native and denatured. Interestingly, the ratio decreased slightly to 0.91 with an increase in x_2. The decrease in the ratio indicates that a stable intermediate exists in the thermal transition. Such an intermediate state induced by DMSO was consistently observed in the increases in ΔH_{cal} and ΔC_p at low x_2. These effects of DMSO on the specific properties at low x_2 and the denaturation mechanism can be explained by the preferential solvation theory, according to the following discussion.

Preferential solvation

The partial specific volume of a protein in a solution at infinite dilution was calculated using the following equations [25]:

$$v^{\circ} = \lim_{c\to 0} v_{app} = \lim_{c\to 0} \frac{1}{c}(1-V_0) \quad (1)$$

$$V_0 = \frac{d-c}{d_0} \quad (2)$$

here v_{app} is the apparent partial specific volume of the protein in the solution; d_0 and d are the densities of the solvent and solution, respectively; and c is the con-

Table 1 Thermodynamic properties for denaturation of lysozyme in binary solutions of water and DMSO at 298.15 K

x_2	T_m/°C	ΔH_{cal}/kJ mol^{-1}	ΔC_p/kJ mol^{-1} K^{-1}	$T_{1/2}$/°C	$\Delta H_{vh}/\Delta H_{cal}$
0.00	77.19±0.01	536.7±2.8	5.36±0.27	6.88±0.01	1.01±0.01
0.05	73.87±0.01	582.6±6.0	5.69±0.49	6.33±0.01	0.97±0.01
0.10	69.80±0.02	571.2±6.3	6.32±0.32	6.82±0.01	0.91±0.01
0.20	60.96±0.01	507.6±4.6	3.49±0.28	7.30±0.01	0.93±0.01
0.30	48.95±0.02	444.7±9.6	0.35±0.30	7.53±0.01	0.91±0.01
0.40	–	–	–	–	–

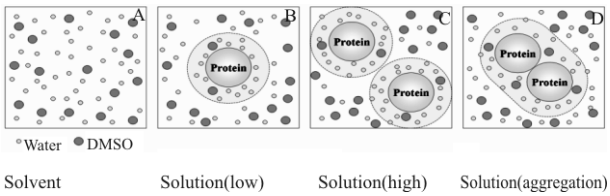

Fig. 4 Model of preferential hydration to protein at different protein concentrations. A – Solvent: binary solution of water and DMSO. B – Solution (low): low concentration of lysozyme in the solvent. C – Solution (high): high concentration of lysozyme in the solvent. D – Solution (aggregation): aggregation model in the solvent

centration of the protein in grams per millilitre of the solution. V_0 is the apparent volume fraction of the solvent in the solution.

When a protein is dissolved in water, the density of bulk solvent will always be constant because water is the only component of the solvent even though the concentration of the protein is changed. However, it should be noted that when a protein is dissolved in a binary solution, the density of the components of bulk solvent will be influenced as a result of preferential solvation or hydration of the protein. Figure 4 shows a model of preferential hydration of a protein at different concentrations in a binary solution of water and DMSO. The dotted cirle shows hidration or solvation area, and the area outside the circle shows bulk solvent. When water binds preferentially to a protein or hydrates it (B), the mole fraction of DMSO in the bulk solvent increases compared to the original solvent (A) because water molecules tend to exist in the solvation shell of the protein due to preferential hydration. When the concentration of the protein increases, the amount of hydration increases, causing an increase in x_2 of bulk solvent (C). The mole fraction of DMSO bulk solvent depends on protein concentration. The change in x_2 of bulk solvent leads to a change in its density. Therefore, the change in x_2 of bulk solvent is reflected by the concentration dependence of the apparent partial specific volume of the protein v_{app} via the change in apparent volume fraction of the solvent in solution V_0, according to Eq. (2). The concentration dependences B were calculated using Eq. (3) with our previous density data in solutions with various x_2 values [17].

$$v_{app} = v^{o} + Bc \tag{3}$$

The obtained B values are listed together with v^o and standard deviation of the fit in Table 2. All standard deviation were significantly small suggesting that the Eq. (3) was appropriate in the concentration ranges used. In water, the concentration dependence B_0 was small, 0.202±0.186 $(cm^3\ g^{-1})^2$, compared to other globular proteins. However, it is interesting that

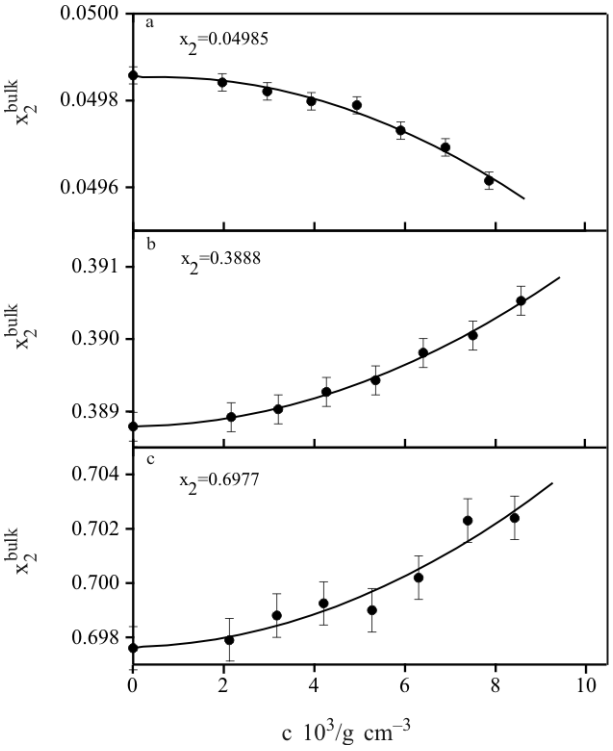

Fig. 5 Expected mole fractions of DMSO in bulk solvent x_2^{bulk} *vs.* the concentration of lysozyme in solutions with typical x_2 values at 298.15 K

the B values varied significantly with x_2. These variations in B indicate that DMSO or water molecules would bind preferentially to lysozyme, and the amount of solvation or hydration would increase with an increase in lysozyme concentration. At a specific mole fraction of DMSO and lysozyme concentration, the density of the bulk solvent d_{x2}^{bulk} that was perturbed by preferential solvation or hydration could be estimated according to Eq. (4) derived from Eq. (1),

$$d_{x_2}^{bulk} = \frac{d - c}{1 - v_{x_2}^{o} c - B_0 c^2} \tag{4}$$

here $v_{x_2}^{o}$ is the partial specific volume of lysozyme at infinite dilution at a specific x_2, and d is the density of solution at a protein concentration c. In this system, the bulk solvent is the binary solution of water and DMSO whose density can be obtained with the Redlich–Kister equation

$$d_{x_2}^{bulk} = (1 - x_2^{bulk}) d_1 + x_2^{bulk} d_{x_1} + x_2^{bulk} (1 - x_2^{bulk}) \sum_{i=1}^{3} \{A_i (1 - 2x_2^{bulk})^{i-1}\} \tag{5}$$

here x_2^{bulk} is the mole fraction of DMSO in the bulk solvent, and d_1 is the density of water. A_1, A_2 and A_3, fit parameters obtained from our previous data, are 0.20965, 0.17394 and 0.07882, respectively. The density can be estimated by using Eq. (5) with an accuracy of 10^{-5} g cm^{-3} at least [17]. According to Eqs (4)

Table 2 Volumetric properties, dependence of mole fraction of bulk solvent on lysozyme concentration and preferential hydration and solvation of lysozyme in binary solutions of water and DMSO at 298.15 K

x_2	v^o/cm^3 g^{-1}	Slope B/(cm^3 g^{-1})^2	10^3(sd)/cm^3 g^{-1}	E	$F\cdot10^8$/cm^3 g^{-1}	$G\cdot10^{10}$/(cm^3 g^{-1})^2	10^3(sd)	r	$\partial g_1/\partial g_3$/g g^{-1}	$\partial g_2/\partial g_3$/g g^{-1}
0	0.712±0.001	0.202±0.186	0.7	–	–	–			–	–
0.0248	0.713±0.001	1.519±0.166	0.9	0.0248	10.47	–287.4	0.01	0.989	–0.63±0.32	0.07±0.04
0.0499	0.713±0.001	1.798±0.194	1.0	0.0499	52.04	–434.9	0.01	0.993	–0.38±0.23	0.09±0.05
0.1299	0.714±0.003	1.244±0.528	2.9	0.1299	69.41	–427.3	0.06	0.874	–0.11±0.07	0.07±0.05
0.1994	0.715±0.003	0.258±0.646	3.0	0.1994	–221.0	298.0	0.08	0.044	0.13±0.03	–0.14±0.03
0.2497	0.719±0.001	1.045±0.171	0.9	0.2497	64.50	–770.1	0.03	0.988	–0.10±0.05	0.14±0.08
0.3004	0.720±0.001	0.080±0.239	1.1	0.3004	28.63	71.1	0.05	0.503	0.01±0.00	–0.02±0.01
0.3465	0.729±0.001	–0.502±0.142	0.9	0.3465	30.72	1077	0.08	0.981	0.10±0.05	–0.23±0.11
0.3888	0.730±0.000	–0.820±0.074	4.6	0.3888	63.10	2258	0.05	0.998	0.18±0.08	–0.49±0.22
0.4326	0.732±0.002	–0.766±0.279	0.4	0.4326	1320	762.2	0.17	0.959	0.18±0.02	–0.61±0.08
0.5046	0.734±0.002	–0.792±0.304	1.5	0.5046	–2350	12605	0.90	0.956	0.45±0.30	–1.97±1.33
0.5478	0.729±0.003	0.266±0.437	2.1	0.5478	3609	–5682	1.78	0.330	0.00±0.12	–0.02±0.64
0.6977	0.711±0.001	0.743±0.218	1.6	0.6976	345.5	6669	0.54	0.969	0.22±0.10	–2.19±0.96
0.7508	0.711±0.001	0.762±0.171	1.0	0.7508	5842	3000	1.01	0.923	0.33±0.04	–4.31±0.49
0.7975	0.707±0.001	0.684±0.169	2.5	0.7975	1842	2116	0.41	0.946	0.12±0.02	–2.09±0.42
0.8989	0.705±0.001	2.116±0.267	0.9	0.8989	3259	20911	0.22	0.999	0.50±0.19	–19.4±7.23

and (5), x_2^{bulk} can be estimated with an accuracy of 10^{-4} at a specific concentration of lysozyme, c, in a solution with specific x_2. In Fig. 5, the obtained x_2^{bulk} values are plotted *vs.* the concentration of lysozyme in some solutions with typical x_2 values. The lines were fitted by the secondary least-squares method where $x_2^{bulk}=E+Fc+Gc^2$, and obtained parameters, standard deviations, and correlation coefficients of the fits, r, are listed in Table 2. The correlation is not good at x_2=0.2, 0.3 and 0.6, because the change in x_2^{bulk} with an increase in c is negligible as the values of slope B in these solutions are similar to that in water, B_0. As shown in Figure 5a, with the low x_2=0.05 solution, the obtained x_2^{bulk} decreased with an increase in c, suggesting that DMSO molecules bind preferentially to lysozyme leading to a decrease in x_2^{bulk}, because the number of DMSO molecules in the bulk solvent decreased due to the specific binding to lysozyme. On the other hand, in the solutions where x_2>0.1, the obtained x_2^{bulk} increased with an increase in c. This suggests that either DMSO molecules are preferentially excluded from the solvation shell of lysozyme or water molecules bind preferentially to lysozyme, leading to the increase in x_2^{bulk}. Such a preferential binding of water or DMSO to lysozyme is called preferential hydration or solvation, respectively, as shown in Fig. 4. The amount of preferential hydration and solvation is calculated from the mass of water (g_1) and DMSO (g_2), respectively, per gram of lysozyme (g_3) transferred from bulk solvent to the hydration or solvation area of lysozyme. The amount of preferential hydration and solvation is denoted ($\partial g_1/\partial g_3$) and ($\partial g_2/\partial g_3$), respectively, and calculated from the difference between the obtained x_2^{bulk} and x_2. The obtained ($\partial g_1/\partial g_3$) and ($\partial g_2/\partial g_3$) in various binary solutions of water and DMSO are listed with their standard deviations in Table 2, and plotted *vs.* x_2 in Fig. 6. Those values were averaged for different concentrations of lysozyme. It is noted that the ($\partial g_2/\partial g_3$) value at the highest x_2=0.8989 was comparable smaller, –19.4±7.2 g g^{-1}, because the Eq. (4) might be inappropriate in this x_2 probably due to a decrease in accessible surface area of lysozyme by a possible soluble aggregation as shown in Fig. 4D. Interestingly, ($\partial g_1/\partial g_3$) and ($\partial g_2/\partial g_3$) were significantly dependent on x_2. In solutions with low x_2, ($\partial g_1/\partial g_3$) was negative and ($\partial g_2/\partial g_3$) was positive, suggesting that water molecules are preferentially excluded from the solvation shell of lysozyme or DMSO molecules bind preferentially to lysozyme. It should be noted that the positive values of ($\partial g_2/\partial g_3$) are consistent with the specific binding of DMSO to lysozyme at low x_2, confirmed by NMR, crystal structure analysis [20–22] and our DSC results. The maximum ($\partial g_2/\partial g_3$) in low x_2 solution was 0.09±0.05 g g^{-1}, corresponding to

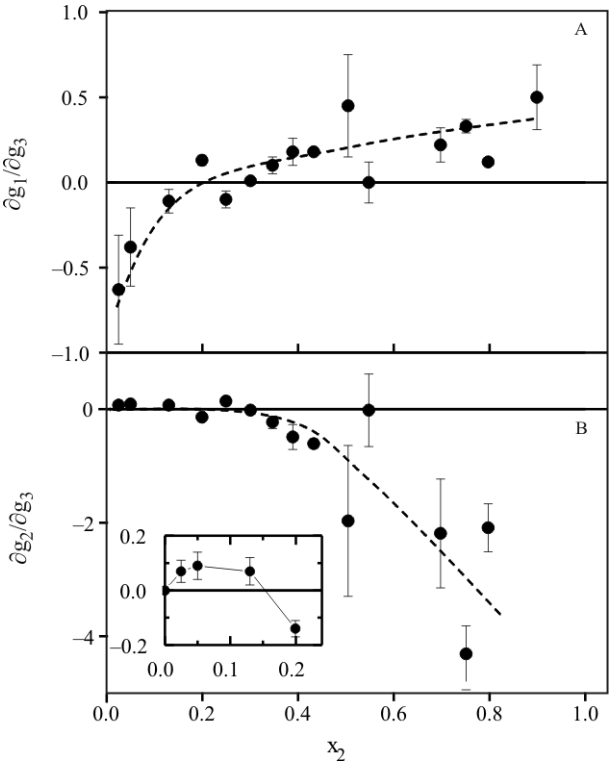

Fig. 6 Plots of amounts of preferential hydration (A) and solvation (B) with lysozyme in binary solutions of water and DMSO *vs.* mole fractions of DMSO, x_2, at 298.15 K. The inset in B shows expanded plots at low x_2

17±9 mol mol^{-1}. Positive ($\partial g_2/\partial g_3$) values indicate that the specific binding of DMSO would be preferred over the exclusion of DMSO around lysozyme by steric exclusion effect proposed by Schachman and Lauffer [8].

When lysozyme was denatured in solutions with high x_2, ($\partial g_1/\partial g_3$) increased to positive values and ($\partial g_2/\partial g_3$) decreased to negative values. DMSO molecules were preferentially excluded from the solvation shell of lysozyme in the denatured state. Timasheff *et al.* determined the amount of preferential solvation of lysozyme by two typical denaturants at high concentration, urea (8 M) and guanidine hydrochloride (6 M), to be 0.05 and 0.09 g g^{-1}, respectively [26]. The positive values for urea and guanidine hydrochloride indicate that these denaturants interact directly with lysozyme while causing denaturation of lysozyme. In contrast, negative ($\partial g_2/\partial g_3$) values were observed when lysozyme was denatured in solutions with high x_2. This suggests that, in contrast to the denaturants, DMSO molecules in high x_2 solutions interact indirectly with lysozyme through interaction with water. These results suggest that a strong interaction exists between water and DMSO molecules, a fact revealed by many studies [5–7]. This interaction influences the conformation of lysozyme and leads to denaturation.

Conclusions

In this study, the stability of lysozyme in binary solutions of water and DMSO was studied by DSC and preferential solvation. The obtained amount of preferential solvation of lysozyme by DMSO ($\partial g_2/\partial g_3$) was positive values at low x_2, and the ($\partial g_2/\partial g_3$) decreased to negative values when lysozyme was denatured at high x_2. DMSO molecules were preferentially bound to lysozyme in its native state and excluded from the solvation shell of lysozyme in its denatured state. Such preferential binding of DMSO reflected on the significant increases in ΔH_{cal} and ΔC_p observed at low x_2. The DMSO molecules interact indirectly with lysozyme leading to denaturation, probably due to a strong interaction between water and DMSO molecules.

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