Volumetric Study of Lysozyme in Dimethyl Sulfoxide + Water Solution at 298.15 K

Tadashi Kamiyama,* Takanori Matsusita, and Takayoshi Kimura

Department of Chemistry, School of Science and Engineering, Kinki University, Kowakae 3-4-1, Higashi-Osaka, Osaka 577-8502, Japan

The densities, *d*, and fluorescence spectra of ternary mixtures of water (1) + dimethyl sulfoxide (2) + lysozyme (3) were measured, and the partial specific volumes of lysozyme, $\bar{v}°$, were determined as the extrapolated values of the apparent partial specific volumes at an infinitely dilute concentration of lysozyme at 298.15 K. A binary mixture of water + dimethyl sulfoxide has a minimum excess volume when the mole fraction of dimethyl sulfoxide (x_2) is in the range 0.25 to 0.42, and lysozyme unfolds and aggregates within this range. This indicates that the strong interaction between water and dimethyl sulfoxide might influence the conformation of lysozyme by interfering water−lysozyme interactions. In the range $x_2 > 0.8$, lysozyme was in a disaggregated−denatured state, having a partial specific volume of (0.706 ± 0.002) $cm^3 \cdot g^{-1}$, which was smaller than that in the native state, (0.712 ± 0.001) $cm^3 \cdot g^{-1}$, probably because the unfolding led to a decreased cavity volume of lysozyme in the solvent.

Introduction

The native conformation of a protein is produced by a delicate balance between covalent bonds and noncovalent bonds such as hydrogen bonds, electrostatic interactions, and hydrophobic interactions. Therefore, the native conformation usually depends not only on temperature and pressure but also on the nature of the solvent, such as its polarity and dielectric constant.

The aprotic solvent dimethyl sulfoxide (DMSO) has both polar and nonpolar groups and is used by the cell as an antifreezing agent and for the dissolution of various substances. Many thermodynamic studies have been carried out for determining properties of DMSO aqueous solutions such as excess enthalpies, excess volumes, and freezing point depression.[1−3] A matter of concern is how the thermodynamic properties of DMSO aqueous solutions can influence a protein's structure when it is in this solution. There are few experimental data regarding protein and amino acids in DMSO aqueous solutions,[4,5] although there have been a few measurements carried out in aqueous solutions with low concentrations of DMSO.[6−9]

The packing density of atoms in a protein molecule is directly related to its volumetric properties. The partial specific volume of a protein in solution involves not only the volume of the protein itself but also the contributions of protein−solvent interactions. Therefore, the partial specific volume of a protein can sensitively reflect conformational changes due to temperature, ligand binding, and cosolvents.[10−12] In this work, hen egg white lysozyme was dissolved in aqueous solutions with various DMSO concentrations, and the solutions were measured with a density meter and fluorescence spectrophotometer at (298.15 ± 0.001) K. To discern the volumetric properties of ternary mixture solutions {water (1) + DMSO (2) + lysozyme (3)} as well as the effects of binary mixture solvents {water (1) + DMSO (2)} on the conformation of lysozyme, the excess volumes of the solvents and solutions and the partial specific volumes of lysozyme were determined in various DMSO concentrations. Lysozyme is a monomeric protein composed of 129 amino acids. Many structural and biophysical studies have been carried out using lysozyme as a model protein.[13,14]

* Corresponding author. E-mail address: kamiyama@chem.kindai.ac.jp. Telephone number: +81(6)-6721-2332 (ext 4111). Fax number: +81-(6)-6723-2721.

Experimental Section

Materials. Hen egg white lysozyme was purchased from Sigma (six times recrystallized, lot no. 90K1922). Dimethyl sulfoxide (DMSO, Kanto Kagaku, spectroscopy grade) was distilled over freshly activated molecular sieves 4A under a reduced pressure of 0.3 kPa at 327 K. Water was distilled twice.

Sample Preparation. In this study, mole fractions of DMSO (x_2) could be regarded as containing two components {water (1) + DMSO (2)} because the amount of lysozyme (3) was negligible as compared to the amounts of DMSO and water used. Several solutions of lysozyme (2 to 10 $mg \cdot cm^{-3}$) in aqueous solutions containing various mole fractions of DMSO ($x_2 = 0$ to 0.9) were prepared by the following procedures. To prepare DMSO at various mole fractions, x_2, the solvents were prepared by diluting DMSO appropriately into water. A series of high-concentration stock lysozyme solutions was prepared by mixing appropriate amounts of lysozyme with water and DMSO, to preserve the exact x_2 of DMSO in the solvent. The serial dilutions of lysozyme were then prepared by diluting the stock solutions into solvents having the same mole fractions of DMSO. The stock solutions, water, DMSO, binary solvent mixtures, and so forth, were weighed after being mixed. Precise x_2 values for the solvents (water + DMSO) and solutions (water + DMSO + lysozyme) were calculated using the gravimetric data for DMSO and water. The concentrations of lysozyme were determined using dilution factors that were obtained from gravimetric and density data for the solvents and solutions. The x_2 values were determined with an accuracy of 10^{-4}, and the concentrations of lysozyme were calculated with an accuracy of 10^{-5}

10.1021/je0340710 CCC: $25.00
Published on Web 08/21/2003

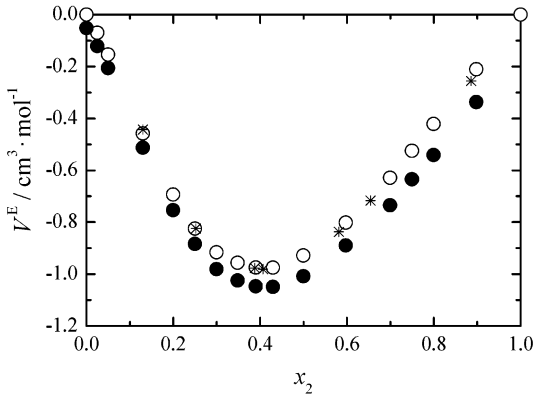

Figure 1. Excess molar volumes of a solvent and solution depending on the mole fraction of dimethyl sulfoxide (x_2): ○, solvent; ∗, solvent (Lau et al.[1]); ●, solution (c = 8.0 mg·cm^{-3}).

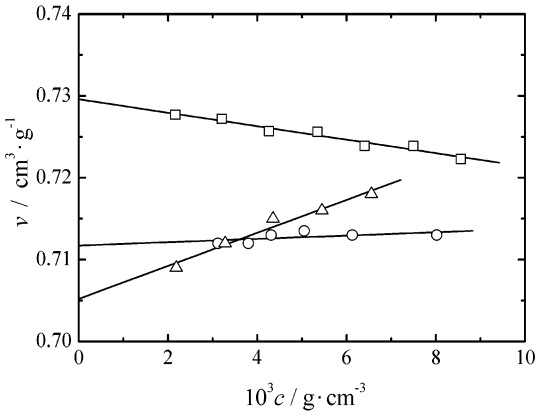

Figure 2. Apparent partial specific volumes of lysozyme depending on the concentration of lysozyme, c, as shown by a few typical mole fractions of DMSO (x_2): ○, x_2 = 0.000; □, x_2 = 0.3896; △, x_2 = 0.8978.

g·cm^{-3}. The differences in x_2 between the solvents and the solutions were accurate to within 10^{-4}. The solutions were incubated for at least 6 h at 278 K to allow the lysozyme to reach a stable state in the DMSO solution.

Apparatus. Densities of solutions were measured with a DMA 512/60 (Anton Paar, precision ± 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. Fluorescence spectra were measured with a fluorescence spectrophotometer F-4500 (HITACHI). The excitation wavelength was 290 nm. Fluorescence changes in this range can reflect changes in the solvent environment of amino acids, especially tryptophan. Temperatures were controlled at (298.15 ± 0.001) K with a TC100 (Tokyo Riko).

Calculating Partial Specific Volumes of Proteins. The partial specific volume of a protein can be derived using the following equation[15]

$$\bar{v}° = \lim_{c \to 0} \frac{1}{c}\left(1 - \frac{d - c}{d_0}\right) \qquad (1)$$

where d is the density of the solution, d_0 is the density of the solvent, c is the concentration of protein in grams per milliliter of solution, and $\bar{v}°$ is the partial specific volume of the protein. The partial specific volume of the protein is expressed as the sum of three contributions:[15] (i) the constitutive volume, estimated as the sum of van der Waals volumes of the constitutive atoms (v_c); (ii) the volume of the cavity or void space in the molecule due to imperfect atomic packing (v_{cav}); and (iii) the volume change due to hydration or solvation ($\Delta_{solv}v$)

$$\bar{v}° = v_c + v_{cav} + \Delta_{solv}v \qquad (2)$$

Results and Discussion

The densities of the solutions and the apparent partial specific volumes, v (= $[1 - (d - c)/d_0]/c$), of lysozyme at various x_2 are listed in Table 1. These results were averages of several measurements. The errors in the estimation of apparent partial specific volumes are 10^{-4} (g·cm^{-3}) because the concentration of lysozyme is given with an accuracy of four significant figures. Figure 1 shows the excess molar volumes, V^E, of solvents and solutions at 8.0 mg·cm^{-3} of lysozyme, as compared to the ideal molar volumes of solvents, which were calculated by the following equation: $V_{(ideal)} = x_1V_{(H_2O)} + x_2V_{(DMSO)}$. The minimum V^E occurred at about x_2 = 0.4 for the solution as well as the solvent, indicating that there were only minor changes of volumetric properties on going from solvents to solutions. However, the small changes in density were very important, because they reflected the apparent partial specific volumes of lysozyme according to eq 1. Figure 2 shows the dependence of the apparent partial specific volume of lysozyme on the concentration of lysozyme at a few typical x_2 values. The partial specific volumes of lysozyme were determined as extrapolated values of the apparent partial specific volumes at infinitely dilute concentration of lysozyme, by a linear least-squares method, $v = \bar{v}° + Bc$. The errors of the extrapolated partial specific volumes were obtained mathematically as intercept errors; in other words, they were the standard deviations of the intercepts. The partial specific volumes of lysozyme calculated at infinite dilution are accurate to about 10^{-3} (g·cm^{-3}). The variability arises due to sample preparation methods, determination of concentration, and the inherent accuracy of various measurements. The partial specific volumes of lysozyme are listed in Table 2 with slopes (B) and standard deviations obtained by the linear least-squares method. It should be noted that the slopes, or B, were significantly dependent on x_2, reflecting conformational changes of lysozyme and/or changes in its interaction with solvent.[16] The partial specific volume of lysozyme in its native state (in water) was (0.712 ± 0.001) cm^3·g^{-1}, which was consistent with values in the literature.[15]

Figure 3 shows that the partial specific volume and the maximum emission wavelength of lysozyme depend on the mole fraction of DMSO, x_2. Four- and three-state transitions were clearly shown, as revealed by the partial specific volumes and the fluorescence values, respectively. It has been suggested that partial specific volumes are more sensitive for detecting conformational changes of proteins, in cases where these changes cannot be observed spectroscopically. When x_2 was less than 0.1, the tryptophan residues were surrounded by DMSO molecules from the water, as revealed by the red shift of the fluorescence peak from 337.5 to 333 nm. However, when x_2 > 0.3, the tertiary structure of the protein is modified, exposing its tryptophan residues to water, as revealed by the blue shift from 333 to 340 nm. The results of these fluorescence measurements indicated that lysozyme was denatured after going through an intermediate state in this denaturation system. The minimum excess volume of the binary solutions is shown at the mole fraction x_2 = 0.4, and lysozyme unfolds and aggregates at this same mole fraction. Water−protein interactions are very important effects for maintaining the native conformation of a protein through hydrogen bonds,

Table 1. Densities of Solutions (d) and Apparent Partial Specific Volumes of Lysozyme (v) in Various Concentrations of Lysozyme (c) and Various Mole Fractions of DMSO (x_2)

$x_2 = 0.0000$			$x_2 = 0.0249$			$x_2 = 0.0499$		
10^2c	d	v	10^2c	d	v	10^2c	d	v
g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}
0.0000	0.997044		0.0000	1.009675		0.0000	1.021541	
0.3120	0.997949	0.7120	0.1997	1.010227	0.7166	0.1967	1.022067	0.7168
0.3801	0.998146	0.7121	0.3011	1.010505	0.7174	0.2963	1.022327	0.7191
0.4310	0.998290	0.7130	0.3994	1.010771	0.7184	0.3934	1.022579	0.7205
0.5051	0.998499	0.7141	0.5025	1.011042	0.7209	0.4940	1.022846	0.7202
0.6128	0.998816	0.7129	0.6007	1.011298	0.7228	0.5903	1.023080	0.7237
0.8020	0.999363	0.7130	0.7050	1.011583	0.7223	0.6892	1.023328	0.7250
			0.8025	1.011817	0.7261	0.7852	1.023551	0.7283
A^a	0.288893		A	0.267396		A	0.263751	
r^b	1.0000		r	0.9999		r	0.9998	

$x_2 = 0.1298$			$x_2 = 0.1996$			$x_2 = 0.2497$		
10^2c	d	v	10^2c	d	v	10^2c	d	v
g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}
0.0000	1.052840		0.0000	1.071804		0.0000	1.081388	
0.2036	1.053342	0.7158	0.2149	1.072306	0.7149	0.2086	1.081849	0.7201
0.3076	1.053591	0.7180	0.3245	1.072559	0.7158	0.3134	1.082073	0.7226
0.4075	1.053814	0.7229	0.4288	1.072816	0.7128	0.4168	1.082300	0.7223
0.5131	1.054093	0.7180	0.5374	1.073028	0.7205	0.5236	1.082512	0.7261
0.6118	1.054301	0.7231	0.6468	1.073296	0.7178	0.6236	1.082732	0.7254
0.7188	1.054584	0.7195	0.7734	1.073616	0.7144	0.7325	1.082944	0.7282
0.8195	1.054766	0.7267				0.8372	1.083160	0.7290
A	0.237080		A	0.232229		A	0.211231	
r	0.9996		r	0.9997		r	0.9999	

$x_2 = 0.2994$			$x_2 = 0.3483$			$x_2 = 0.3896$		
10^2c	d	v	10^2c	d	v	10^2c	d	v
g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}
0.0000	1.088196		0.0000	1.092428		0.0000	1.095074	
0.2152	1.088658	0.7217	0.3082	1.093062	0.7272	0.2165	1.095514	0.7277
0.3170	1.088878	0.7213	0.4098	1.093274	0.7264	0.3203	1.095727	0.7272
0.4268	1.089123	0.7192	0.4967	1.093460	0.7251	0.4265	1.095950	0.7257
0.5312	1.089340	0.7211	0.5148	1.093486	0.7272	0.5352	1.096174	0.7256
0.6397	1.089571	0.7215	0.6163	1.093714	0.7244	0.6401	1.096401	0.7239
0.7546	1.089831	0.7198	0.6800	1.093844	0.7248	0.7498	1.096629	0.7239
0.8581	1.090040	0.7215	0.7195	1.093915	0.7262	0.8561	1.096863	0.7223
			0.8189	1.094135	0.7246			
			1.0225	1.094575	0.7232			
A	0.215551		A	0.209346		A	0.208942	
r	1.0000		r	0.9999		r	0.9999	

$x_2 = 0.4296$			$x_2 = 0.4993$			$x_2 = 0.5971$		
10^2c	d	v	10^2c	d	v	10^2c	d	v
g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}
0.0000	1.096889		0.0000	1.098451		0.0000	1.098833	
0.2092	1.097303	0.7314	0.1046	1.098656	0.7319	0.3445	1.099511	0.7308
0.3124	1.097516	0.7289	0.2138	1.098872	0.7310	0.4571	1.099742	0.7290
0.4183	1.097732	0.7281	0.3146	1.099070	0.7313	0.5713	1.099973	0.7283
0.5219	1.097944	0.7274	0.4221	1.099267	0.7343	0.6832	1.100171	0.7318
0.6264	1.098142	0.7294	0.5278	1.099510	0.7278	0.7992	1.100406	0.7309
0.7310	1.098362	0.7280	0.6342	1.099707	0.7300			
0.8335	1.098604	0.7241	0.7414	1.099953	0.7259			
			0.8438	1.100151	0.7270			
A	0.204098		A	0.201939		A	0.196836	
r	0.9998		r	0.9998		r	0.9999	

$x_2 = 0.6993$			$x_2 = 0.7495$			$x_2 = 0.7992$		
10^2c	d	v	10^2c	d	v	10^2c	d	v
g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}	g·cm^{-3}	g·cm^{-3}	cm^3·g^{-1}
0.0000	1.098365		0.0000	1.097814		0.0000	1.097288	
0.2122	1.098827	0.7121	0.2137	1.098277	0.7139	0.2049	1.097747	0.7074
0.3172	1.099047	0.7146	0.3180	1.098511	0.7112	0.3056	1.097967	0.7090
0.4204	1.099267	0.7151	0.4247	1.098705	0.7198	0.4102	1.098191	0.7109
0.5271	1.099502	0.7141	0.5317	1.098940	0.7180	0.4532	1.098291	0.7097
0.6301	1.099714	0.7155	0.6351	1.099134	0.7216	0.6178	1.098638	0.7123
0.7383	1.099927	0.7178	0.7314	1.099347	0.7201	0.7211	1.098869	0.7116
0.8418	1.100150	0.7173	0.8464	1.099593	0.7195	0.8241	1.099093	0.7118
A	0.211817		A	0.208183		A	0.218235	
r	0.9999		r	0.9996		r	0.9999	

Table 1 (Continued)

x_2= 0.8978			x_2 = 1.0000		
10^2c / g·cm^{-3}	d / g·cm^{-3}	v / cm^3·g^{-1}	10^2c / g·cm^{-3}	d / g·cm^{-3}	v / cm^3·g^{-1}
0.0000	1.096282		0.0000	1.095560	
0.2184	1.096766	0.7098			
0.3281	1.097001	0.7122			
0.4353	1.097225	0.7146			
0.5452	1.097452	0.7164			
0.6557	1.097680	0.7177			
A	0.212657				
r	0.9998				

[a] A is the slope; $d = d_0 + Ac$. [b] r is the correlation coefficient.

Table 2. Partial Specific Volumes of Lysozyme, Slope *B*, and Standard Deviations at Various x_2

x_2	$\bar{v}°$ / cm^3·g^{-1}	B / (cm^3·g^{-1})^2	10^3(SD) / cm^3·g^{-1}
0.0000	0.712 ± 0.001	0.202 ± 0.186	0.739
0.0249	0.713 ± 0.001	1.519 ± 0.166	0.886
0.0499	0.713 ± 0.001	1.798 ± 0.194	1.010
0.1298	0.714 ± 0.003	1.244 ± 0.528	2.866
0.1996	0.715 ± 0.003	0.258 ± 0.646	2.990
0.2497	0.719 ± 0.001	1.045 ± 0.171	0.949
0.2994	0.720 ± 0.001	0.080 ± 0.239	1.087
0.3483	0.729 ± 0.001	−0.502 ± 0.142	0.876
0.3896	0.730 ± 0.000	−0.820 ± 0.074	0.419
0.4296	0.732 ± 0.002	−0.766 ± 0.279	1.537
0.4993	0.734 ± 0.002	−0.792 ± 0.304	2.079
0.5971	0.729 ± 0.003	0.266 ± 0.437	1.570
0.6993	0.711 ± 0.001	0.743 ± 0.218	0.958
0.7495	0.711 ± 0.001	0.762 ± 0.171	0.950
0.7992	0.707 ± 0.001	0.684 ± 0.169	0.927
0.8978	0.705 ± 0.001	2.116 ± 0.267	0.920

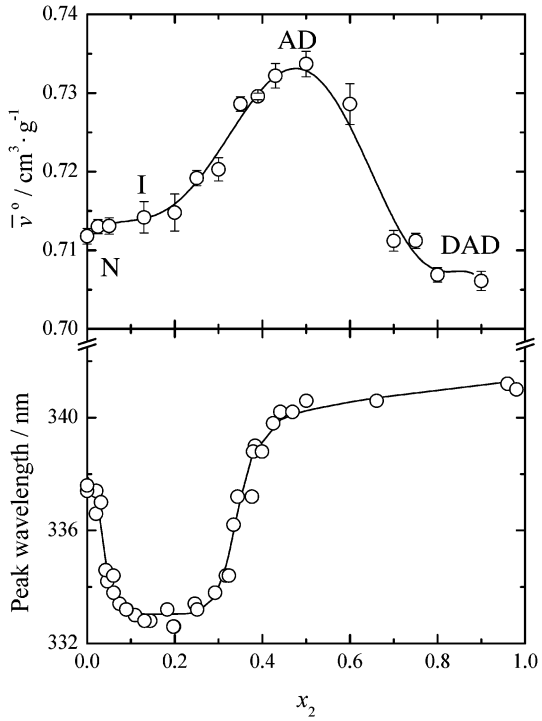

Figure 3. Partial specific volumes and peak emission wavelengths of lysozyme depending on the mole fraction of dimethyl sulfoxide (x_2): N, native state; I, intermediate state; AD, aggregated denatured state; DAD, disaggregated and denatured state.

electrostatic interactions, and hydrophobic interactions. The experimental results described above indicate a strong interaction of water with DMSO. This was also observed in many previous measurements of DMSO aqueous solutions.[1−3] This strong interaction also might interfere with the water−protein interactions, thus destabilizing the native, folded state of the protein.

The changes in partial specific volume of a protein can be explained by changes in two terms, v_{cav} and $\Delta_{solv}v$, because the van der Waals volumes of its constituent atoms are not dependent on conformational changes of the protein. The partial specific volume of lysozyme at the intermediate state was (0.714 ± 0.001) cm^3·g^{-1}, which was slightly increased from the partial specific volume of the native state (+0.002 cm^3·g^{-1}). This increase might be caused by the formation of new cavities and by dehydration due to the binding of several DMSO molecules, as observed in X-ray and neutron diffraction studies.[6,7] In general, the partial specific volume of a denatured protein decreases, because unfolding will decrease the internal cavity volume. It will also increase the extent of hydration, due to the increased surface area of the unfolded state.[11] However, when lysozyme was denatured in the range x_2 = 0.2 to 0.42, there was a significant increase in the partial specific volume of about +0.023 cm^3·g^{-1}, as compared to the case of the native state. The volume increase might occur because new cavities are formed as a result of protein aggregation. Aggregation in this system was followed using quasielastic light scattering measurements.[4] A large decrease of partial specific volume was observed for mole fractions greater than x_2 = 0.5, probably because the cavities disappeared upon disaggregation. However, when $x_2$was greater than 0.8, the partial specific volumes were almost the same. The partial specific volume of the disaggregated, denatured state was about (0.706 ± 0.002) cm^3·g^{-1}. This was smaller than that of the native state (−0.006 cm^3·g^{-1}). The decrease may have been caused by the reduction of cavity volume that accompanies disaggregated unfolding. Infrared radiation spectra of lysozyme in high concentrations of DMSO showed a collapse of β structure in the interior of the molecule,[5] consistent with a decrease in cavity volume inside the hydrophobic core of the lysozyme. The partial specific volume of the disaggregated and denatured state induced by DMSO was larger than that of the denatured state induced by guanidine chloride (0.579 cm^3·g^{-1}).[17] This suggests that the DMSO-induced disaggregated and denatured state might retain many cavities between and within the lysozyme molecules, as compared with the case of the denatured state induced by guanidine chloride.

In this study, lysozyme was gradually denatured with increasing DMSO concentrations. The protein was seen to have an intermediate state and an aggregated denatured state, and both of these states were accompanied by changes of partial specific volume. The strong interaction between water and DMSO thus appears to influence the conformation of lysozyme. Precision densimetry of ternary

solutions containing protein is an effective technique for investigating conformational changes of protein, as well as interactions between proteins and binary mixtures of solvents.

Literature Cited

(1) Lau, C. F.; Wilson, P. T.; Fenby, D. V. Excess volumes of dimethyl sulfoxide mixtures. *Aust. J. Chem.* **1970**, *23*, 1143−1148.

(2) Cowie, J. M. G.; Toporowski, P. M. Association in the binary liquid system dimethyl sulfoxide-water. *Can. J. Chem.* **1961**, *39*, 2240−2243.

(3) Havemeyer, R. N. Freezing point curve of dimethyl sulfoxide-water solutions. *J. Pharm. Sci.* **1966**, *55*, 851−853.

(4) Ma, R. J.; Wang, C. H. Studies of the Protein−Protein interaction of lysozyme in dimethyl sulfoxide-water solutions by quasielastic light scattering. *J. Phys. Chem.* **1983**, *87*, 649−682.

(5) Pinf, H.; Aichun, D.; Winslow, S. C. Effects of dimethyl sulfoxide, glycerol and ethylene glycol on secondary structures of cytochrome *c* and lysozyme as observed by infrared spectroscopy. *J. Pharm. Sci.* **1995**, *84*, 387−392.

(6) Mande, S. C.; Sbhia, M. E. Structural characterization of protein-denaturant interactions: crystal structures of hen egg-white lysozyme in complex with DMSO and guanidinium chloride. *Prot. Eng.* **2000**, *13*, 133−141.

(7) Lehmann, M. S.; Stansfield, R. F. D. Binding of dimethyl sulfoxide to lysozyme in crystal, studied with neutron diffraction. *Biochemistry* **1989**, *28*, 7028−7033.

(8) Hohannesson, H.; Denisov, V. P.; Halle, B. Dimethyl sulfoxide binding to globular proteins: A nuclear magnetic relaxation dispersion study. *Prot. Sci.* **1997**, *6*, 1756−1763.

(9) Shen, J. L.; Li, Z. F.; Wang, B. H.; Zhang, Y. M. Partial molar volumes of some amino acids and a peptide in water, DMSO, NaCl, and DMSO/NaCl aqueous solutions. *J. Chem. Thermodyn.* **2000**, *32*, 805−819.

(10) Chalikian, T. V.; Gindikin, V. S.; Breskauer, K. J. Volumetric characterization of the native, molten globule and unfolded states of cytochrome *c* at acidic pH. *J. Mol. Biol.* **1995**, *250*, 291−306.

(11) Tamura, Y.; Gekko, K. Compactness of thermally and chemically denatured ribonuclease A as revealed by volume and compressibility. *Biochemistry* **1995**, *34*, 1878−1884.

(12) Kamiyama, T.; Gekko, K. Effect of Ligand Binding on the Flexibility of Dihydrofolate Reductase as Revealed by Compressibility. *Biochim. Biophys. Acta* **2000**, *1478*, 257−266.

(13) Radford, S. E.; Dobson, C. M.; Evans, P. A. The folding of hen lysozyme involves partially structured intermediates and multiple pathways. *Nature* **1992**, *358*, 302−307.

(14) Pfeil, W.; Privalov, P. L. Thermodynamic investigations of proteins. I. Standard functions for proteins with lysozyme as an example. *Biophys. Chem.* **1976**, *4*, 23−32.

(15) Gekko, K.; Hasegawa, Y. Compressibility-structure relationship of globular proteins. *Biochemistry* **1986**, *25*, 6563−6571.

(16) Millero, F. J.; Surdo, A. L.; Shin, C. The apparent molal volumes and adiabatic compressibilities of aqueous amino acids at 25 °C. *J. Phys. Chem.* **1978**, *82*, 784−792.

(17) Kamiyama, T.; Gekko, K. Compressibility and volume changes of lysozyme due to guanidine hydrochloride denaturation. *Chem. Lett.* **1997**, 1063−1064.

Received for review April 16, 2003. Accepted July 6, 2003.

JE0340710