Volume Changes Associated with Guanidine Hydrochloride, Temperature, and Ethanol Induced Unfolding of Lysozyme

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We studied the guanidine hydrochloride (GdnHCl)-, temperature-, and ethanol-induced unfolding of lysozyme using high-precision densitometric measurements, aiming to characterize and compare the volume changes, $\Delta\nu^o$, accompanying the unfolding of a protein simultaneously by different means, that is, by GdnHCl, temperature, and an organic cosolvent, EtOH. The data obtained are also compared with other means of unfolding, such as high-pressure- and dimethyl sulfoxide (DMSO)-induced denaturation. To aid in interpreting the temperature dependence of the apparent specific volume of lysozyme, we have also carried out pressure perturbation (PPC) and differential scanning calorimetry (DSC) measurements under the same solution conditions. The PPC method allows the detection of very small volume changes with high accuracy. Next to the strong temperature dependence of $\Delta\nu^o$, the volume changes associated with the unfolding of the protein are found to be very sensitive to the type of denaturation. The apparent specific volume decreases upon the heat- and GdnHCl-induced denaturation. The observed volume change for the GdnHCl-induced denaturation is 60% larger (i.e., more negative) than that obtained for thermal denaturation. Conversely, the apparent specific volume increases by an order of magnitude and becomes positive upon ethanol-induced denaturation, similar to the aprotic organic solvent, DMSO. Hence, depending on the type of denaturant (temperature, pressure, chemical denaturants, or cosolvents), positive and negative volume changes of unfolding are found, which can—at least in part—be attributed to the formation of different unfolded state structures (including clustering) of lysozyme. The standard Gibbs energy changes upon denaturation, ΔG_D^o, for the various perturbation parameters are found to be similar, however, if extrapolated to zero cosolvent concentration.

1. Introduction

The study of protein folding and unfolding is an ongoing active field of investigation.[1−6] A detailed characterization of the unfolded states and the unfolding process under various environmental conditions is required for constructing the conformational and free energy landscape of proteins. Furthermore, such knowledge is needed for understanding the pathology of diseases originating in environmentally induced protein misfolding.[2,7,8] One of the parameters accompanying protein unfolding reactions, the volume change upon unfolding, is an important thermodynamic quantity directly related to the compactness or globularity of the protein molecule and is generally thought to arise from a combination of factors.[9−18] The elimination of cavities and internal voids appears to represent a major negative contribution to the value of the volume change of unfolding. The disruption of electrostatic interactions also leads to a marked decrease in volume caused by the electrostriction of water molecules around the unpaired charged residues. The hydration of charged and polar groups causes a decrease in volume. On the other hand, the volume changes found associated with the exposure of hydrophobic groups depend on the model compounds selected and fall into the range from small negative to positive values, and it is not clear whether the volume changes associated with the exposure of hydrophobic groups upon protein unfolding are net negative or positive and if the volume change associated with hydrophobic hydration plays an important role in the total volume change of unfolding. What seems to be clear, however, is that these effects largely compensate for the increase in volume as the crystalline-like state of the protein interior is disrupted and exposed to solvent upon unfolding.

Chemical denaturants, temperature, and organic solvents are well-established as powerful tools in the study of protein folding and unfolding reactions.[19−24] The thermal unfolding of proteins is caused by the heat-induced conformational transitions in the protein molecule. This phenomenon has been extensively studied, and its mechanism is rather well-understood.[25−27] Upon heating, the protein molecule unfolds because of the heat-induced disruption of the delicate balance of various noncovalent interactions that maintain the native structure at room temperature. It is generally known that a considerable amount of nonpolar groups is exposed to the solvent on thermal denaturation, as revealed by the large increase in heat capacity.[25] However, an appreciably residual protein structure remains after the heat-induced unfolding, as compared with guanidine hydrochloride-induced unfolding of lysozyme or ribonuclease.[28]

Protein unfolding may also be induced by using substances that directly bind to particular groups on the protein's surface, for example, to hydrophobic regions in the case of organic solvents[22,29−33] or to peptide groups in the case of guanidine hydrochloride and urea.[23,34,35] Guanidine hydrochloride (GdnHCl) is a widely used protein denaturant.[19,23,24,36] It offers several advantages over other means of unfolding. First, the degree of

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unfolding is maximal. Second, unfolding is more likely to approach a two-state mechanism. Third, denaturation is more likely to be completely reversible. The unfolded state in the presence of 6 M GdnHCl has been taken as an initial reference for protein folding.

Owing to the ability to vary the size, polarity, and strength of hydrogen bonds, organic solvents, including alcohols, are used in biophysical chemistry and biotechnology to selectively modulate the properties of a protein system. As an example of an innovation-promising scientific area, enzymatic catalysis in nonaqueous media (including organic solvents, ionic liquids, and supercritical fluids) has been intensively developed, recently.[37−44] There are several advantages in employing nonaqueous media for biocatalysis, including the high solubility of hydrophobic reagents, the synthesis of useful chemicals, the suppression of undesirable side reactions caused by water, and enhanced thermostability. High selectivity (perhaps the most attractive feature of enzymes in organic liquids) can be markedly affected, and sometimes even inverted, by the solvent. Notably, the protein activity and structure in organic solvents depend in a complicated way on the water content and the hydration "history" of an enzyme.[30−33,40,41,44,45]

This "organic solvent" approach can be successfully extended to other, noncatalytic, biochemical processes, such as protein folding and unfolding reactions.[46] A detailed understanding of the volume change upon protein unfolding in the presence of organic solvents is still elusive. Only limited data have been reported as regards the concentration dependence of the volume change that accompanies protein unfolding in a wide range of organic solvent concentrations.[47] To our knowledge, no attempt has been undertaken to study simultaneously the volume changes associated with chemical denaturant-, temperature-, and organic solvent-induced unfolding of proteins. This comparison has been in the focus of this work. We used hen egg-white lysozyme as a model protein. Lysozyme is one of the most studied enzymes in biochemistry and enzymology.[6,48,49] It is a small monomeric protein composed of 129 amino acids and contains four disulfide bonds. Ethanol was used as a model organic solvent. It is a typical protic solvent having both polar and nonpolar groups. It is an important solvent in chemical synthesis, biotechnology, and medicine for the dissolution of various biochemical substances.

2. Experimental Section

2.1. Materials. Hen egg-white lysozyme (EC 3.2.1.17, L6876, thrice crystallized, dialyzed, and lyophilized), ethanol, guanidine hydrochloride, and glycine were purchased from Sigma and used without further purification.

2.2. Density Measurements. The density of sample solutions and solvents were measured with a precision densitometer, the DMA-5000 (Anton Paar, Austria). The accuracy of the measurements was 1×10^{-6} g mL^{-1}. The instrument constant was determined by calibration measurements with NaCl solutions of known density. For the thermal denaturation studies, the density of the solvent, d_0, was measured at various temperatures. The apparent specific volume of lysozyme, ν^o, at each temperature was calculated from eq 1[50] according to standard procedures:

$$\nu^o = \frac{1}{d_0} - \frac{d - d_0}{d_0 c} \quad (1)$$

where d and d_0 are the densities of the protein solution and the solvent, respectively; c is the specific protein concentration of in grams per milliliter of solution.

In a similar way, GdnHCl denaturation of lysozyme was monitored by density measurements of the sample using solvent solutions at various GdnHCl concentrations at pH 2.5 (0.02 M glycine buffer) and $T = 25$ °C. The glycine buffer was used to be able to compare our data with those available for related systems (see below).

For the ethanol denaturation studies, the density of the solvent, d_0, was measured at various organic solvent concentrations. The apparent specific volume of lysozyme, ν^o, at each concentration of ethanol was calculated from eq 1 according to standard procedures, in which d_0 of the sample solution was obtained from the concentration dependence of the solvent density. The ethanol-induced denaturation of lysozyme was also performed at pH 2.5 (0.02 M glycine buffer) and $T = 25$ °C.

2.3. Differential Scanning Calorimetry (DSC) and Pressure Perturbation Calorimetry (PPC). The thermal unfolding of lysozyme was determined by DSC. DSC measurements were carried out using a MicroCal (Northampton, MA) VP-DSC instrument. Runs were carried out at protein concentrations ranging from 0.5 to 2 wt % and in 20 mM glycine buffer at pH 2.5. No significant changes were observed either in the onset temperature or in the value of the enthalpy change ΔH between different concentrations.

The same instrument, supplemented by the MicroCal PPC accessory, was used in the PPC experiments. Experimental details can be found in refs 51 and 52. As the PPC technique is comparatively new, a brief description is given here. PPC measures the heat consumed or released by a sample after small isothermal pressure jumps. In the differential PPC experiment, two cells of equal volume (here 0.514 mL), containing the protein solution and the buffer, respectively, are subjected to the same small pressure jump. In a decompression step, a pressure of 5 bar (here by using nitrogen gas) is applied and then released to ambient pressure. After equilibration, the gas pressure is used to initiate a compression step. During the pressure jumps, a constant temperature is achieved by active compensation of the heat changes. Integration of the supplied power yields the heat released or consumed. The heat peaks in the compression and decompression steps should be equal in value but are of opposite sign. The amount of heat released or absorbed in a reversible process, Q_{rev}, varies linearly with the pressure change Δp, and the constant of proportionality includes the coefficient of thermal expansion of the solution. Thermodynamics relates the pressure coefficient $(\partial Q_{rev}/\partial p)_T$ of the heat Q_{rev} exchanged in a reversible process to the coefficient of thermal expansion of the sample volume (eq 2):

$$\alpha = \frac{1}{\nu^o}\left(\frac{\partial \nu^o}{\partial T}\right)_p \quad (2)$$

To correct for small differences in the volume of the two cells, heats obtained in a buffer/buffer run were subtracted from those in a sample/buffer run over the temperature range of interest. When determining the thermal expansion coefficient for a buffer (sample cell) from the known thermal expansion coefficient of pure water (reference cell), heats from those for a water/water run were subtracted from a buffer/water run. Data from control runs were fit to third-order polynomials in temperature, with coefficients stored for later use in similar experiments. All calculations were carried out using a modified version of the Origin software for PPC supplied by MicroCal.

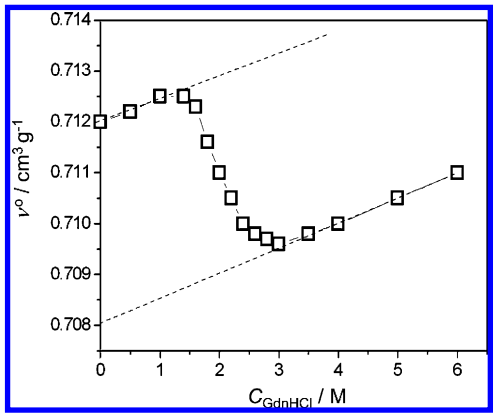

Figure 1. GdnHCl concentration dependence of the apparent specific volume, $\Delta\nu^o$, of lysozyme at pH 2.5 and 25 °C. The $\Delta\nu^o$ values were determined within the experimental error of 0.001 cm^3 g^{-1}.

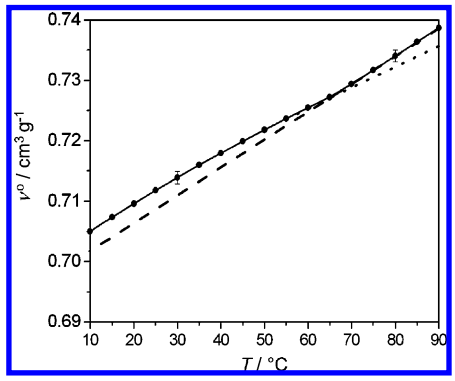

Figure 2. Temperature dependence of the apparent specific volume, $\Delta\nu^o$, of lysozyme (pH 2.5). The $\Delta\nu^o$ values were determined within the experimental error of 0.001 cm^3 g^{-1}.

3. Results

3.1. Guanidine Hydrochloride-Induced Denaturation of Lysozyme. Figure 1 shows the GdnHCl concentration dependence of the apparent specific volume, ν^o, of lysozyme at pH 2.5 and 25 °C. The transition region occurs at about the same GdnHCI concentration as the transition observed from spectroscopic measurements.[36,53] This indicates that all of these techniques probe the same transition in lysozyme. As shown in Figure 1, the ν^o value of the native protein is larger than that of the denatured one at any GdnHCl concentration examined, indicating a negative volume change upon GdnHCl denaturation for that temperature (25 °C). Since the apparent specific volume observed involves the contribution of GdnHCl bound to the protein at a given concentration of GdnHCl, the intrinsic volume change of unfolding in pure buffer solution was estimated from the extrapolated values of the two linear lines for the pre- and post-transition regions to infinite GdnHCl dilution. By linear extrapolation of the data shown in Figure 1, we estimated the volume change ν^o to be −0.004 (±0.001) cm^3 g^{-1} or −57.2 cm^3 mol^{-1}.

3.2. Heat-Induced Denaturation of Lysozyme. Figure 2 depicts the temperature dependence of the apparent specific volume of lysozyme at pH 2.5. Inspection of Figure 2 reveals that the heat-induced denaturation of lysozyme leads to an increase in the ν^o values. In the pretransitional region, the ν^o values increase with increasing temperature in a gradual, nonlinear fashion, with a slope that decreases with increasing temperature. In the post-transitional region, a gradual linear increase of ν^o was observed. A similar trend was observed for lysozyme at pH 1.9.[54] The ν^o values of lysozyme in the native region are in good agreement with previously published data.[17]

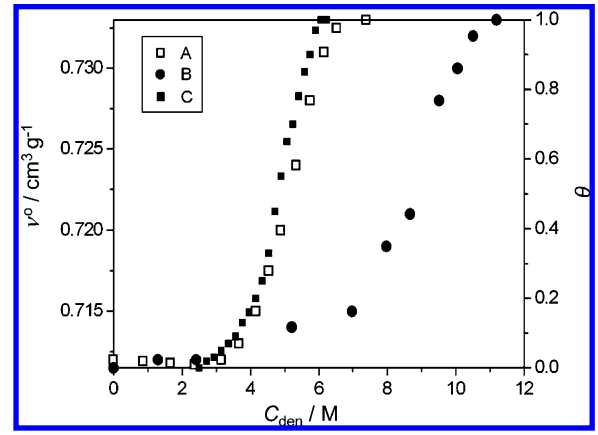

Figure 3. Apparent specific volume, $\Delta\nu^o$, of lysozyme as a function of organic solvent concentration, C_{den}: (A) Ethanol, pH 2.5 and 25 °C (this work); (B) DMSO, 25 °C (adapted from ref 45). The extent of denaturation, θ, of lysozyme as a function of ethanol concentration estimated from the enthalpy data at pH 2.0 and 40 °C, adapted from ref 55.

By linear extrapolation of the data shown in Figure 2, we estimated the volume change, $\Delta\nu^o$, accompanying the heat-induced denaturation of lysozyme at pH 2.5 and 25 °C to be −0.0025 (±0.001) cm^3 g^{-1}. The temperature dependence of the apparent volume of the solute, dν^o/dT, is its apparent specific expansibility, e^0. From the data presented in Figure 2, we estimated the e^0 value for the native region, which is 4.2 × 10^{-4} cm^3 g^{-1} K^{-1} at 25 °C. This was done graphically by drawing a tangent to the curve at 25 °C. This e^0 value falls within the 3.5 × 10^{-4} to 4.6 × 10^{-4} cm^{3}g^{-1} K^{-1} range previously reported for expansibility values of other globular proteins.[9,55,56]

3.3. Ethanol-Induced Denaturation of Lysozyme. Figure 3 displays the ethanol concentration dependence of the apparent specific volume, ν^o, of lysozyme at pH 2.5 and 25 °C. At low denaturant concentrations (C_{den} < 3 M), the ν^o values do not depend markedly on the ethanol content. However, at a critical C_{den} value of ∼3 M, the ethanol-induced denaturation of lysozyme leads to a large increase in the ν^o value ($\Delta\nu^o$ = 0.02 cm^3 g^{-1}). This sharp increase reaches a maximum at C_{den} ≈ 6.5 M. Endothermic heat effects were observed by isothermal calorimetry upon the interaction of lysozyme with water−ethanol mixtures in this concentration region.[57] The position and width of this transition are in good agreement with the ethanol-induced transition observed from isothermal calorimetry data.

4. Discussion

4.1. Heat-Induced Denaturation. A negative volume change has been observed upon heat-induced unfolding of lysozyme (Figure 2). The observed volume change for the GdnHCl denaturation at 25 °C is larger (more negative) than that obtained for thermal denaturation. This difference may be due to the fact that some residual protein structure remains after the heat-induced unfolding, as compared with GdnCl-induced unfolding of lysozyme.[28] At higher temperatures, $\Delta\nu^o$ will become even less negative and may also change sign for the heat-induced unfolding process.

To aid in interpreting the temperature dependence of the apparent specific volume of lysozyme, we have carried out DSC and PPC measurements under the solution conditions which were used for the volumetric studies. According to our DSC results (Figure 4), the thermal unfolding of lysozyme occurs at T_m = 65.5 (±0.2) °C with an enthalpy change ΔH of 475 (±10)

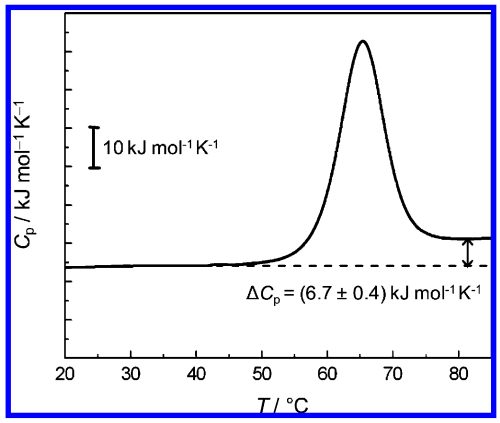

Figure 4. DSC trace of a 2.0 wt % solution of lysozyme at pH 2.5 in glycine buffer (scan rate: 1 °C min^{-1}).

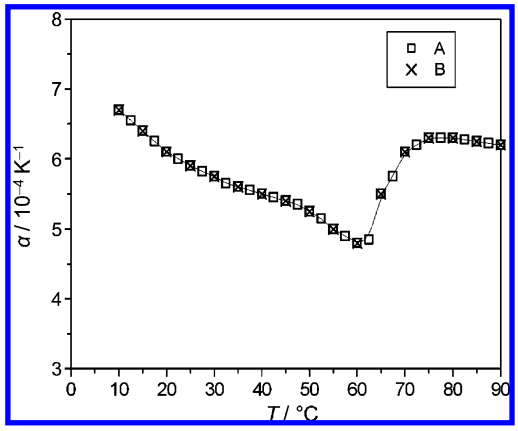

Figure 5. Coefficient of thermal expansion, α, of the apparent specific volume of lysozyme at pH 2.5 (the α-values were determined within the experimental error of 2.0 × 10^{-5} K^{-1}): (A) from PPC data, (B) from densitometry.

kJ mol^{-1} and an increase in heat capacity ΔC_P of 6.7 (±0.4) kJ mol^{-1} K^{-1}. The transition parameters obtained here are in good agreement with previously published data.[58,59]

The temperature dependence of the thermal expansion coefficient, α, of lysozyme is presented in Figure 5. In the low temperature region, where the native state is stable (as assessed by the DSC measurements (Figure 4)), lysozyme displays a negative slope and a positive curvature in the α-temperature plot. As concluded by Brandts et al.,[51] the strong temperature dependence of α is largely controlled by solvation effects. Hydrophilic groups known to act as structure breakers in water have a large negative slope and positive curvature in the α-temperature plots. For comparison, structure-making hydrophobic groups exhibit an opposite behavior. Owing to the dominance of hydrophilic groups, both native and unfolded lysozyme exhibit a net structure-breaking profile, similar to that observed for other proteins such as SNase and RNase A.[60]

To demonstrate the reliability of our findings, we present in Figure 5 the α-temperature curve additionally calculated from the volumetric data using eq 2. As can be concluded from Figure 5, there is good agreement between the curves obtained by the two independent methods. The thermal expansion coefficient of the native protein, α, of about (5.9 ± 0.2) × 10^{-4} K^{-1} at 25 °C is on the order of what is generally observed for native proteins.[15,51]

The midpoint of the thermal unfolding transition is indicated by a negative peak in $\Delta\nu^o$ (Figure 5), which occurs near 65 °C. The T_m value indicated from PPC agrees closely with that found for the same sample in a parallel study using the DSC mode (Figure 4). The fractional volume change for lysozyme unfolding, $\Delta\nu^o/\nu^o$, was obtained by integrating $\alpha(T)$ over the temperature range where the transition occurs. Baselines in the pretransition and post-transition region were projected into the transition region, which are then used to form a progress baseline needed for the integration. For lysozyme, this procedure leads to a small and negative relative volume change of −0.09% (corresponding to $\Delta\nu^o$(65 °C) = −0.0007 cm^3 g^{-1}, again demonstrating the strong temperature dependence of $\Delta\nu^o(T)$). This PPC result for $\Delta\nu^o/\nu^o$ is in very good agreement with similar data obtained from PPC (−0.08%, ref 51) and high pressure (−0.1%, ref 61) studies.

As previously discussed by Chalikian et al.,[9−18] changes in the apparent specific volume of proteins contain three different contributions:

(i) ν_{intr}: the intrinsic volume, which originates from the van der Waals volume of the constituent atoms plus the volume of intrinsic voids within the water-inaccessible protein interior;

(ii) ν_{solv}: the hydration or solvation volume, describing the solvent volume associated with the hydration (solvation) of solvent-accessible protein atomic groups, that is, from solute−solvent interactions around the charged (via electrostriction), polar (e.g., hydrogen-bonding), and nonpolar (hydrophobic hydration) atomic groups on the protein surface;

(iii) υ_{therm}: the thermal volume, which results from thermally induced mutual molecular vibrations and reorientations.

Since the constitutive atomic volume of lysozyme does not change significantly with temperature, the observed temperature dependence of the apparent specific volume of lysozyme can be essentially ascribed to changes of the internal void volume, that is, $\Delta\upsilon_{intr} \approx \Delta\upsilon_{void}$, to the hydration changes on the protein surface (with respect to the bulk), and to the changes in the thermal volume (eq 3):

$$\Delta\nu^0 = \Delta\nu_{void} + \Delta\nu_{solv} + \Delta\nu_{therm} \quad (3)$$

Hence, the latter (positive) term must be responsible for the strong temperature dependence of $\Delta\nu^o$.

4.2. Guanidine Hydrochloride-Induced Denaturation. It is well-known that addition of GdnHCl causes preferential binding of GdnHCl to the protein.[23] Negative volume changes have been observed upon the GdnHCl-induced unfolding of lysozyme. For example, Skerjanc and Lapanje[62] estimated from dilatometric measurements the volume change of lysozyme upon GdnHCl-induced unfolding to be −54 cm^3 mol^{-1} at pH 5.2 and 25 °C. Sasahara and Nitta studied the pressure-induced unfolding of lysozyme in the presence of GdnHCl by ultraviolet spectroscopy.[24] They estimated the volume change of lysozyme unfolding to be between −40 and −50 cm^3 mol^{-1} at pH 4 and 25 °C. These values are in good agreement with our results (Figure 1).

In general, the apparent specific volume of the GdnHCl-denatured protein decreases because unfolding will decrease the internal cavity volume, that is, $\Delta\nu_{void} < 0$. It will also increase the extent of solvation, due to the increased exposed surface area of the unfolded state, which, because of the electrostriction effect, will lead to a decrease of ν^o as well.

4.3. Ethanol-Induced Denaturation. The changes in the apparent specific volume of lysozyme upon ethanol-induced unfolding exhibits a different scenario. When lysozyme is denatured in the range C_{den} = 3−6.5 M, a significant increase in the apparent specific volume of about +0.020 cm^3 g^{-1} is

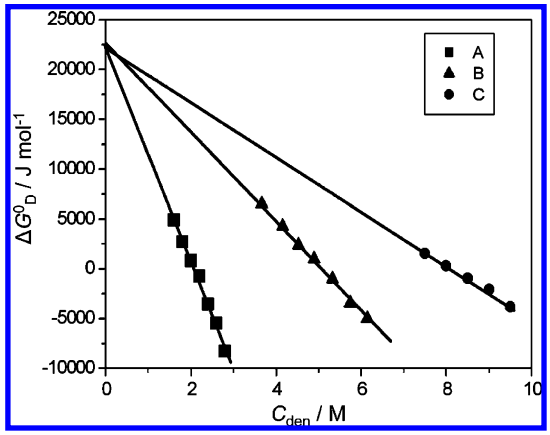

Figure 6. Dependences of the standard Gibbs free energy of unfolding of lysozyme, ΔG^o_D, on the denaturant concentration: (A) GdnHCl, (B) EtOH, (C) DMSO.

observed as compared to the native state. $\Delta\nu^o$ may be explained by changes in two terms, $\Delta\nu_{void}$ and $\Delta\upsilon_{solv}$, because the $\Delta\nu_{therm}$ value is zero at constant temperature (25 °C). The volume increase might occur because new cavities are formed as a result of lysozyme clustering or aggregation. In fact, aggregation in this system was followed using light scattering measurements.[63] It was concluded that lysozyme molecules form "temporary clusters" at intermediate ethanol concentrations ($C_{den} \sim 7.5-10$ M or 45−60 % v/v). Significant changes in the solvation or hydration of the protein surface may also occur. The contribution of the $\Delta\upsilon_{solv}$ term is not clear, however, because the organic solvent and water contributions may have opposite signs (similar to the preferential solvation/hydration contributions). To resolve this question, molecular dynamics simulations might be helpful in future studies.

To corroborate this conclusion, we may refer to data of a related system. Similar changes were observed upon dimethyl sulfoxide (DMSO)-induced denaturation of lysozyme from volumetric measurements.[47] DMSO is a typical aprotic organic solvent having both polar (S═O) and nonpolar (CH_3) groups. The adapted ν^o-C_{DMSO} curve is presented in Figure 3 as well. There is a sharp increase in the ν^o values at C_{DMSO} larger than 5 M, which reaches a maximum at $C_{DMSO} = 9-10$ M ($\nu^o = 0.732$ cm^3 g^{-1}). This ν^o value is close to that observed for ethanol-induced unfolding at $C_{EtOH} \approx 6.5$ M (Figure 3). This observation was interpreted as a result of lysozyme aggregation as well.[47] Additionally, from light scattering measurements, Potekhin et al. concluded that, at $C_{DMSO} \sim 9.8$ M (corresponding to 70 % v/v of DMSO), lysozyme forms dimeric species.[64] The state of lysozyme was characterized as a partially unfolded one with an increased α-helical content.

These results are also consistent with a thermodynamic study of excess partial molar enthalpies of ethanol in aqueous solutions.[65] It has been concluded from these studies that, at the lowest ethanol concentrations, the water hydrogen bond network is bond-percolated. Above a critical concentration ($C_{den} > 3$ M), a transition to a different mixing scheme occurs where two kinds of clusters appear, rich in H_2O or ethanol, respectively. This transition occurs in a rather narrow concentration region ($C_{den} \sim 3-6.5$ M), and the bond-percolation nature of the water hydrogen bond network is disrupted in this concentration region. Interestingly, in this concentration region where the solute molecules have a tendency to cluster,[66,67] we observe a marked increase in the ν^o value (Figure 3). It thus appears that the conformation of the organic solvent-denatured lysozyme (Figure 3) is quite different from those of the thermally and GdnHCl unfolded states (Figures 1 and 2).

4.4. Linear Extrapolation Model. Assuming a model in which lysozyme can exist in only two states (native and denatured), that is, N (native) ↔ D (denatured), with different volumetric properties, we calculated the fraction of each state present at different denaturant concentrations. For a two-state transition, the equilibrium constant, K, and hence the corresponding standard Gibbs energy change, ΔG^o_D, of unfolding (denaturation, D) can be calculated from the volumetric data, using the eq 4:

$$\Delta G^o_D = -RT\ln K = -RT\ln(f_D/f_N) \approx -RT\ln((\nu^o - \nu^o_N)/(\nu^o_N - \nu^o)) \quad (4)$$

where R is the gas constant; ν^o_N and ν^o_D are the apparent specific volumes for the native and denatured states, respectively, and ν^o is the observed specific volume in the transition region. ν^o_N and ν^o_D at a given denaturant concentration were estimated by assuming the same linear dependence of the apparent specific volume in the transition region as in the pure native (pretransition region) and denatured (post-transition region) state (an example is given in Figure 1). The values of ΔG^o_D calculated using eq 4 are plotted as a function of the denaturant concentration in Figure 6.

To estimate the standard free energy change of unfolding at zero concentration of the denaturant, $\Delta G^o_D(C_{den}\rightarrow 0)$, we used the linear extrapolation model, assuming a linear relationship between ΔG^o_D and the molarity of the denaturant, C_{den},[36] for eq 5:

$$\Delta G^o_D(0) = \Delta G^o_D + m\cdot C_{den} \quad (5)$$

where m is a measure of the degree of stabilization ($m > 0$) or destabilization ($m < 0$) of a protein at constant temperature and pressure, and $\Delta G^o_D(0)$ is the extrapolated value of ΔG^o_D for $C_{den} = 0$.

The thermodynamic parameters calculated using eq 5 are given in Table 1. As can be concluded from Table 1, the $\Delta G^o_D(0)$ value calculated in this work is in good agreement with the previously published data where available. Additionally, we find that the $\Delta G^o_D(0)$ value does not seem to depend on the type of denaturant (see Figure 6). Conversely, the different denaturants

TABLE 1: Thermodynamic Parameters of Lysozyme Denaturation

conditions	$\Delta G^o_D(0)$ (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	$\Delta\nu^\circ$ (cm^3 g^{-1})	ref
GdnHCl, pH 2.5, 25 °C	22.3 (1.4)	−9.8 (0.4)	−0.004	this work
GdnHCl, pH 2.9, 25 °C	24.3[a]	−7.5[a]	-	34
GdnHCl, pH 2.0, 25 °C	19.1[a]	−8.5[a]	-	51
EtOH, pH 2.5, 25 °C	23.7 (1.0)	−4.7 (0.3)	+0.020	this work
DMSO, pH 2.5, 25 °C	21.3 (1.7)	−2.6 (0.3)	+0.020	this work

[a] Errors are not available.

have a profound effect on the m value. The most significant destabilizing effect was obtained for GdnHCl ($m = -9.8$ kJ mol^{-1} M^{-1}), and a minimal destabilizing effect was obtained for DMSO ($m = -2.6$ kJ mol^{-1} M^{-1}). Largely different is also the accompanying volume change of unfolding, which can at least in part be attributed to the formation of different unfolded state structures of lysozyme induced by GdnHCl, EtOH, or DMSO, respectively. The denatured state obtained in GdnHCl has a maximal degree of unfolding as compared with the scenario observed in EtOH at $C_{EtOH} \sim 6.5$ M or in DMSO at $C_{DMSO} = 9-10$ M, respectively, where some degree of clustering occurs, which lead to $\Delta\nu^o$ values of > 0, contrary to what is observed for the temperature induced unfolding at sufficiently low temperatures.

To conclude, lysozyme was gradually denatured with temperature and increasing concentrations of different denaturants, and high-precision measurements of the volume change of unfolding by PPC were carried out, complemented by DSC and densimetric measurements. The volume changes associated with protein unfolding were observed to be sensitive to the type of denaturation. Positive and negative volume changes of unfolding were recorded, depending on the type of the denaturant, which can be essentially attributed to the formation of different structures of the unfolded state ensemble of lysozyme. Whereas the $\Delta G^o_D(0)$ values are found to be similar, the volume changes that accompany unfolding transitions of proteins under various environmental conditions, such as temperature, pressure, chaotropic salts, or cosolvents, may vary significantly.

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