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Thermodynamic Characterization of the Osmolyte Effect on Protein Stability and the Effect of GdnHCl on the Protein Denatured State

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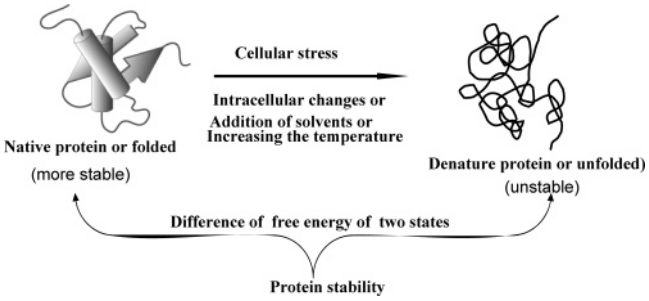
To understand the biomolecular interactions of osmolytes or guanidine hydrochloride (GdnHCl) with protein functional groups, we have determined the apparent transfer free energies ($\Delta G'_{tr}$) of a homologous series of cyclic dipeptides (CDs) from water to aqueous solutions of osmolytes or GdnHCl through solubility measurements, as a function of osmolyte or GdnHCl concentration at 25 °C under atmospheric pressure. The materials investigated in the present study included the CDs of cyclo(Gly-Gly), cyclo(Ala-Gly), cyclo(Ala-Ala), cyclo(Leu-Ala), and cyclo(Val-Val), the osmolytes of trimethylamine *N*-oxide (TMAO), sarcosine, betaine, proline, and sucrose, and the denaturant of GdnHCl. We observed positive values of $\Delta G'_{tr}$ for CDs from water to osmolyte, indicating that interactions between osmolytes and CDs are unfavorable. In contrast, negative $\Delta G'_{tr}$ contributions were observed for CDs from water to GdnHCl, revealing that favorable interactions are predominant. The experimental results were further used to estimate the transfer free energies ($\Delta g'_{tr}$) of the peptide bond (−CONH−), the peptide backbone unit (−CH_2C═ONH−), and various functional groups from water to aqueous solutions of osmolyte or GdnHCl.

Introduction

Proteins are very sensitive and are highly complex systems, exhibiting a substantial degree of structural variability in their folded state. The underlying principles of protein folding and unfolding have been a subject of intense study over the past several decades. In 1959, Kauzmann[1] introduced the use of thermodynamic data for model compounds in elucidating the interactions contributing to protein stability and concluded that the hydrophobic effect, as manifested in the burial of nonpolar groups in the native protein, was the dominant contribution to protein stability. A more quantitative analysis was performed by Tanford,[2] who accepted the paradigm of transfer between two phases and employed data on the solubility of amino acids in water and in ethanol to estimate the hydrophobic contribution to the stability of proteins. Since then, a number of experimental studies have been performed to provide accurate measures of the protein folding/unfolding.[3−11] These investigations have focused on elucidating the protein stability by the addition of solvents, and such studies have attributed the effects to hydrophobicity, hydrogen bonding, and aqueous solvation of proteins. However, the detailed contribution of functional groups and side chains, which are normally buried interior segments of the protein, at higher concentration of solvents has been rather scarcely investigated up to now.

Protein stability and associations depend on the electrostatic, hydrophobic, van der Waals, and steric interactions of the protein with itself and with solvents.[12] In other words, water molecules are preferentially attracted by the surface of a protein in solution, and this preferential hydration excludes other solutes. Alternatively, solutes may be excluded from the immediate vicinity of the protein due to the contributions from steric repulsions. The phenomena are often denoted as "excluded-volume effects", because they arise from the fact that two molecules cannot occupy the same space at the same time.[13−15] The exclusion of solutes (osmolytes) from the protein surface will lead to protein stabilization. Protein stability or its denaturing was estimated mainly through the transfer free energy measurements from water to solvents. Virtually, the net stability of a protein is defined as the difference in the free energy (ΔG) between the native (folded) and denatured (unfolded) states, which is shown below:



We can represent the equilibrium between these two states with a simple mechanism as

$$\text{F (folded)} \overset{K}{\leftrightarrow} \text{U (unfolded)} \qquad (1)$$

and define the net protein stability as

$$\Delta G = G_U - G_F = -RT \ln K = -RT \ln \frac{[U]}{[F]} \qquad (2)$$

where [U] and [F] and G_U and G_F denote the concentrations and the free energies of the unfolded and folded states, respectively. K is the equilibrium constant for eq 1.

Many organisms and cells accumulate small organic molecules, which comprise the bulk of the osmotically active

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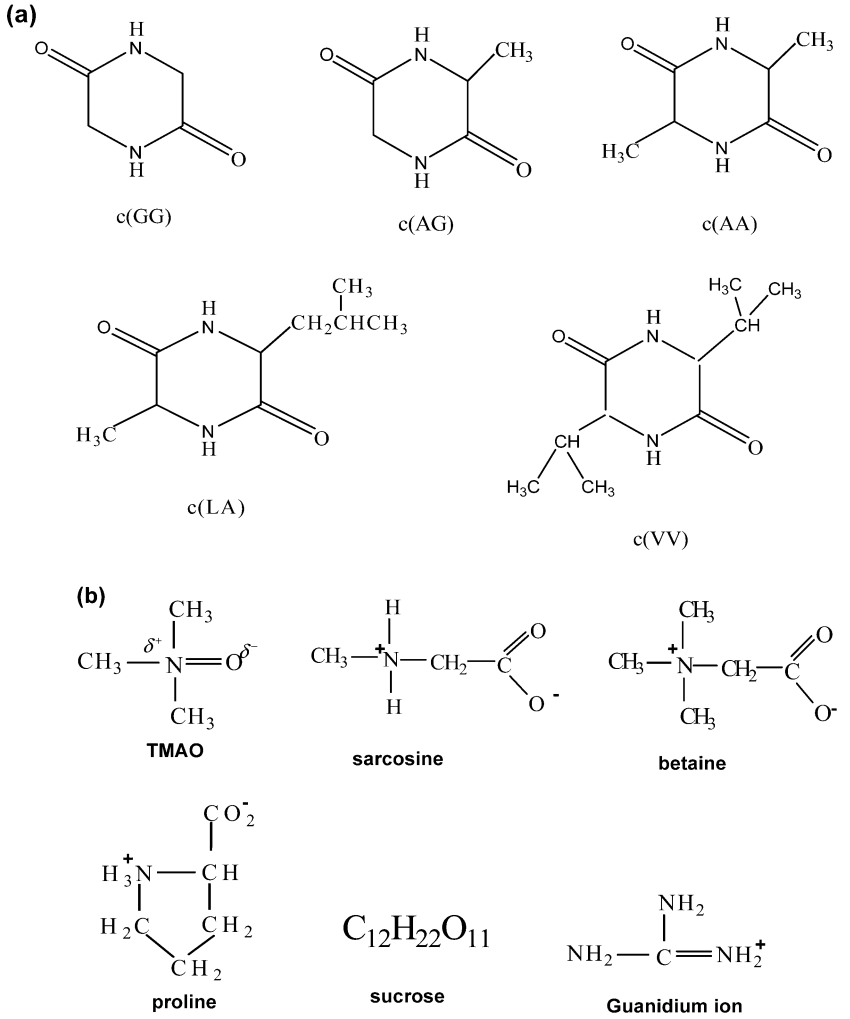


Figure 1. Schematic structures for (a) CDs [c(GG), c(AG), c(AA), c(LA), and c(VV)] and (b) osmolytes or GdnHCl.

solutes, termed as osmolytes (or osmoprotectants), to protect either external osmotic stresses such as dehydration, temperature variations, variable pH, freezing, and high salinity or internal stress such as high concentrations of protein denaturants.[16,17] These osmolytes fall into the following categories: polyhydric alcohols (polyols), such as glycerol, trehalose, and sucrose; free amino acids and amino acid derivatives (taurine and β-alanine, proline); and methylamines, such as trimethylamine *N*-oxide (TMAO), betaine, sarcosine, and urea.[17] These osmolytes have been found to stabilize the conformations of proteins without substantial changes in the function of proteins. This stabilizing ability is denoted as the osmolyte effect. In order to explore the osmolyte effect on proteins and for the sake of clarity and comparison, we have chosen at least one osmolyte from each of the three chemical categories of osmolytes, as follows: TMAO, sarcosine, and betaine from the methylamines; proline from the amino acid derivatives; and sucrose from the polyols. With regard to osmolyte induced stability, Bolen and co-workers[18−22] have proposed the osmophobic effect, which delineates significant protein stability by the addition of osmolytes, due to a solvophobic thermodynamic force. On the other hand, the solution thermodynamics of biomolecules and osmolyte mixtures have been characterized and appeared in the literature.[23−26] However, the origin of osmolyte compatibility at higher concentrations, particularly for various functional group contributions, yet remains to be understood.

The unfolding of proteins by the classical chemical denaturants, such as urea and guanidine hydrochloride (designated GdnHCl), has long been considered to arise because of the favorable interactions of these reagents with the protein segments.[27,28] The basis of biomolecular interactions for destabilization by these denaturants has been generally attributed to direct ligand binding with the protein surface[29−31] or the effects of the denaturants on the structure and dynamics of water molecules, which are involved in the denaturation of protein.[32−36] These denaturants can influence not only the protein unfolding but also the ensemble of the native structure.

The interactions between solvent and the various constituent groups of a protein, such as the peptide bond (−CONH−), the peptide backbone unit (−CH_2C═ONH−), and the amino acid side chains, play a central role in the structure, the conformation, and the function of proteins in aqueous solution. Cyclic dipeptides (CDs), whose organic backbone is the six-membered diketopiperazine ring, should offer several advantages in the thermodynamics of protein model compounds and in the protein stability. They have no free carboxylate or amine terminus within these structures, and their conformational rigidities are small. A further advantage of CDs is that they have been shown to be reasonable model compounds for the solid-like core of globular proteins.[37−39] To date, thermodynamic experimental evidence was conventionally interpreted by various research groups[37−42] to understand the group additivity properties of CDs in aqueous solution and in a few aqueous solvents. Nevertheless, a molecular description of the interactions of osmolytes or GdnHCl with CDs is lacking and numerous issues remain unresolved on CDs' stabilization or destabilization by naturally

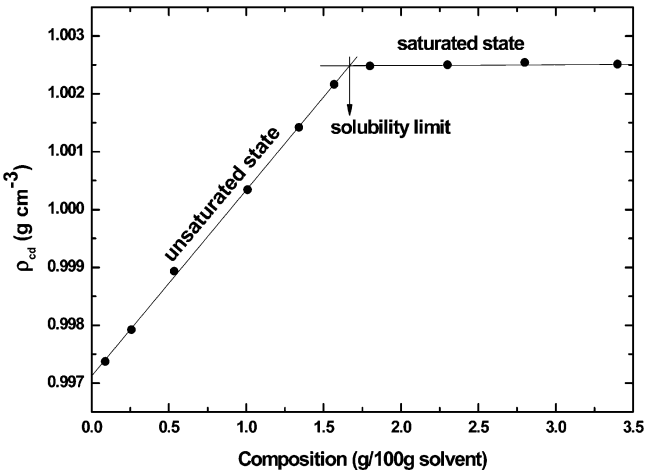


Figure 2. Densities of c(GG) in water vs composition of c(GG) in water at 25 °C. The solubility limit of the model compound was determined at the point of the intersection of the two fitted lines in each system.

occurring osmolytes or GdnHCl. Since CDs can serve as good model compounds, it is obviously of interest to understand the basis of the physical effects and characterization of the osmolyte effect on CDs' stability and the effects of GdnHCl on CDs' denatured state.

In this study, we have focused on understanding the principles of folding or unfolding of a homologous series of cyclic dipeptides with aliphatic nonpolar side chains (glycine, alanine, valine, and leucine) combination in the presence of osmolytes (TMAO, sarcosine, betaine, proline, and sucrose) or GdnHCl, using the transfer free energies, which were obtained from solubility measurements, as a function of osmolyte or GdnHCl concentration at 25 °C under atmospheric pressure. Five cyclic dipeptides, including cyclo(Gly-Gly), cyclo(Ala-Gly), cyclo(Ala-Ala), cyclo(Leu-Ala), and cyclo(Val-Val), were investigated. These CDs are abbreviated as c(GG), c(AG), c(AA), c(LA), and c(VV), respectively. The schematic chemical structures of the CDs as well as the osmolytes or GdnHCl are shown in Figure 1. The experimental results allow us to investigate the individually estimated transfer free energy ($\Delta g'_{tr}$) contribution of apolar hydrogen, the peptide bond, the peptide backbone unit, and various functional groups from water to osmolytes or GdnHCl. Group additivity relationships have also been utilized to obtain the transfer free energies of the peptide bond as well as apolar hydrogen.

Materials

The cyclic dipeptides c(GG) and c(LA) were purchased from Sigma Chemical Co., USA. c(AG), c(AA), and c(VV) were obtained from Bachem Chemical Co., Switzerland. The osmolytes TMAO, betaine, proline, and sucrose and GdnHCl were purchase from Sigma Chemical Co. Sarcosine was supplied from Fluka Biochemical Co., Switzerland. All these purchased materials were used as received. High purity water used for preparing the aqueous osmolyte or GdnHCl solutions was obtained from a NANO pure−Ultra pure water system. The purified water can be distilled and deionized with a resistance of 18.3 MΩ. All solutions were prepared gravimetrically.

Methods

The solubility of CDs was obtained from density (ρ_{cd}) measurements, which is similar to the method of Nozaki and Tanford.[43−45] The detailed procedure and apparatus used in this work have been delineated in our earlier article.[46] To each of the sample vials containing a fixed amount of solvent (water or aqueous osmolyte or GdnHCl solution) was added a weighed amount of a model compound of CDs to provide a series of mixtures with increasing composition of CDs mass. Each of the vials was then sealed with a Teflon coated screw cap, and the cap was folded with Parafilm to produce an airtight and watertight seal. The vials were completely immersed in a low-temperature shaker equipped with a water bath (BT-350R, Yih-Der, Taiwan). The bath temperature was controlled to 25.00 ± 0.01 °C. The water bath was housed in a constant temperature room, also maintained at near 25 °C. At least ten samples were prepared for each system. Among these sample vials, five to six were prepared with unsaturated solutions and the remaining vials with saturated solutions. In the present study, water and different concentrated osmolyte or GdnHCl aqueous solutions were chosen as solvents. After 36−48 h, the shaker was stopped, and the supernatant of each solution was removed with a syringe and filtered through a 0.47 μm disposal filter (Millipore, Millex-GS) before performing the density measurements. The densities (ρ_{cd}) of the solutions were measured with a high precision vibrating tube digital densitometer (model 4500, Anton Paar, Austria), with an uncertainty of ± 5 × 10^{-5} g cm^{-3}, and temperature was controlled to within ±0.02 °C. The uncertainty of the solubility is lower than ±0.8%. The densitometer has a built-in thermostat for maintaining the desired temperatures within the temperature range 0−90 °C. The instrument was calibrated with air and degassed distilled water.

Results

Solubility of CDs in Aqueous Osmolyte or GdnHCl Solutions. To obtain thermodynamic characterization of the osmolyte or GdnHCl effect on the representative model compounds, we measured the solubilites of CDs in the absence and in the presence of osmolyte or GdnHCl. An example of the measured densities of the samples was plotted as a function of the composition of CDs in osmolyte or GdnHCl aqueous solutions as shown in Figure 2. The solubility limit of CDs was determined at the point of the intersection of the two fitted lines

TABLE 1: Molar Mass (M) of Water, Osmolytes, or GdnHCl and Densities (ρ) of Water and Aqueous Solvents at 25 °C[a]

		ρ (g cm^{-3})				
compound	M (g mol^{-1})	0 M	0.5 M	1 M	2 M	4 M
water	18.02	0.99704				
TMAO	111.14		0.99819	0.99957	1.00327	1.01444
sarcosine	89.10		1.01003	1.02258	1.04924	1.09564
betaine	117.15		1.00635	1.01571	1.03468	1.07511
proline	115.13		1.01366	1.02840	1.06064	1.12092
sucrose	342.30		1.06259	1.12701	1.09502*	
GdnHCl	95.53			1.02362	1.04862	1.09567

[a] * indicates 0.75 M sucrose.

TABLE 2: Cyclic Dipeptide Solubilities (S_{cd}) in Water or in Aqueous Osmolyte or GdnHCl Solutions and Densities (ρ^*_{cd}) at Solubility Limits at 25 °C[a]

	S_{cd} (g/100 g of solvent)				ρ^*_{cd} (g cm^{-3})			
solvent	0 M	1 M	2 M	4 M	0 M	1 M	2 M	4 M
				c(GG) (MW = 114.10 g mol^{-1})				
water	1.68[b]				1.00250			
TMAO		1.25	0.902	0.452		1.00377	1.00619	1.01564
sarcosine		1.48	1.18	0.801		1.02818	1.05280	1.09778
betaine		1.34	1.16	0.780		1.02012	1.03797	1.07715
proline		1.43	1.18	0.803		1.03383	1.06406	1.12281
sucrose		1.36	1.47*	1.42**		1.13039	1.06690*	1.09918**
GdnHCl		1.97	2.24	2.86		1.03004	1.05581	1.10394
				c(AG) (MW = 128.13 g mol^{-1})				
water	7.31[c]				1.01549			
TMAO		5.58	3.86	1.69		1.01335	1.02125	1.03024
sarcosine		6.07	4.75	2.72		1.03654	1.05963	1.10092
betaine		6.02	4.46	2.48		1.03139	1.04501	1.07996
proline		5.81	4.01	1.83		1.04433	1.07119	1.12572
sucrose		5.48	6.34*	5.92**		1.13598	1.07606*	1.10611**
GdnHCl		8.36	9.59	11.97		1.04349	1.06947	1.11891
				c(AA) (MW = 142.16 g mol^{-1})				
water	2.55[d]				1.00238			
TMAO		1.94	1.26	0.482		1.00353	1.00624	1.01566
sarcosine		1.94	1.46	0.682		1.02633	1.05182	1.09691
betaine		1.97	1.43	0.725		1.01973	1.03759	1.07632
proline		2.19	1.68	0.957		1.03358	1.06355	1.12216
sucrose		1.82	2.08*	1.94**		1.12905	1.06565*	1.09785**
GdnHCl		2.95	3.43	4.02		1.02930	1.05465	1.10142
				c(LA) (MW = 184.24 g mol^{-1})				
water	0.328				0.99750			
TMAO		0.265	0.178	0.0756		0.99990	1.00354	1.01455
sarcosine		0.235	0.140	0.0482		1.02291	1.04959	1.09586
betaine		0.253	0.178	0.0851		1.01614	1.03508	1.07536
proline		0.256	0.182	0.0860		1.03005	1.06090	1.12108
sucrose		0.265	0.298*	0.280**		1.12711	1.06282*	1.09519**
GdnHCl		0.382	0.446	0.576		1.02419	1.04915	1.09656

	S_{cd} (g/100 g of solvent)				ρ^*_{cd} (g cm^{-3})			
solvent	0 M	0.5 M	1M	2 M	0 M	0.5 M	1 M	2 M
				c(VV) (MW = 198.27 g mol^{-1})				
water	0.0273[e]				0.99711			
TMAO		0.0258	0.0241	0.0200		0.99824	0.99966	1.00336
sarcosine		0.0252	0.0225	0.0181		1.01015	1.02271	1.04936
betaine		0.0254	0.0230	0.0201		1.00655	1.01589	1.03484
proline		0.0265	0.0256	0.0232		1.01374	1.02989	1.06076
sucrose		0.0250	0.0219	0.0234**		1.06267	1.12707	1.09509**
GdnHCl[f]		0.0405	0.0519	0.0798		1.02375	1.04883	1.09594

[a] * indicates 0.5 M sucrose. ** indicates 0.75 M sucrose. [b] S_{cd} =1.68;[19,21] 1.66;[40] 1.69.[41] [c] S_{cd} = 7.26.[41] [d] S_{cd} = 2.52.[41] [e] S_{cd} = 0.0258.[41] [f] 1, 2, and 4 M.

for each system, as illustrated in this figure. Table 1 provides the molar mass (M) of the solvents and the densities (ρ) of the aqueous solutions at 25 °C. The solubility limits of each of the five CDs in the presence and in the absence of osmolyte or GdnHCl aqueous solutions, expressed as grams of CD per 100 grams of solvent and the densities (ρ^*_{cd}) of the saturated solutions at the solubility limit are summarized in Table 2 and graphically represented in Figure 3. From Table 2, one can clearly see that the obtained solubility limits of CDs in water are in good agreement with the literature values at 25 °C.

Transfer Free Energy (ΔG_{tr}) of CDs from Water to Aqueous Osmolyte or GdnHCl Solutions. The solubility data were used to determine the transfer free energy of the model compounds from water to the aqueous osmolyte or GdnHCl solutions at 25 °C under atmospheric pressure. The detailed description of obtaining ΔG_{tr} has been previously reported in our earlier work.[46] At the solubility limit, solid and liquid phases are at equilibrium, and thus, the fugacities of component i in the solid and the liquid phases should be equal.[47] Assuming that the solid phase is pure compound i, the fugacity equality becomes

$$f_{i(\text{pure solid})} = \hat{f}_{i(\text{solute } i \text{ in liquid solution})} \tag{3}$$

or

$$f_{i(\text{pure solid})} = x_i \gamma_i f_i^{\circ} \tag{4}$$

where x_i is the solubility of component i in water or in the aqueous solutions, γ_i is the activity coefficient of component i in the liquid phase, and f_i° is the standard-state fugacity to which γ_i refers. As a consequence, the transfer free energy of CDs from water to the aqueous osmolyte or GdnHCl solutions, ΔG_{tr}, can be calculated from the following equation:

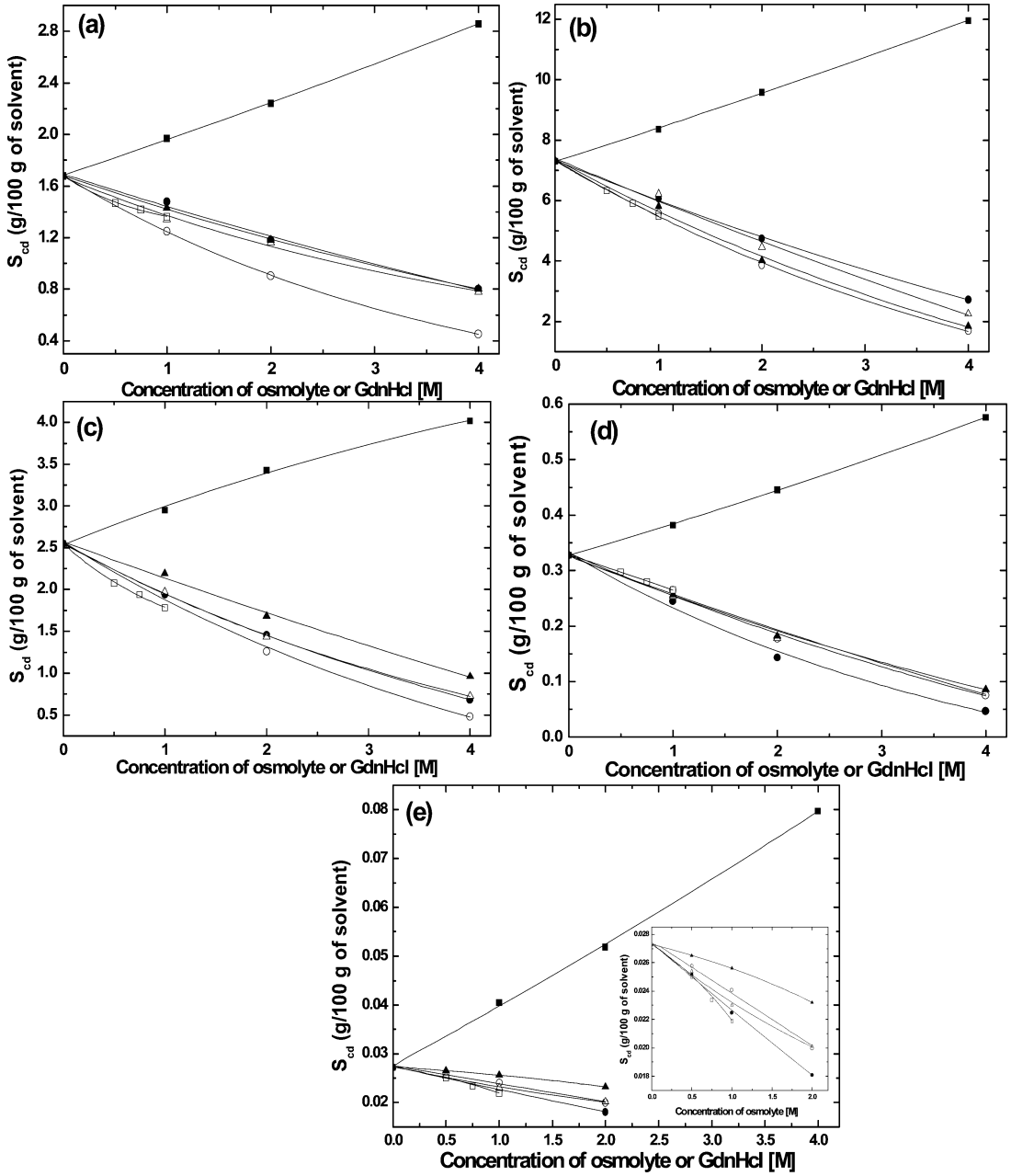


Figure 3. Solubility limits for CDs of (a) c(GG), (b) c(AG), (c) c(AA), (d) c(LA), and (e) c(VV) in an aqueous solution of osmolyte or GdnHCl at 25 °C: (○) TMAO; (●) sarcosine; (△) betaine; (▲) proline; (□) sucrose; (■) GdnHCl. The inset in part e represents the data of only aqueous osmolyte solutions at 25 °C. Solid lines show the smoothness of the solubility points.

$$\Delta G_{tr} = \bar{G}^{\infty}_{cd,ws} - \bar{G}^{\infty}_{cd,w} = \mu^{\infty}_{cd,ws} - \mu^{\infty}_{cd,w}$$

$$= RT \ln\left(\frac{f^{\infty}_{cd,ws}}{f^{\infty}_{cd,w}}\right) = RT \ln\left(\frac{x_{cd,w}\gamma^{*}_{cd,w}}{x_{cd,ws}\gamma^{*}_{cd,ws}}\right) =$$

$$RT \ln\left(\frac{x_{cd,w}}{x_{cd,ws}}\right) + RT \ln\left(\frac{\gamma^{*}_{cd,w}}{\gamma^{*}_{cd,ws}}\right) \quad (5)$$

where the subscript w refers to the aqueous, i.e., the osmolyte- or GdnHCl-free, systems and ws refers to the aqueous osmolyte or GdnHCl solution.

Equation 5 can also be expressed in terms of molar concentration,

$$\Delta G_{tr} = RT \ln\left(\frac{C_{cd,w}}{C_{cd,ws}}\right) + RT \ln\left(\frac{\gamma^{\#}_{cd,w}}{\gamma^{\#}_{cd,ws}}\right) \quad (6)$$

Note that Cohn and Edsall[48] and Tanford[49] used mole fraction scale, while Robinson and Jencks[50,51] and Bolen and co-workers[19,20] used the molarity scale for determining the transfer free energies that we are used. On the basis of a statistical standard thermodynamic treatment, Ben-Naim[52] has strongly suggested in favor of the molarity scale for obtaining transfer free energies.

In 1960−1970, Nozaki and Tanford[43−45] noted that the activity coefficient term is a self-interaction coefficient term and found that the ratio of the activity coefficient term makes only a small contribution to ΔG_{tr}; thereby, this effect of the simplification is negligible and also not much greater than the experimental uncertainty. Therefore, many researchers[19−21,43−45,51,53−55] have ignored the activity coefficient term on the right-hand side of eq 6 for determining transfer free energies of various solutions, because rigorously obtaining the activity coefficients of proteins in multicomponent liquid mixtures from phase

TABLE 3: Apparent Transfer Free Energies of CDs from Water to Aqueous Osmolyte or GdnHCl Solutions at 25 °C and at Atmospheric Pressure[a]

		$\Delta G'_{tr}$ (J mol^{-1})		
CD	solvent	1 M	2 M	4 M
c(GG)	TMAO	719.23	1513.60	3192.14
	sarcosine	246.65	742.20	1589.54
	betaine	509.07	819.23	1701.93
	proline	317.02	715.80	1527.54
	sucrose	218.36	171.54*	182.21**
	GdnHCl	−454.88	−827.97	−1529.21
c(AG)	TMAO	622.75	1487.96	3461.38
	sarcosine	381.12	903.33	2142.12
	betaine	489.54	1087.09	2416.18
	proline	465.01	1278.67	3047.75
	sucrose	393.67	186.78*	278.61**
	GdnHCl	−376.02	−749.27	−1357.54
c(AA)	TMAO	660.11	1706.70	4046.56
	sarcosine	604.42	1236.58	3000.30
	betaine	583.12	1321.06	2896.79
	proline	292.59	866.51	2110.87
	sucrose	523.34	341.92*	437.44**
	GdnHCl	−417.27	−839.77	−1326.66
c(LA)	TMAO	521.20	1496.53	3589.75
	sarcosine	761.87	1979.95	4513.76
	betaine	595.84	1419.84	3152.23
	proline	533.00	1303.73	3022.98
	sucrose	224.35	79.79*	159.45**
	GdnHCl	−441.91	−884.04	−1624.48

		$\Delta G'_{tr}$ (J mol^{-1})		
CD	solvent	0.5 M	1 M	2 M
c(VV)	TMAO	137.24	302.67	755.67
	sarcosine	166.15	416.39	891.95
	betaine	155.39	378.53	666.72
	proline	32.72	79.16	249.91
	sucrose	60.25	242.50	149.66**
	GdnHCl[b]	−1042.80	−1717.32	−2891.98

[a] * indicates 0.5 M sucrose. ** indicates 0.75 M sucrose. [b] 1, 2, and 4 M.

equilibrium data without using solution theory models is extremely problematic and difficult. When the activity coefficient term was neglected, ΔG_{tr} is better denoted as apparent transfer free energies ($\Delta G'_{tr}$). On the other hand, the ΔG_{tr} is valid at infinite dilution while the $\Delta G'_{tr}$ is valid at the solubility limit. The uncertainty of $\Delta G'_{tr}$ is lower than ±1.6%. The obtained $\Delta G'_{tr}$ values are reported in Table 3 as well as graphically represented in Figure 4 as a function of osmolyte or GdnHCl concentration at 25 °C. This transfer free energy represents the change in free energy of each model compound of CDs upon transferring from water (0 M) to an aqueous osmolyte or GdnHCl solution at a specific concentration.

Transfer Free Energy ($\Delta g'_{tr}$) Contribution of the Peptide Bond (Amide Unit) and Apolar Hydrogens from Water to Aqueous Osmolyte or GdnHCl Solutions. The transfer free energies of a given model compound are usually interpreted in terms of group additivity, which is a good approximation method to obtain the contribution of a functional group that is assumed to be independent of neighboring functional groups.[56−58] The values of $\Delta G'_{tr}$ were then used to calculate the contribution of the peptide bond (−CONH−) and apolar hydrogens (N_{aH}) from water to various concentrations of aqueous osmolyte or GdnHCl solutions. This contribution is designated as $\Delta g'_{tr}$.

The peptide bond consists of the amide linkage, including the carbonyl carbon, carbonyl oxygen, amide nitrogen, and amide hydrogen. Apolar hydrogens (denoted as aH) are defined as hydrogen atoms bonded to carbon and are related to the hydrophobic surface area of peptides.[26] The results in Figure 4a−e clearly show that the $\Delta G'_{tr}$ values for CDs in osmolyte solutions increase linearly with increasing osmolyte concentrations in all cases. In contrast, the $\Delta G'_{tr}$ values for CDs in aqueous GdnHCl solutions were found to decrease linearly with increasing GdnHCl concentrations, as shown in Figure 4f. The slopes of these lines (in Figure 4) represent the excess free energies $\Delta G'_{ex}$[26] of CDs from water to 1 M osmolyte or 1 M GdnHCl aqueous solution, and these values are changing with the size of the hydrophobic side chain. The slopes of the fitted lines of c(GG), c(AG), and c(AA) are plotted versus the number of apolar hydrogens (N_{aH}) in Figure 5. However, our results show that the combination of the Ala-Leu-Val side chains of c(LA) and c(VV) does not apply for a simple additivity scheme, and this is consistent with the results of solublity measurements of five cyclic dipeptides in water or aqueous urea solutions by Sijpkes et al.,[41] since the solubilities of c(LA) or c(VV) in solvents are very low. The intercept of each line in Figure 5 provides the $\Delta g'_{tr}$ contribution for two peptide bonds (−CONH−), and the slope of each line will then give the hydrophobic contribution per aH, which is the contribution of one apolar hydrogen. The fitted values of the intercept and slope are collected in Table 4.

The $\Delta g'_{tr}$ Contribution of the Peptide Backbone Unit and Various Functional Groups from Water to Aqueous Osmolyte or GdnHCl Solutions. c(GG) was chosen as a model compound of typical interactions found in proteins because it is the cyclic dimer of glycine. The hydrogen-bonded structure makes c(GG) a good model compound for hydrogen bonding which takes place within the peptide backbone of a protein. Several authors[19,40,42,46] have confirmed that c(GG) is a good peptide model for the peptide backbone unit since it contains two peptide groups in the structure that expose the peptide unit to the solvent. Consequently, the $\Delta G'_{tr}$ value of c(GG) from water to osmolyte or GdnHCl aqueous solutions is divided by two to obtain the contribution of one peptide backbone unit as shown in Scheme 1. In a similar fashion, to estimate $\Delta g'_{tr}$ of the alanyl side chain contribution ($-CH_3$), we have subtracted the corresponding values of $\Delta G'_{tr}$ of c(GG) from c(AG). Subsequently, we obtained the alanyl side chain contribution from the combination of $\Delta G'_{tr}$ values of c(AA) and c(AG) and also of c(GG) and c(AA), as presented in Scheme 2.

In order to obtain the Leu side chain contribution of $\Delta g'_{tr}$, we have made a subtractional construct that consists of subtracting the $\Delta G'_{tr}$ values of c(AG) from those of c(LA), as shown in Scheme 3. Furthermore, the $\Delta G'_{tr}$ values of c(AA) or c(VV) from water to the aqueous osmolyte or GdnHCl solutions are divided by two to obtain the contribution of an alanyl residue or a valyl residue, respectively, as illustrated in Scheme 4. The calculated values of these constituent functional group contributions $\Delta g'_{tr}$ at 25 °C are collected in Table 5. All of the obtained values of Scheme 2 provide the $\Delta g'_{tr}$ contribution of the alanyl side chain from water to the aqueous osmolyte or GdnHCl solutions with reference to the difference of their definitions as well as their interactions with different solvents.

Discussion

Solubilities of CDs in Water and Aqueous Osmolyte or GdnHCl Solutions. Table 2 demonstrates qualitatively the effects of the molecular structure of CDs on solubility in water. It shows that the highest solubility in water is obtained for c(AG) and substantially lower solubilities in water are obtained for c(LA) or c(VV), due to increasing the number of CH_2 groups in the side chains of Leu and Val, varying the shape of c(LA) or c(VV), and correspondingly increasing the hydrophobic

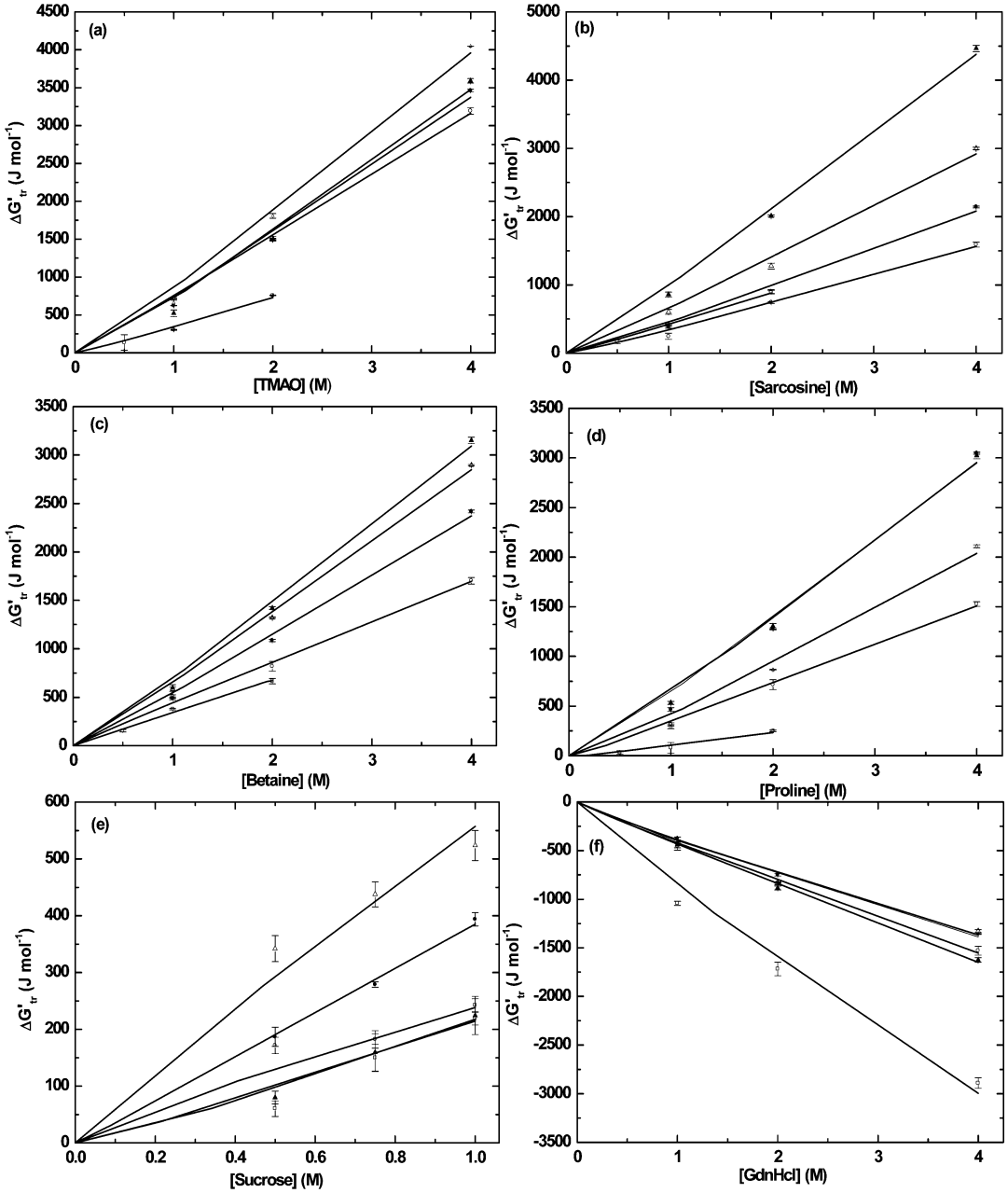


Figure 4. Transfer free energies ($\Delta G'_{tr}$) of cyclic dipeptides of c(GG) (○), c(AG) (●), c(AA) (△), c(LA) (▲), and c(VV) (□) as a function of osmolyte or GdnHCl concentration at 25 °C under atmospheric pressure: (a) TMAO; (b) sarcosine; (c) betaine; (d) proline; (e) sucrose; (f) GdnHCl. The slope of each of the fitted lines represents the excess free energy ($\Delta G'_{ex}$) of each CD from water to 1 M osmolyte or GdnHCl.

character (see Figure 1). In addition, when the long chain residues are in an aqueous medium, it forces the neighboring water molecules to form a cagelike structure or "clathrate", which restricts the motion and the number of possible arrangements of the water molecules and lowers their solubilities.[59] Naturally, when dissolving the compound in water, a molecule must occupy a certain volume, thereby disrupting the water structure at least within that space. However, the space that is occupied by such a molecule in solution reflects not only the atomic volume of the molecule but also any changes of rearranging the liquid around it. The simplest amino acid residues, Gly and Ala, have 57.8 and 86.4 Å^3 (ref 13) partial volumes in solution, which are smaller than those for the more hydrophobic residues Leu and Val: 164.6 and 136.8 Å^3, respectively.[13] For these reasons, the combinations of simple residues of Gly and Ala CDs, such as c(GG), c(AG), and c(AA), are very soluble in water; thereby, we observed higher solubilities (see Figure 3) when compared with those of the more hydrophobic CDs of c(LA) and c(VV). Furthermore, the hydrophobicities of Gly and Ala (0 and −2.09 kJ mol^{-1}) are lower as compared to those of the side chains of Leu and Val (−7.53 and −6.28 kJ mol^{-1}).[13]

As seen from the experimental results in Figure 3, the solubilities of the model compounds are significantly affected by the addition of the solvents. It is systematically clear that the solubilities of the five investigated CDs decrease monotonically with increasing concentrations of aqueous osmolyte solutions. In contrast, the solubilities of CDs in GdnHCl solution increase linearly with increasing GdnHCl concentrations, as also shown in Figure 3. These CDs unfold with the addition of GdnHCl, but often with drastic consequences for their solubility.

Apparent Transfer Free Energies of CDs from Water to Aqueous Osmolyte or GdnHCl Solutions. Transfer free energy is the best suited property for identifying the favorability or

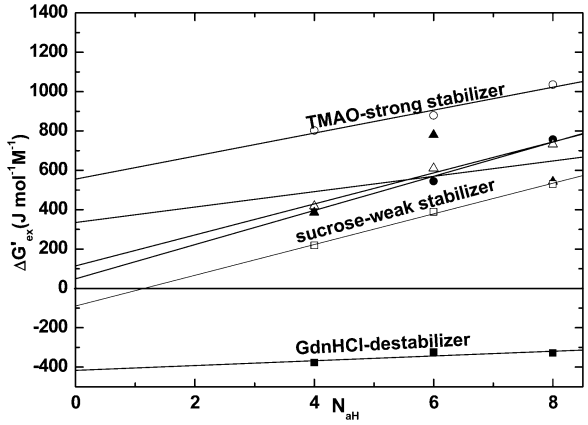


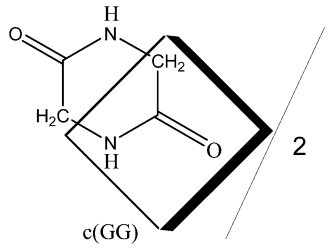
Figure 5. Excess free energies ($\Delta G'_{ex}$) of CDs c(GG), c(AG), and c(AA) from water to 1 M osmolyte (TMAO (○), sarcosine (●), betaine (△), proline (▲), and sucrose (□)) or 1 M GdnHCl (■) as a function of the number of apolar hydrogens (N_{aH}) (i.e., hydrogens bonded to carbon; four, six, and eight bonds for c(GG), c(AG), and c(AA), respectively).

TABLE 4: Transfer Free Energy ($\Delta g'_{tr}$) Contribution of Functional Groups of CDs from Water to Aqueous Osmolyte or GdnHCl Solutions at 25 °C

	$\Delta g'_{tr}$ (J mol^{-1})	
solvent	one peptide bond[a] (−CONH−)	one apolar hydrogen (N_{aH})[b]
TMAO	278.01 ± 10.46	58.29 ± 3.89
sarcosine	24.29 ± 10.12	86.84 ± 3.76
betaine	57.00 ± 9.32	78.81 ± 3.46
proline	167.52 ± 80.06	39.10 ± 30.85
sucrose	−44.65 ± 4.08	78.01 ± 1.51
GdnHCl	−208.25 ± 7.82	12.13 ± 2.90

[a] The linear regression fit analysis of the data of the excess free energies ($\Delta g'_{ex}$) of CDs in Figure 5. The intercept value of each line gives two peptide bonds, and here we present one peptide bond value; error indicates the standard deviation of linear fit propagated analysis. [b] The slope of each line of Figure 5 gives one apolar hydrogen contribution, and error indicates the standard deviation of linear fit propagated analysis.

SCHEME 1 : Schematic Illustration of the Contribution of the Peptide Backbone Unit of c(GG)



unfavorability of transferring protein functional groups from water to other media. We analyzed the osmolyte effect on CD stability and the effect of GdnHCl on CD denaturation with the aid of the transfer free energies from water to aqueous osmolytes or GdnHCl solution. Virtually, the unfavorable interactions of solvents with proteins account for the increase of protein stability, indicating that solubilites decrease with increasing concentration of solvents, as well as the positive contribution of transfer free energy. The importance of this mechanism is that the aqueous osmolyte solutions stabilize the proteins, while not interfering with the functional activity of macromolecules. In contrast, transfer free energy that has a negative sign and the solubility increase with increasing concentration of denaturant indicate favorable interactions. These denaturants are perturbing the structures of proteins and eventually destabilize the proteins.

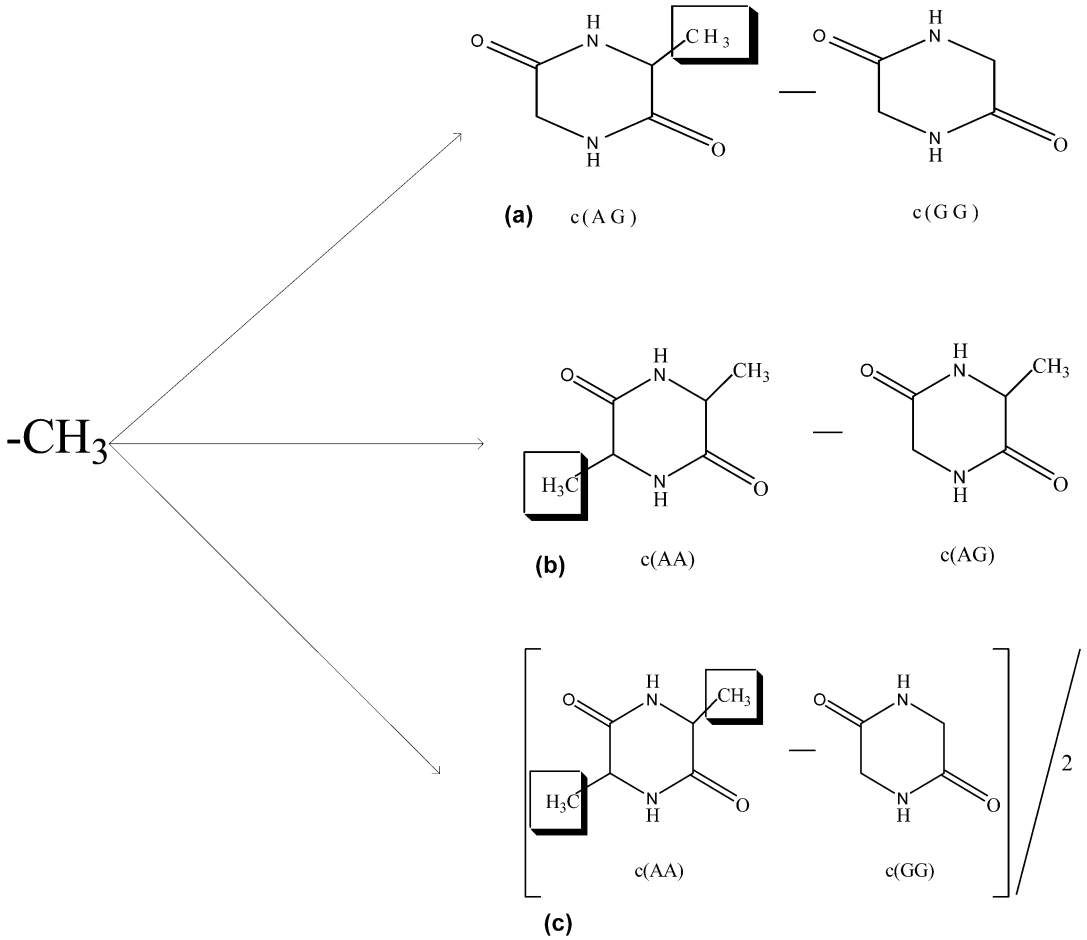
The results in Figure 4 and Table 3 clearly show that the apparent transfer free energies ($\Delta G'_{tr}$) are positive for CDs with osmolytes, whereas the $\Delta G'_{tr}$ values are negative for the CDs in aqueous GdnHCl solutions. The differences in signs and magnitudes reflect differences of interactions among water and CDs or CDs and solvents. The representative $\Delta G'_{tr}$ data, plotted in Figure 4a−e, indicate that the $\Delta G'_{tr}$ values linearly increase with increasing osmolyte concentration. This explicitly shows that unfavorable interactions between osmolytes and CDs occurred. Jaravine et al.[60] also found a similar trend: that free energies calculated from hydrogen exchange measurements on cold-shock protein A vary linearly with TMAO concentration.

It is noteworthy to compare the results of the three osmolytes of methylamines at all concentrations. The transfer free energy values of TMAO with each of c(AG), c(GG), and c(AA) are significantly larger, and there is a large increase with increasing TMAO concentrations compared to the cases of the other two osmolytes (sarcosine and betaine) (Table 3). Interestingly, this behavior is sharply reversed for the more hydrophobic character of c(LA) or c(VV), and we observed larger $\Delta G'_{tr}$ values in sarcosine with these hydrophobic CDs.

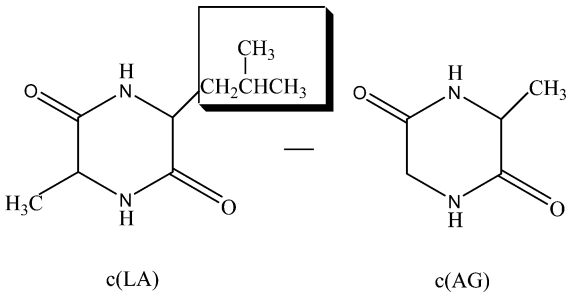
On the other hand, the sensitivity of the transfer free energies of the five CDs in sarcosine solutions is greater than that in betaine solutions. The possible explanation is that the betaine molecule is more hydrophobic than sarcosine because two more bulky CH_3 groups are attached to a nitrogen atom of betaine (Figure 1). According to the cosphere model[8] (betaine and sarcosine), both molecular structures have hydrophobic hydration cospheres of alkyl side chains and hydrophilic hydration cospheres of charged zwitterionic head groups. The overlap of the cospheres of these solutes with those of the exposed nonpolar residues of proteins would squeeze out water from their hydrophobic hydration cospheres.[8] Obviously, this effect is larger in betaine, since two additional methyl groups are attached. The enhanced hydrophobic interactions between the betaine and the nonpolar residues of the protein reduce its stabilizing effect; therefore, we found lower $\Delta G'_{tr}$ values in betaine with all CDs, except that the values of c(GG) in betaine at all concentrations (for example, 509.07 J mol^{-1} for 1 M betaine) are larger than those in sarcosine at all concentrations (246.65 J mol^{-1} for 1 M sarcosine). This unexpected situation may be attributed to the fact that the additional side chains are attached to the nitrogen atoms within the diketopiperazine rings of c(AG), c(AA), c(LA), and c(VV). The absence of the side chains in c(GG) prevents the hydrophobic interactions with betaine; hence, betaine is a stronger stabilizer than sarcosine for c(GG). The other two osmolytes (proline and sucrose) also interact unfavorably with these proteins and were found to have different positive $\Delta G'_{tr}$ values. In other words, there is no longer any regular order between these two osmolytes because their solublities are not in a regular order. The evidence for osmolyte stabilization of CDs emphasizes a dependence on different aspects of CD and solvent structures.

The effects of the osmolytes on protein stability can be interpreted essentially in terms of the preferential interactions of the osmolytes with the aqueous interface and with the CD surface. As mentioned before, the osmolytes increase the stability of the CDs and also tend to decrease their solubilities. Such osmolytes are excluded from the surfaces of CDs, where their concentrations near the CD are lower than those in the bulk solution. Osmolyte enhances the water structure and forms the hydration layer with water molecules. Apparently, the

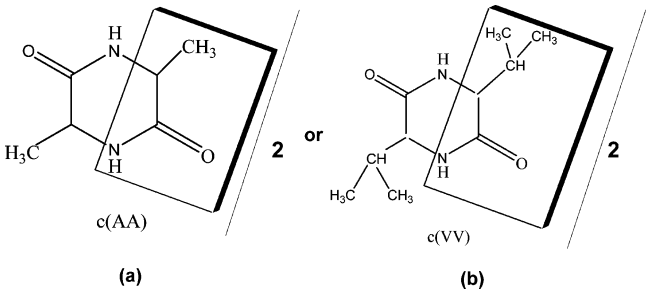
SCHEME 2 : Schematic Illustration of the Contribution of the Alanyl Side Chain (−CH_3)



SCHEME 3 : Schematic Illustration of the Contribution of the Leu Side Chain



SCHEME 4 : Schematic Illustration of the Contribution of an Alanyl Residue or a Valyl Residue of c(AA) or c(VV), Respectively



peptide bond of CDs is less able to interact with hydrated water around the osmolyte and, therefore, there is negative binding between the osmolyte and the CD. Accordingly, unfavorable interactions between CDs and osmolytes are primarily responsible for the model compound stability. During this period, water interacts more favorably with the surface of the CD. Meanwhile, osmolyte can be excluded from the CD surface due to the steric repulsion from water molecules. Based on the molecular dynamics modeling of protein/water/TMAO or urea, Bennion and Daggett[61] recently concluded that the water structure was enhanced by TMAO. Subsequently, the backbone of the protein is unable to interact with the organized water around TMAO (hydrated). In other words, TMAO interacts unfavorably with protein surfaces that assist in forming the hydration layer around the protein, further stabilizing it. This result is corroborated with the observations of this study. Moreover, our results are consistent with a simple statistical mechanics backbone salvation model, in which the protecting osmolytes raise the free energy of the unfolded state, favoring the folded population.[62]

Turning to a discussion of the transfer thermodynamics of GdnHCl with these model compounds, the absolute negative values were found to increase with increasing GdnHCl concentration in water, and these results show that GdnHCl molecules interact favorably with CDs (Figure 4f). Preliminary explanations of these effects are that GdnHCl perturbs the surface of CDs. Moreover, GdnHCl enhances CDs' solubility and interacts preferentially with the CDs' surface, thus appearing to be bonded, and the CD is noted to be preferentially binding. Therefore, GdnHCl interacts more favorably with the CD surface and eventually destabilizes the CDs. The binding interactions overcome the stabilizing osmolyte effects, causing the CDs to be denatured. Very recently, Sherman and Haran[63] concluded that GdnHCl directly binds to the protein, in particular to its backbone, thereby promoting unfolding of the protein. Interest-

TABLE 5: Some Other Functional Group Contributions of Transfer Free Energy ($\Delta g'_{tr}$) of CDs from Water to Aqueous Osmolyte or GdnHCl Solutions at 25 °C[a]

		$\Delta g'_{tr}$ (J mol^{-1})		
scheme	solvent	1 M	2 M	4 M
1	TMAO	359.61	756.80	1596.07
	sarcosine	123.34	371.12	794.79
	betaine	254.55	409.61	850.98
	proline	158.53	357.90	763.79
	sucrose	109.20	85.77*	91.13
	GdnHCl	−227.44	−413.96	−764.63**
2a	TMAO	−96.48	−25.648	269.24
	sarcosine	134.47	161.13	552.58
	betaine	−172.59	267.86	957.55
	proline	147.99	562.87	1520.21
	sucrose	175.31	15.23*	96.40**
	GdnHCl	78.87	78.70	171.67
2b	TMAO	37.36	218.74	585.17
	sarcosine	223.30	333.26	858.18
	betaine	246.65	233.97	237.316
	proline	−172.42	−412.17	−936.88
	sucrose	129.66	155.14*	158.82**
	GdnHCl	−41.25	−90.50	30.88
2c	TMAO	−29.58	96.57	427.23
	sarcosine	178.91	247.19	705.38
	betaine	37.03	250.91	597.43
	proline	−12.22	75.35	291.67
	sucrose	152.51	85.17*	127.61**
	GdnHCl	18.83	−5.90	101.29
3	TMAO	−101.55	8.58	128.36
	sarcosine	277.73	1006.60	2423.54
	betaine	259.37	332.75	492.75
	proline	67.99	25.06	−24.77
	sucrose	−169.33	−106.98*	−119.16**
	GdnHCl	−65.90	−134.77	−266.94
4a	TMAO	330.08	853.37	2023.3
	sarcosine	302.21	618.31	1500.17
	betaine	291.58	660.53	1448.42
	proline	146.31	433.25	1055.46
	sucrose	261.67	170.96*	218.74**
	GdnHCl	−208.66	−420.07	−663.33

		$\Delta g'_{tr}$ (J mol^{-1})		
scheme	solvent	0.5 M	1 M	2 M
4b	TMAO	68.62	151.34	377.86
	sarcosine	83.09	208.20	445.97
	betaine	77.70	189.28	333.38
	proline	16.36	39.58	124.98
	sucrose	30.12	121.25	74.85**
	GdnHCl[b]	−521.41	−858.68	−1446.00

[a] * indicates 0.5 M sucrose. ** indicates 0.75 M sucrose. [b] 1, 2, and 4 M.

ingly, our analysis suggests that changes in water−CD as well as GdnHCl−CD binding interactions can favor the denatured state of the protein. Furthermore, this behavior is consistent with the single-molecule studies of the molecular dynamics of a small enzyme, ribonuclease HI (RNase H), in the presence of the chemical denaturant of GdnHCl.[64] The difference between preferential binding and preferential hydration is shown schematically in Figure 6.

Peptide Bond and Apolar Hydrogen Contributions. We used group additivity to obtain the contribution of peptide bonds or apolar hydrogen relating to the CDs folding or unfolding by the addition of the solvents. As seen from Figure 5, the transfer free energy ($\Delta G'_{ex}$) is a linear function of the number of apolar hydrogens (N_{aH}) and these values are directly proportional to the hydrophobic surface area. The sign and magnitude of the intercept (two peptide bonds) and the slope (apolar hydrogens) of each linear fitted line of c(GG), c(AG), and c(AA) in the presence of five different osmolytes or GdnHCl clearly reveal that the assumption of the group additivity is valid (Figure 5). However, this assumption is not satisfied for proline, in which the N_{aH} of c(AG) is a little far away from the straight line. Table 4 shows that the $\Delta g'_{tr}$ values for one peptide bond as well as one apolar hydrogen are positive, and therefore, the $\Delta g'_{tr}$ contribution is unfavorable for peptide bonds or for apolar hydrogen groups with osmolytes. However, sucrose interacts slightly favorably with the peptide bond, because of a small negative (-44.65 ± 4.08 J mol^{-1}) contribution to $\Delta g'_{tr}$. The observed $\Delta g'_{tr}$ contribution for the peptide bond with TMAO is large with a value 278.01 ± 10.46 J mol^{-1}, and that for one apolar hydrogen is 58.29 ± 3.89 J mol^{-1}. Our results reveal that TMAO is a strong stabilizer while sucrose is a weak stabilizer. The other three osmolytes (betaine, proline, and sarcosine) have a moderate effect on the stabilizing ability of the peptide bond or apolar hydrogen groups of CDs.

On the other hand, GdnHCl exhibits a significantly favorable interaction with peptide bonds, because the $\Delta g'_{tr}$ contribution is negative (-208.25 ± 7.82 J mol^{-1}), but it exhibits slightly unfavorable interactions with apolar hydrogen groups, because of a small positive (12.13 ± 2.90 J mol^{-1}) contribution to the $\Delta g'_{tr}$. The negative value indicates that a specific interaction occurs between the peptide bonds of CDs and GdnHCl since the surface of the peptide can be perturbed by denaturant and new hydrogen bonds are formed between the molecules. This favorable interaction of GdnHCl with the surface of the peptide unit reveals that GdnHCl effectively unfolds the peptide bonds of CDs.

Peptide Backbone Unit (or Glycyl Residue) Contribution. To obtain a thermodynamic quantity capable of describing the effects of osmolytes or GdnHCl on protein process, we further calculated the peptide backbone unit ($-CH_2C{=}ONH$) transfer free energy ($\Delta g'_{tr}$) contribution from $\Delta G'_{tr}$ values of c(GG) with osmolytes or GdnHCl using Scheme 1. As seen from the results of this scheme in Table 5, it is clear that the $\Delta g'_{tr}$ values for the peptide backbone unit from water to osmolytes are positive and are observed to increase with increasing osmolyte concentration in solution. In the case of the peptide backbone unit of c(GG) with GdnHCl, negative $\Delta g'_{tr}$ values were obtained and were also found to increase with increasing GdnHCl concentrations. These findings provide an unfavorable transfer free energy of the peptide backbone unit of c(GG) from water to osmolytes but a favorable transfer free energy of the peptide backbone unit from water to GdnHCl. Within the series of the osmolytes, TMAO is the strongest protein stabilizer, because high positive $\Delta g'_{tr}$ values (for example, 359.61 J mol^{-1} for 1 M TMAO) were obtained, and sucrose is the weakest stabilizing osmolyte, since its contribution is very small (for example, 109.20 J mol^{-1} for 1 M sucrose). The remaining three osmolytes (betaine, proline, and sarcosine) have a moderate effect on the stabilizing ability of the peptide backbone unit of c(GG). From Table 5 (Scheme 1), it can be seen that the values of $\Delta g'_{tr}$ of the peptide backbone unit of c(GG) from water to GdnHCl decrease with increasing concentration of GdnHCl (−227.44, −413.96, and −764.63 J mol^{-1} for 1, 2, and 4 M, GdnHCl, respectively); that is, the denature effect increases. It is also interesting to note that urea interacts favorably with the peptide backbone unit of c(GG) and the denaturing effect of urea increases with increasing concentration of urea (−182.72 J mol^{-1} (ref 21) and −288.70 J mol^{-1} (ref 20) for 1 and 2 M urea, respectively). The comparison presented here of the contributions of the peptide backbone unit with these denaturants shows that both denaturants have similar action on CDs and that GdnHCl is more effective as a denaturant than urea. These results show that the

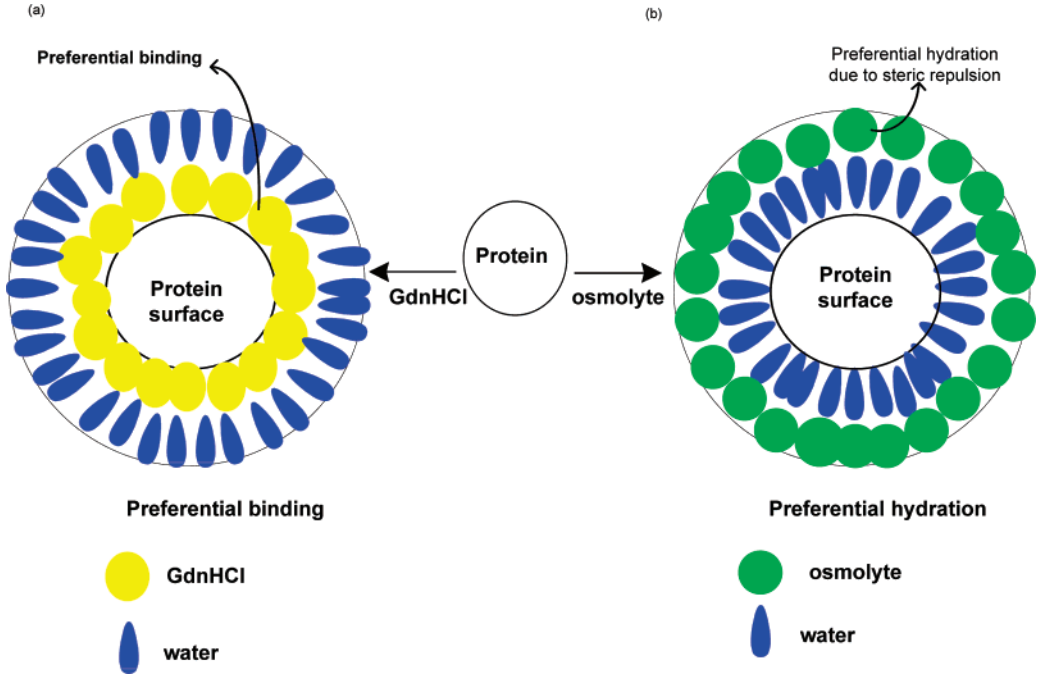


Figure 6. Schematic depiction of protein (a) preferential binding and (b) preferential hydration by the presence of solvents.

value of $\Delta g'_{tr}$ for the peptide backbone unit is very sensitive to the nature of the neighboring groups and is dependent on the denaturant strength of the solutions.

Alanyl and Leucyl Side Chain Contributions. The $\Delta g'_{tr}$ values of the alanyl side chain contribution were calculated from Scheme 2 and were found to be both positive and negative from water to osmolytes or GdnHCl solutions. The results of Scheme 2a in Table 5 explicitly reveal that TMAO (−96.48 and −25.64 J mol^{-1} for 1 and 2 M TMAO, respectively) and betaine (−172.59 J mol^{-1} for 1 M betaine) interact favorably with alanyl side chains, and interestingly, the interactions became unfavorable at higher concentrations of TMAO and betaine. In fact, the other three osmolytes interact unfavorably with alanyl side chains. By contrast, in Scheme 2b and c, the $\Delta g'_{tr}$ values of all osmolytes were found to be positive, except in the cases of proline at all concentrations of Scheme 2b, at 1 M TMAO of Scheme 2c, and at 1 M proline of Scheme 2c. In the case of an effective denaturant of GdnHCl, positive $\Delta g'_{tr}$ values were observed for the alanyl side chains, except at 1 and 2 M GdnHCl of Scheme 2b and also at 2 M GdnHCl of Scheme 2c, indicating that the alanyl side chain interacts unfavorably with this solvent. For the $\Delta g'_{tr}$ values of the leucyl side chain, similar discrepancies were observed for different solvents. It was observed that the results of Scheme 3 in Table 5, for 1 M TMAO and sucrose at all concentrations, differ in the signs of the $\Delta g'_{tr}$ values of the leucyl side chain. Apparently, alanyl and leucyl side chains contribute favorably for some solvents and unfavorably for others. These types of interactions are described as providing an increase in structural order of the solvent systems, the structural arrangements, and the positions of the side chains in the structure of the CDs. The results of the $\Delta g'_{tr}$ values of the side chains show marginal free energy changes at low concentrations of some solvents that become larger at higher concentrations. One of the important conclusions drawn from the results of Schemes 2 and 3 is that some of the protecting osmolytes interact favorably with some of the side chains while the denaturant GdnHCl interacts unfavorable with some of the side chains. This behavior is quite contrasting to the peptide bond or peptide backbone unit contributions, which exhibit unfavorable interactions with protecting osmolytes and favorable interactions with denaturants. Note that the peptide bond or the peptide backbone unit (containing amide) is the most numerous group, responsible for protein stability and the feature of importance in a protein. However, side chains play a less significant role.

Alanyl or Valyl Residue Contribution. As seen from Table 5, Scheme 4 explains that the $\Delta g'_{tr}$ values of both these residues from water to osmolyte solutions are positive, while the $\Delta g'_{tr}$ values are negative for GdnHCl solutions. The magnitude and sign of these values have no significant difference from those of the peptide backbone unit of c(GG). The contribution, for each CD, (absolute value of $\Delta g'_{tr}$) increases systematically with increasing osmolyte or GdnHCl concentrations. It is clear that osmolytes interact unfavorably with the alanyl or valyl residue, similar to the case of interactions between the glycine residue and osmolytes. On the other hand, GdnHCl interacts favorably with the alanyl or valyl residue.

Conclusions

The transfer free energies were determined at 25 °C under atmospheric pressure from solubility measurements for homologous series of five CDs from water to aqueous osmolyte or GdnHCl solutions. These results make it easy to understand the influence of CDs with osmolytes or GdnHCl on the conformational peptide bond, the peptide backbone unit, and various functional groups. The contributions of the peptide group (−CONH), apolar hydrogen, the peptide backbone unit, the alanyl and leucyl side chains, and the alanyl or valyl residue were systematically predicted from the $\Delta G'_{tr}$ measurements. This detailed information points to the conclusion that osmolyte can stabilize and classical chemical denaturants can destabilize the CDs.

Based on the analyses of $\Delta g'_{tr}$ values for peptide bonds as well as one apolar hydrogen from water to osmolyte (which are positive, except for that of sucrose), the $\Delta g'_{tr}$ contribution is unfavorable for peptide bonds and for apolar hydrogen groups from water to osmolytes, whereas GdnHCl exhibits significantly favorable interaction with peptide bonds because the $\Delta g'_{tr}$ contribution is negative, but it interacts slightly unfavorably with

apolar hydrogen groups. In addition, the $\Delta g'_{tr}$ values of the peptide backbone unit of c(GG), the alanyl residue of c(AA), or the valyl residue of c(VV) from water to osmolytes are positive and were observed to increase with increasing osmolyte concentration in solution while those of $\Delta g'_{tr}$ are negative in the case of GdnHCl. These findings show that the small molecules of osmolytes that have unfavorable interactions with these residues appear to be an excellent strategy toward CDs stabilization. By contrast, favorable $\Delta g'_{tr}$ values were observed for the peptide backbone unit, the alanyl residue, or the valyl residue from water to GdnHCl. Within the series of the osmolytes, TMAO is the strongest protein stabilizer and sucrose is a weak stabilizer. The rest of the three osmolytes (betaine, proline, and sarcosine) have a moderate effect on the stabilizing ability of CDs. Interestingly, alanyl and leucyl side chains contribute favorably for some osmolytes, and some of those contribute unfavorably for GdnHCl. The results of the $\Delta g'_{tr}$ values of side chains show marginal free energy changes at low concentrations of some solvents that become larger at higher concentrations. The results for the alanyl side chain seem to show that different chemical models give different transfer free energies. Our observations concluded that the peptide bond, the peptide backbone unit, the alanyl residue, and the valyl residue (containing amide) are the most numerous groups, responsible for the protein folding/unfolding and the feature of importance in a protein; however, side chains play a less significant role in protein folding/unfolding.

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