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Structural Basis for the Enhanced Stability of Protein Model Compounds and Peptide Backbone Unit in Ammonium Ionic Liquids

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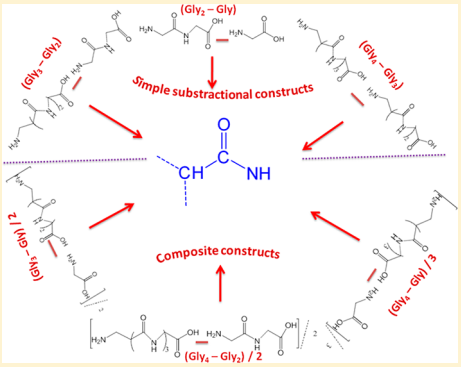
Supporting Information

ABSTRACT: Protein folding/unfolding is a fascinating study in the presence of cosolvents, which protect/disrupt the native structure of protein, respectively. The structure and stability of proteins and their functional groups may be modulated by the addition of cosolvents. Ionic liquids (ILs) are finding a vast array of applications as novel cosolvents for a wide variety of biochemical processes that include protein folding. Here, the systematic and quantitative apparent transfer free energies ($\Delta G'_{tr}$) of protein model compounds from water to ILs through solubility measurements as a function of IL concentration at 25 °C have been exploited to quantify and interpret biomolecular interactions between model compounds of glycine peptides (GPs) with ammonium based ILs. The investigated aqueous systems consist of zwitterionic glycine peptides: glycine (Gly), diglycine (Gly_2), triglycine (Gly_3), tetraglycine (Gly_4), and cyclic glycylglycine (c(GG)) in the presence of six ILs such as diethylammonium acetate (DEAA), diethylammonium hydrogen sulfate (DEAS), triethylammonium acetate (TEAA), triethylammonium hydrogen sulfate (TEAS), triethylammonium dihydrogen phosphate (TEAP), and trimethylammonium acetate (TMAA). We have observed positive values of $\Delta G'_{tr}$ for GPs from water to ILs, indicating that interactions between ILs and GPs are unfavorable, which leads to stabilization of the structure of model protein compounds. Moreover, our experimental data $\Delta G'_{tr}$ is used to obtain transfer free energies ($\Delta g'_{tr}$) of the peptide backbone unit (or glycyl unit) (—CH_2C═ONH—), which is the most numerous group in globular proteins, from water to IL solutions. To obtain the mechanism events of the ILs' role in enhancing the stability of the model compounds, we have further obtained *m*-values for GPs from solubility limits. These results explicitly elucidate that all alkyl ammonium ILs act as stabilizers for model compounds through the exclusion of ILs from model compounds of proteins and also reflect the effect of alkyl chain on the stability of protein model compounds.



1. INTRODUCTION

The ability of amino acids to form a three-dimensional protein structure is significantly interesting and fascinating that combines the aspects of biophysical properties, structural features, and the conformation of certain functional groups of biomolecules. Proteins are virtually unique in being linear macromolecules with a nonrepetitive, specific covalent structure with the capability of adopting a relatively three-dimensional structure/conformation.[1−4] The three-dimensional structure arises particularly because of sequences in amino acids of the polypeptide chains that fold to generate compact domains. The nature and the arrangement of the amino acid side-chain along with the protein backbone are responsible for the individual characteristics of the macromolecule, and it has been recognized that all the information pertaining to protein is implicit in the amino acid sequence.[5,6] Basically, these bonds/backbones are influenced by the aqueous solutions; hence, it is very much important to have a clear idea on the solubility, stability, and thermodynamic properties of these amino acids/backbone in aqueous solution. Although protein model compounds containing amino acids of zwitterionic glycine peptides (GPs) are the simplest and highly interesting molecules that are of fundamental importance in science and are currently under active research.

Most proteins found in nature must adopt a specific conformation, called the folded or native state, to function properly. Under physiological conditions, protein of amino acid residues can undergo a reversible disorder ↔ order transition called protein folding.[7−9] Protein folding plays a central and vital role in the properties of biomolecules in solution and poses remarkable challenges in both modern biophysical and pharmaceutical contexts.

The solubility and stability of biomolecules under cosolvent conditions are dependent on the nature of the cosolvent, which can alter protein's properties and structural effects through biomolecular interactions between its functional groups and the cosolvent molecules.[10−15] For ions containing charged groups, the role of ion−ion interactions is predominant for protein stability.[16,17] The stability of protein is essentially and marginally stable against environmental stress, such as temperature, pressure, or cosolvent. Various studies have been made in

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improving the protein stability in cosolvents, and it has been established that the protein stability is balanced between intramolecular interaction of protein functional groups and their interaction with solvent environment.[18−21]

In recent years, a number of cosolvents have been used for different processes in academics and industries. However, because of new environmental regulations, the challenge of using green solvents has promoted a great development to protect the environment. In this context, ILs have emerged as a certain new class of cosolvents which considerably replace the volatile, conventional, environmentally harmful organic solvents in chemical and biological processes to balance both economic and environmental requirements.[7,22−25] The ILs completely consist of weakly coordinating ions such as organic cation and inorganic or organic anion that have designable properties, which are liquids at or close to room temperature.[26−28] Obviously, an interesting aspect is that ILs being able to influence not only physicochemical properties of mixtures[29−35] but also protein folding studies[36−43] and even a slight variation in their ions can lead to substantial differences in their properties. ILs are emerging as more promising solvents in the various fields such as organic synthesis, catalysis, materials science, electrochemistry, and separation technology.[42−53]

ILs are easy to handle and have very attractive solvent media for the assay of proteins.[54] As solvents, they have been used to solubilize protein and DNA, increase protein activity, selectivity, refolding for biocatalysis, biosensors, and also preserve enzyme stability.[29,44,43,50−52] The stability of proteins is among the most interesting and important emerging scientific research areas, while ILs are emerging tools in bioscience. Apparently, protein stability in the presence of ILs is in its infancy. However, attention must be paid to see the influence of IL solubilization on the higher order structure of the proteins/enzymes.[50−52] Moreover, the stability of simple GPs, which are building blocks of globular proteins, is entirely different from globular proteins, and the relationship of glycine structures and their stability in ILs is not fully understood and has remained elusive until now. This uncovers the discussion of great interest not only from the protein biophysics point of view but also from the more general point of view of the physical chemistry of solutions and solvation. To accomplish this, complete understanding of the influence of ILs on model protein compounds and to reveal the molecular interactions between ILs and protein functional groups, a more detailed thermodynamic study is still essentially required.

In the present study, we have reported the effect of six ILs such as diethylammonium acetate (DEAA), diethylammonium hydrogen sulfate (DEAS), triethylammonium acetate (TEAA), triethylammonium hydrogen sulfate (TEAS), triethylammonium dihydrogen phosphate (TEAP), and trimethylammonium acetate (TMAA) on the GP compounds of glycine (Gly), diglycine (Gly_2), triglycine (Gly_3), tetraglycine (Gly_4), and cyclic glycylglycine c(GG). To interpret the influence of ammonium based ILs on protein model compounds, the values of apparent transfer free energy ($\Delta G'_{tr}$) measurements are determined. In $\Delta G'_{tr}$ analyses, preferential interactions of ions of ILs with GPs have been determined from solubility measurements and dissected. Further, we have estimated the transfer free energy ($\Delta g'_{tr}$) contribution of the glycyl residue (—CH_2C═ONH—) from water to IL solutions by using the $\Delta G'_{tr}$ values of respective GPs. Moreover, the influence of ILs on the GPs is quantified by *m*-value analysis, which provides a impact of solvent-induced solvation effects on protein stability. Our studies have highlighted the role of the cation or anion of ILs on the biomolecular interactions and how such interactions affect the protein stability.

2. EXPERIMENTAL SECTION

2.1. Materials. The highest purity of all GPs was purchased from different commercial sources and used without further purification. Gly and Gly_2 were purchased from Acros organics (USA) chemical company. The c(GG), Gly_3, and Gly_4 were supplied by Sigma-Aldrich Chemical Co (USA). High purity water was obtained from a Nano pure-ultra pure water equipment, which was distilled and deionized with a resistivity of 18.3 Ω·cm for making the aqueous IL solutions. All the samples were carefully prepared by mass using a Mettler Toledo analytical balance, which measured with a precision of ±0.0001 g. The ammonium based ILs used in the present study—DEAA, DEAS, TEAA, TEAS, TEAP, and TMAA—are synthesized and purified as per the procedure delineated in our previous papers.[28,34,40,42]

2.2. Methods. *2.2.1. Solubility Measurements.* Solubility is a measure of all forces maintaining protein molecules in the solution are at a thermodynamic equilibrium with the crystal phase. The solubilities of model proteins were performed as described by Nozaki and Tanford.[55−57] The detailed procedure used in the present study has been depicted elsewhere.[8,39,40,58,59] The concentrated ammonium based IL samples were used as cosolvents for GPs. Ten glass vials, each containing a fixed weight of aqueous ILs, were added to a weighed amount of GPs to provide a series of mixtures with increase in the composition of GPs. Each vial was closed with a Teflon-coated screw cap, and then, the cap was sealed with parafilm to ensure an airtight and water tight seal as well as to prevent evaporation. Accordingly, the samples were prepared such that approximately five vials would ultimately result in unsaturated solutions and the remaining vials in saturated solution. The vials containing the solutions were placed in a low temperature shaker equipped with a water bath (Metrex, New Delhi, India). The water bath temperature was retained at 25 ± 0.01 °C with a constant shaking rate. After 36−48 h, the shaker was stopped and the supernatant of each vial was removed through a syringe and filtered by 0.47 μm disposal filter (Millipore, Millex-GS), before measurements. The ρ values of the samples were measured at 25 °C with a precision of ±0.00005 g cm^{-3} using a vibrating tube densimeter (DMA-4500 M, Anton-Parr, Austria). The instrument was equipped with a built-in solid-state thermostat and a resident program with an accuracy of temperature of ±0.03 K. The densimeter was initially calibrated by measuring the ρ of atmospheric air and double distilled water, according to manufacture recommendations. The precision of the solubility limit is lower than ±1.2%.

3. RESULTS AND DISCUSSION

3.1. Solubility of Glycine Peptides (GPs) from Water to Ammonium ILs. Interactions of ammonium IL with selected, relatively soluble, model compounds were quantified as a function of concentrations of ILs by solubility and apparent transfer free energy ($\Delta G'_{tr}$) measurements. The densities (ρ) of the samples of the GP in water and in aqueous ammonium IL solution have been measured. The ρ for each value was plotted as a function of concentration of GP, and the solubility limit of the GP was obtained at the point of intersection of the two

fitted lines for each system, as displayed in Figure 1. The solubility limits (S_{GP}) of GPs at 25 °C under atmospheric

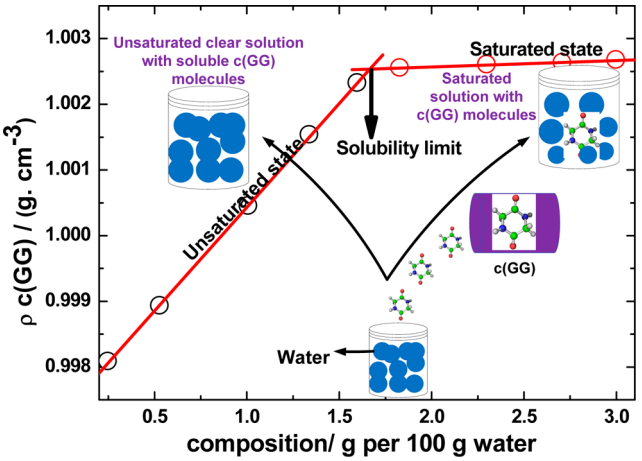

Figure 1. The schematic depiction to obtain the solubility limit of c(GG). Densities of c(GG) in water vs composition of c(GG) in water at 25 °C. The solubility limit of the model compound was obtained at the point of the intersection of the two fitted lines in each system. For the sake of simplicity and clarity of presentation, all the solubility curves for other systems are not shown.

pressure were plotted as a function of the concentration of ammonium based IL, in the five panels of Figure 2. The densities (ρ^*) of samples at the solubility limits, and the solubility limits of GPs in solvents are also tabulated in Table 1S (Supporting Information). Our solubilities of GPs in aqueous solutions are in excellent agreement with the data reported by earlier workers.[55−65] The solubility of GPs in water decreases from Gly to Gly_4 and c(GG). It shows that the highest solubility in water is obtained for Gly and a substantially lower solubility in water is obtained for Gly_4. This is because, when long chain residues are in an aqueous medium, formation of a cage-like structure or "clathrate" takes place which restricts the motion and the number of possible arrangements of the water molecules, thereby lowering their solubility.[4]

Analysis of the solubilities in Figure 2 reveals that S_{GP} values decrease monotonically with an increase in concentrations of ILs in GPs, which indicates that the salting-out effect is dominant. The salting-out of the solute reflects the increase in stability of the native structure of GPs in the presence of ILs. Therefore, the IL-induced solubility decrease is likely due to its effects on the glycine parts of the molecules in the solution. The magnitude of this salting-out effect depends on the nature of ILs and generally follows the physical interface of GPs. The salting-out effect mainly indicates that ILs increase the stability of the structure of GPs. To the best of our knowledge, no solubility data of GPs in ammonium ILs have been reported in the literature; therefore, our results cannot be compared with the literature values.

3.2. *m*-Values. Experimentally, it is observed that S_{GP} depends linearly on the IL concentration. The slope is generally known as the *m*-value and is the characterization property of a given model protein.[66−68] *m*-Values for protein folding and other protein model compound processes are quantitatively interpreted and predicted using the solubility limit.[66,67] Cosolvents effects on biomolecules such as amino acids or protein model compounds are quantified by *m*-values, derivatives with respect to cosolvent concentration (m_3) of the observed standard free energy change for the process ($\Delta G^\circ_{obs} = RT \ln K_{obs}$), where K_{obs}, the equilibrium concentration quotient for the process, is independent of choice of initial model compound concentrations[66] but varies with the solute concentration because it is expressed in terms of amino concentrations and not activities:

$$\begin{aligned} m\text{-values} &= \frac{\partial \Delta G^\circ_{obs}}{\partial m_3} \\ &= -RT\frac{\partial \ln K_{obs}}{\partial m_3} \\ &= RT\frac{\partial \ln K_\gamma}{\partial m_3} \\ &= RT\Delta\left(\frac{\partial \ln \gamma_2}{\partial m_3}\right) \\ &= \Delta\mu_{23} \end{aligned} \quad (1)$$

where K_γ is the equilibrium constant in terms of model protein activity coefficients and the subscripts 1, 2, and 3 refer to water, model compounds and ILs, respectively. Equation 1 is valid for experimental conditions where $m_3 > m_2$ and the partial derivatives are at constant m_2, temperature, and pressure. The chemical potential derivative μ_{23} is

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{m_2} = RT\left(\frac{\partial \ln \gamma_2}{\partial m_3}\right)_{m_2} \quad (2)$$

where γ_2 is the activity coefficient of component 2; μ_{23} is negative when there is a favorable preferential interaction between the ILs and amino acids and positive when there is an unfavorable interaction between the ILs and amino acids.[66,67]

To understand preferential interactions or preferential exclusion (based on favorable or unfavorable interactions) of ILs with protein model compounds, values of μ_{23} are determined for the effects of the ILs on the chemical potentials of appropriately chosen model compounds.[67] In transfer-free energy analyses, preferential exclusion of ILs with amino acids and small peptides has been determined from solubility measurements. The outcome of ILs on the solubility of the model compound gives the μ_{23} values for the exclusion of ILs with the surface area of the model compound from a macroscopic phase. In general, $\mu_{23} = -\mu_{22}(\partial m_2/\partial m_3)_{\mu_2}$, where

$$\mu_{23} = -\mu_{22}\left(\frac{\partial m_2}{\partial m_3}\right)_{\mu_2} \quad (3)$$

Evaluated at the solubility of component 2 (designated m_2^*),

$$\mu_{23} = -RT\left(1 + \left(\frac{\partial \ln \gamma_2}{\partial \ln m_2}\right)_{m_3}\right)\frac{d\ln m_2^*}{dm_3} \approx -RT\frac{d\ln m_2^*}{dm_3} \quad (4)$$

The solubility *m*-value ($d\ln m_2^*/dm_3$) therefore yields μ_{23}/RT directly if the solubility of the model compound is adequately small that its saturated solution is an ideal dilute solution in the absence of ILs, in which $((\partial \ln \gamma_2/\partial \ln m_2)_{m_3})$ evaluated at m_2^* is negligibly small.[66,67] Values of μ_{23}/RT obtained from the slopes of linear fits of the logarithm of the ratio of solubilities of amino acids in the absence and presence of ILs (S_0/S) are plotted as a function of IL concentration, illustrated in Figure 3 and listed in

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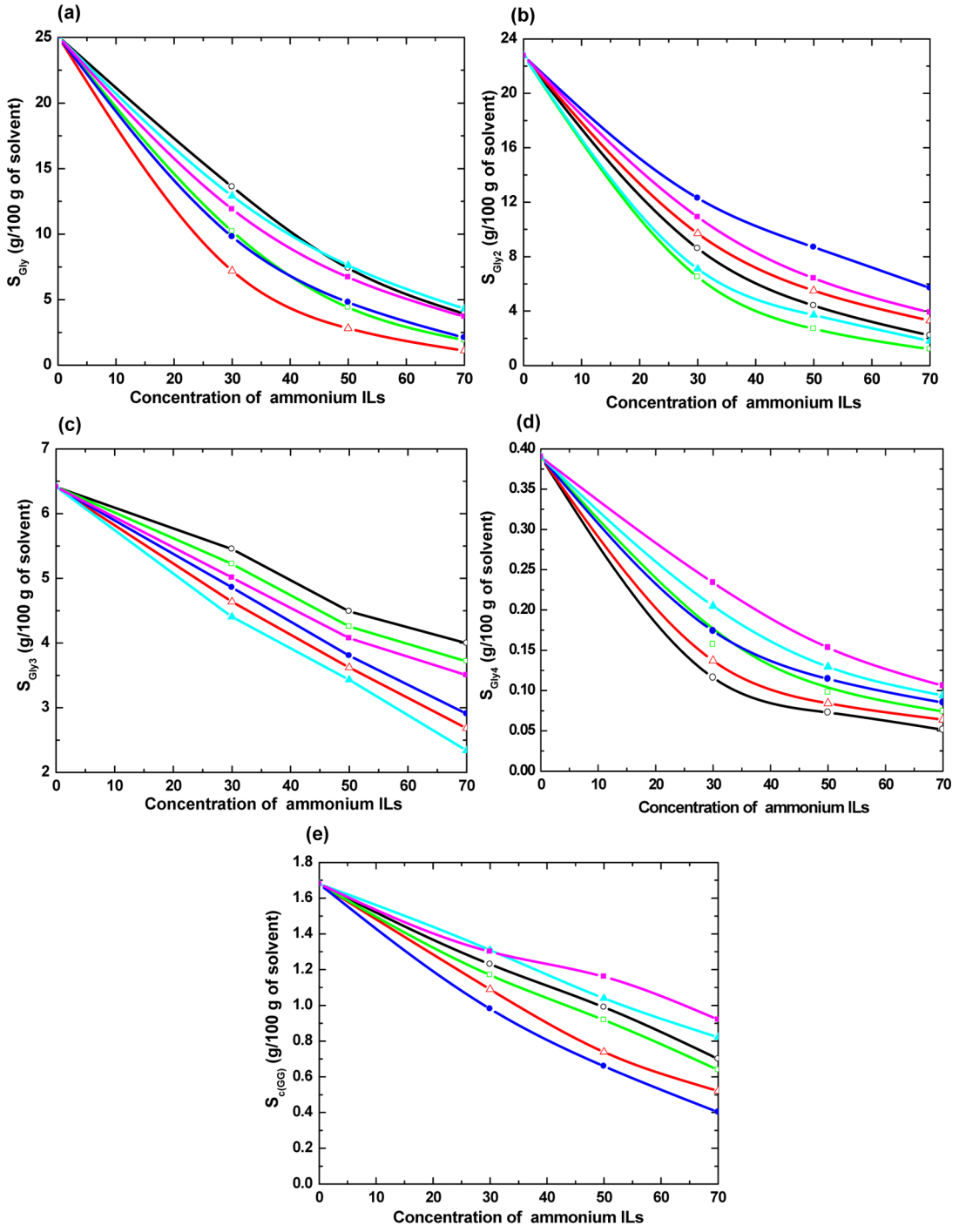

Figure 2. Solubility limits for GPs of (a) Gly, (b) Gly_2, (c) Gly_3, (d) Gly_4, and (e) c(GG) in an aqueous or aqueous IL solution at 25 °C: (○) DEAA; (△) DEAS; (□) TEAA; (●) TEAS; (▲) TEAP; (■) TMAA. Solid lines show the smoothness of the solubility points.

Table 1. The obtained *m*-values are positive; therefore, we observed unfavorable interactions between ILs and the surface of GPs. It is quantified by the *m*-values that ILs are preferentially excluded from the protein model compound surface.

3.3. Apparent Transfer Free Energy ($\Delta G'_{tr}$) of GPs from Water to Ammonium ILs. Attempts to understand and describe the thermodynamic contribution of solute−solvent versus solute−cosolvent interactions have attracted considerable effort from both academic and industrial applications. These efforts have begun to provide us with the structural and thermodynamic details needed for elucidating the molecular origins of the stabilizing/destabilizing action of various ILs. New biophysical methods such as NMR, fluorescence, or ellipticity as measured by circular dichroism have been developed for examining proteins in their natural environment, but such techniques may not be feasible or adequate for characterizing all simple protein model compounds which mimic solvent-accessible protein groups.[68−70] Traditionally, the evaluating of transfer free energy measurements (ΔG_{tr}) is based on measuring the differential solubility of model compounds in a water−cosolvent mixture.[55−57,59−70] The ΔG_{tr} measurements

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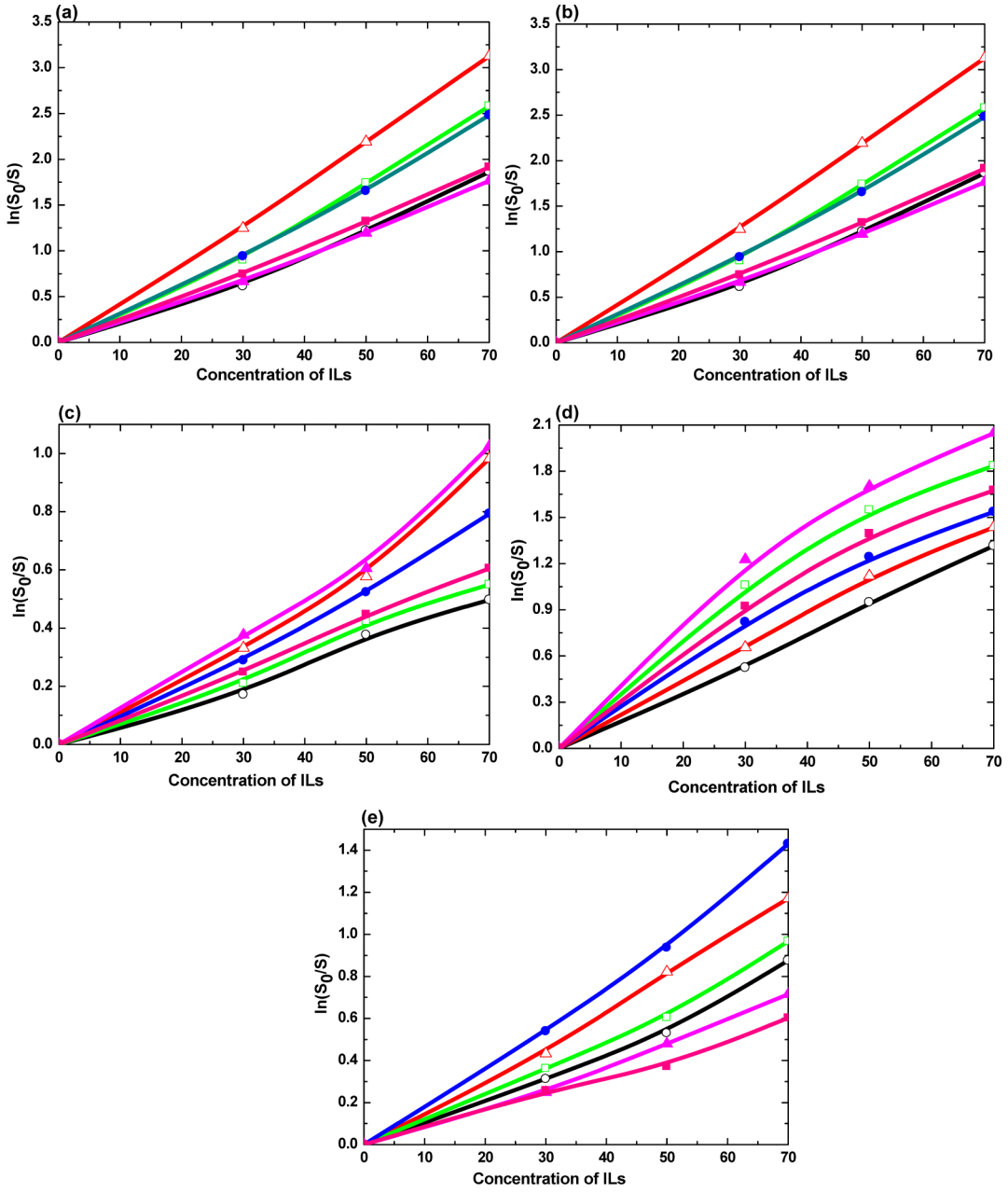

Figure 3. The logarithm of the ratio of solubilities of amino acids in the absence and presence of ILs (S_0/S) plotted as function of IL concentration: (a) Gly, (b) Gly_2, (c) Gly_3, (d) Gly_4, and (e) c(GG) in an aqueous or IL solution at 25 °C: (○) DEAA; (△) DEAS; (□) TEAA; (●) TEAS; (▲) TEAP; (■) TMAA. Solid lines show the linear fit of $\ln(S_0/S)$.

have played a key role in understanding the effect of stabilization or destabilization of protein model compounds and their functional groups in cosolvents.[14,40,55−57,61−67]

Nozaki and Tanford[55−57,62] developed a transfer free energy model that has been utilized to assess the transfer free energies of protein from water to cosolvents. The apparent transfer free energy values ($\Delta G'_{tr}$) of GPs from water to ammonium ILs at 25 °C under atmospheric pressure have been obtained by means of the solubility measurements. A detailed description of the evaluating $\Delta G'_{tr}$ has been described in our previous articles.[8,14,17,39,40,58,59] The uncertainty in $\Delta G'_{tr}$ is to be ±1.5%. In Figure 4, we have displayed the dependencies of the $\Delta G'_{tr}$ for GPs from water to IL solution as a function of IL concentration and also listed in Table 2S (Supporting Information). Importantly, these dependencies of $\Delta G'_{tr}$ are strictly positive values. Figure 4 illustrates that the addition of IL strongly and linearly increases the $\Delta G'_{tr}$ values of GPs. The increase in linearity indicates that there is unfavorable interaction between ILs and GPs that leads to stabilizing the structure of GPs.

The use of ILs as cosolvents for biomolecule solvation is an application that is encouraged by the favorable properties bestowed on the biomolecules by ILs. The order of stability for ammonium ILs on Gly is as follows: DEAS > TEAA > TEAS > DEAA > TMAA > TEAP (Figure 4a). The $\Delta G'_{tr}$ of the ILs for the simple Gly amino acid shows that DEAS is a powerful stabilizer, while TEAP is a weak stabilizer.

It is noteworthy to compare the stability between the common acetate anion with various alkyl chain length cations of ammonium ILs. Triethylammonium substituted cation

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Table 1. *m*-Values (μ_{23}/RT) for ILs in GPs Surface

GPs	ILs	experimental μ_{23}/RT (m^{-1}) ± SD[a]
glycine	DEAA	0.026 ± 0.006
	DEAS	0.044 ± 0.007
	TEAA	0.036 ± 0.009
	TEAS	0.035 ± 0.003
	TEAP	0.025 ± 0.002
	TMAA	0.027 ± 0.003
Gly_2	DEAA	0.033 ± 0.005
	DEAS	0.027 ± 0.007
	TEAA	0.044 ± 0.008
	TEAS	0.019 ± 0.005
	TEAP	0.036 ± 0.001
	TMAA	0.025 ± 0.002
Gly_3	DEAA	0.007 ± 0.003
	DEAS	0.013 ± 0.006
	TEAA	0.008 ± 0.001
	TEAS	0.011 ± 0.003
	TEAP	0.014 ± 0.002
	TMAA	0.008 ± 0.007
Gly_4	DEAA	0.018 ± 0.009
	DEAS	0.020 ± 0.008
	TEAA	0.026 ± 0.005
	TEAS	0.022 ± 0.002
	TEAP	0.029 ± 0.004
	TMAA	0.024 ± 0.003
c(GG)	DEAA	0.001 ± 0.001
	DEAS	0.001 ± 0.005
	TEAA	0.002 ± 0.001
	TEAS	0.002 ± 0.001
	TEAP	0.001 ± 0.006
	TMAA	0.001 ± 0.001

[a]Error estimated provided are the larger of 5%.

$[TEA]^+$ such as TEAA acts as a strong stabilizer, while DEAA is a moderate stabilizer. On the other hand, trimethyl ammonium substituted cation $[TMA]^+$, i.e., TMAA, behaves as a weak stabilizer for Gly. Accordingly, we have observed more unfavorable interactions in the case of TEA^+ as compared to TMA^+ on the surface of Gly. It is clear that ILs bearing a long alkyl chain in their cations perform as a strong stabilizer for amino acid. Further, Gly tends to stabilize ILs in the following order for TEA cation with different anions: TEAA > TEAS > TEAP. Obviously, acetate anion is a strong stabilizer; sulfate is a moderate stabilizer, while phosphate ion is a weak stabilizer for Gly. The Hofmeister series order of anion stability of ILs might be $F^- \approx SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > NO_3^- > Br^- > ClO_3^-$. However, our results show that acetate anions show more unfavorable interaction with Gly as compared to HPO_4^{2-} contribution to Gly. Ammonium family ILs fail to follow the Hofmeister series, as it is not suitable for explaining protein behavior in hydrophilic ILs. In contrast, Constantinescu et al.[37] concluded that ILs follow the Hofmeister series on stability of RNase A. These discrepancies are mainly due to the weak hydration as well as an increase in hydrophobicity of the anion which has a stabilizing effect on the native state of the protein.

The results in Figure 4b evidently reveal that the Gly_2 structure of the folding state is not altered by the addition of ILs, as we observed positive $\Delta G'_{tr}$ values. The stability abilities of the native state of Gly_2 vary from IL to IL; therefore, the efficiency of stabilizing effects follows the trend TEAA > TEAP > DEAA > DEAS > TMAA > TEAS. As we know that ΔG is a better indication of folding studies, hence TEAA has the highest $\Delta G'_{tr}$ value of 2901.84 J·mol^{-1} and TEAS has the lowest value of 1102.79 J·mol^{-1} for 30% (v/v), as shown in Table 2S (Supporting Information), which represents that, as the $\Delta G'_{tr}$ value increases, the protein becomes more stable. Interestingly, the $\Delta G'_{tr}$ order of the folding formed by the ILs shows that TEAA is the strongest stabilizer, while TEAS is a weak stabilizer for Gly_2. The results indicate that ILs interact unfavorably with the surface of Gly_2 and these ILs stabilize the folded structure of Gly_2, while these do not interfere with the surface of Gly_2. It is interesting to compare the results between the common acetate anion with various alkyl chain lengths of cation. The results show that the higher alkyl chain TEA^+ cation of IL is a strong stabilizer, the DEA^+ cation of IL is a moderate stabilizer, and the lower alkyl chain TMA^+ cation of IL is a weak stabilizer for Gly_2. More hydrophobic groups of TEA^+ and DEA^+ cations are strong stabilizers as compared to the cation of TMA^+ IL. Moreover, in the triethylammonium cation family, the stability on the Gly_2 structure is of the order TEAA > TEAP >TEAS; at this point, the acetate anion is dominated on stabilization of Gly_2 structure over phosphate or sulfate anion.

The results in Figure 4c reveal that $\Delta G'_{tr}$ values of Gly_3 are significantly affected by the addition of ILs. Figure 4c depicts that ILs rapidly increase $\Delta G'_{tr}$ positive values of Gly_3 with increasing IL concentration and keep the Gly_3 in folded form. The results indicate that ILs interact unfavorably with the surface of Gly_3 and stabilize the folded structure of Gly_3. The stability abilities of the structure of Gly_3 vary from one IL to another. The efficiency of stabilizing effects of ILs follows the trend TEAP > DEAS > TEAS > TMAA > TEAA > DEAA. Interestingly, the $\Delta G'_{tr}$ of the folding formed by the ILs shows that TEAP is a stronger stabilizer, while DEAA is a weak stabilizer. The larger $\Delta G'_{tr}$ value for Gly_3 in TEAP has revealed that protein retains much of its conformation and more compatible stability. The evidence for IL stabilization of Gly_3 emphasizes a dependence on various aspects of Gly_3 and solvent environment. Apparently, the variation of the alkyl chain at the substituted ammonium cations leads to variation in the efficacy of the ILs as stabilizers for the protein. Therefore, it is noteworthy to compare the stabilizing ability between the alkyl chains at the substituted ammonium cations with combination of acetate anion of ILs. Trimethylammonium substituted cation such as $[TMA^+]$ acted as a strong stabilizer, while on the other hand triethylammonium substituted cation, i.e., $[TEA^+]$, behaved as a moderate stabilizer and diethylammonium cation $[DEA^+]$ acts as a weak stabilizer for Gly_3 with the same anion. More hydrophobic groups of TEA^+ and DEA^+ cations are lower stabilizers as compared to the cation of TMA^+ ILs. Subsequently, for the triethylamine family, the order of stability for Gly_3 structure is TEAP > TEAS > TEAA, whereby it is observed as earlier that phosphate ion is dominated over the sulfate and acetate ion.

Figure 4d demonstrates that ILs increase $\Delta G'_{tr}$ values of Gly_4 structure with increasing concentration of IL. Through these effects, we can infer that the ILs increase the $\Delta G'_{tr}$ values of biomolecule and keep it in the folded state. The observation evidently explains that the Gly_4 structure of the folding state is not altered by the addition of ILs. Our experimental results reveal that the stabilization order for Gly_4 structure is followed as TEAP > TEAA > TEAS > TMAA > DEAS > DEAA, which shows that the variation in ammonium cation from triethyl to trimethyl amine to diethyl amine changes the protein stabilization process. Interestingly, this order shows that

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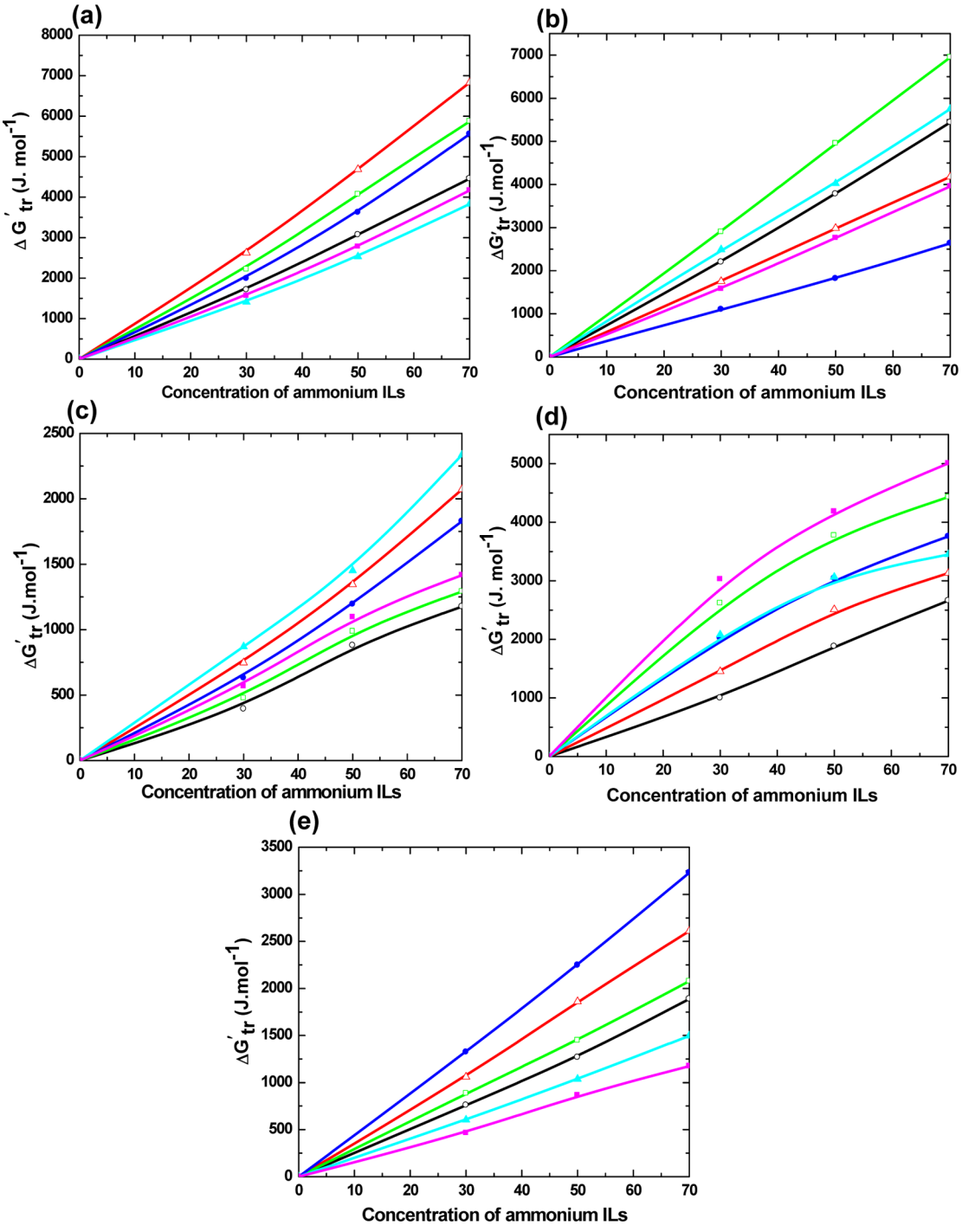

Figure 4. Representation of apparent transfer free energy measurements of GPs: (a) Gly, (b) Gly_2, (c) Gly_3, (d) Gly_4, and (e) c(GG) from water to ammonium IL solution at 25 °C: (○) DEAA; (△) DEAS; (□) TEAA; (●) TEAS; (▲) TEAP; (■) TMAA. Solid lines show the smoothness of the solubility points.

TEAP is the strongest stabilizer, while DEAA is a weak stabilizer. The larger $\Delta G'_{tr}$ values for Gly_4 structure in TEAP disclose the fact that Gly_4 retains much of its native conformation and more compatible stability. From the triethylammonium (TEAA, TEAS, TEAP) family, ILs fail to follow the Hofmeister series, as it is not suitable for explaining protein behavior in hydrophilic ILs (Table 2S, Supporting Information). Further, it is interesting to compare the results between the common acetate anion with various alkyl chain lengths of cation. The results show that the TEA^+ cation of IL is a strong stabilizer, the TMA^+ cation of IL is a moderate stabilizer, and the lower DEA^+ cation of IL is a weak stabilizer for Gly_4.

The stabilizing abilities of ILs on c(GG) follow the trend TEAS > DEAS > TEAA > DEAA > TEAP > TMAA (Figure 4e). c(GG) tends to stabilize ILs in the following order for TEA^+ with different anions: TEAS > TEAA > TEAP. Obviously, sulfate anion is a strong stabilizer; acetate is a moderate stabilizer, while phosphate ion is a weak stabilizer for c(GG). As a result, it is significant to compare the stabilizing

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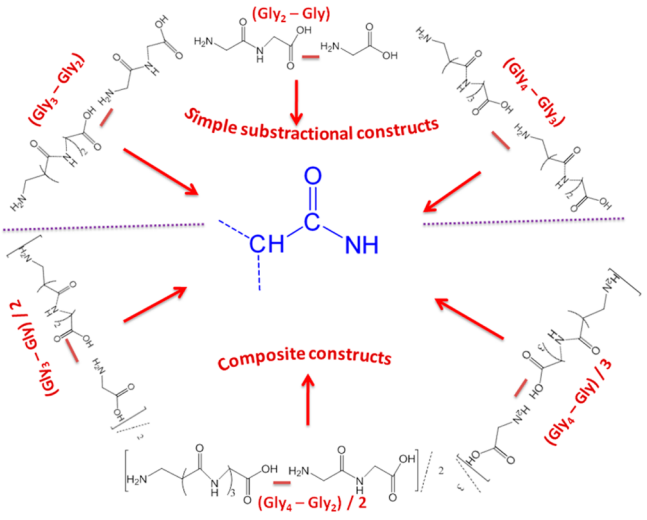
ability between the alkyl chains at the substituted ammonium cations with combination of acetate anion of ILs. Triethylammonium substituted cation such as [TEA^+] acts as a strong stabilizer, while on the other hand diethylammonium substituted cation, i.e., [DEA^+], behaves as a moderate stabilizer and trimethylammonium cation [TMA^+] acts as a weak stabilizer for c(GG) with the same anion. More hydrophobic groups of TEA^+ and DEA^+ cations are strong stabilizers as compared to the cation of TMA^+ ILs. In addition to this, our results show that acetate anions show more unfavorable interaction with c(GG) as compared to HPO_4^{2-} and SO_4^{2-} show more unfavorable interaction than acetate anion; hence, SO_4^{2-} contributes more to the stabilization of c(GG).

Overall, our results clearly show that acetate common anion with alkyl ammonium substituted cations have comparable abilities on stabilizing the GPs. The cation $[TEA]^+$ IL such as TEAA acts as a strong stabilizer, while DEAA is a weak stabilizer for all investigated GPs. Whereas the ability of sulfate common anion with alkyl ammonium substituted cations follows DEAS > TEAS in all studied GPs, except Gly_4 and c(GG), it mainly depends upon the nature of interaction between both alkyl cation and GPs. These results have emphasized that the stability order of the GPs or protein with ILs mainly depends upon the nature of ILs and GPs. Ammonium family ILs fail to follow the Hofmeister series, as it is not suitable for explaining model protein behavior in ammonium based ILs. Our results explicitly elucidate that anion variation has significantly more influence on GP stability efficiency than cation variation. It can be concluded from our experimental findings that a critical role is played by the anion and alkyl chain length of the cation of the ILs in controlling the model protein stability. Clearly, there is no regular order for ammonium ILs in stabilizing the GPs; this may be due to variation in the structural arrangements of ILs as well as model compounds.

3.4. The Contribution of Peptide Backbone Unit (or Glycyl Residue) of Transfer Free Energies from Water to IL 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.[71,72] One aim of this work is to elucidate the influence of ILs on peptide backbone unit, i.e., the glycyl residue ($-CH_2CONH-$). This transfer free energy represents the change in free energy of glycyl residue upon transferring from water (0 M) to 1, 2, and 4 M concentrations of each of the six ILs. The values of $\Delta G'_{tr}$ are then used to calculate the contribution of peptide backbone unit, from water to ILs and designated by $\Delta g'_{tr}$. The implication of these results is that the peptide backbone of amino acid interacts with the ILs, which indicates that the protein has been stabilized or destabilized.

3.4.1. Transfer Free Energy Contribution of Peptide Backbone Unit from Zwitterionic Glycine Peptides. The chemical structures of glycine peptides are as follows: H_2NCH_2COOH (Gly), $H_2NCH_2CONHCH_2COOH$ (Gly_2), $H_2NCH_2[CONHCH_2]_2COOH$ (Gly_3), $H_2NCH_2[CONHCH_2]_3COOH$ (Gly_4). The transfer free energy contribution of the peptide backbone unit of GPs from water to the aqueous IL solutions at 25 °C was obtained in the manner of Talukdar et al.[63] and Nozaki and Tanford.[55−57,62] To obtain $\Delta g'_{tr}$ of the glycyl residue, these researchers subtracted the corresponding values of glycine peptides, as presented in Scheme 1, which depicts two types of mathematical constructs. Among them, the simple subtractional

Scheme 1. Schematic Illustration of the Contribution of the Peptide Backbone Unit of Glycine Peptides



constructs (denoted as SSC) consist of subtracting the $\Delta G'_{tr}$ of two glycines, such as Gly_2 and Gly; Gly_3 and Gly_2; Gly_4 and Gly_3. In other words, the composite constructs (denoted as CC) obtained by subtracting the $\Delta G'_{tr}$ or ΔG_{tr} of two glycines that differ in chain length by more than one peptide unit, such as Gly_4 and Gly, then dividing the difference by 3; the number of remaining peptide units, $[(Gly_4 - Gly)/3]$. Similarly, for other two differ chain length peptide units, we would get $[(Gly_4 - Gly_2)/2]$ and $[(Gly_3 - Gly)/2]$. This subtraction provides a mathematical means to eliminate the transfer free energy contribution of end groups and evaluate the transfer free energy of peptide backbone unit. In order to evaluate the peptide backbone unit contribution, we have used Scheme 1 for zwitterionic glycine peptides and the values are listed in Table 2. All these mathematical constructs for each scheme of the model compounds provide a determination for the peptide backbone unit transfer free energy contribution from water to IL solutions with reference to the difference of their definitions as well as their interactions with different ILs.

The results in Table 2 show the $\Delta g'_{tr}$ contribution of peptide backbone from water to IL solutions; herein, we have observed both positive and negative $\Delta g'_{tr}$ values, and the values increase with increasing IL concentration. Apparently, different values are observed for the glycine residue contribution, depending on the molecule into which the glycine group is inserted. These results show that the value of $\Delta g'_{tr}$ for peptide group is very sensitive to the nature of neighboring groups and dependent on the ionic strength of the solutions. The negative contribution indicates that the interactions between the IL and glycyl residue are favorable. The observed negative $\Delta g'_{tr}$ values for glycine residue from water to IL systems suggest that the zwitterionic glycine peptides are significantly denatured by the IL and the destabilization of these glycine peptides increases with increase in the strength of ILs. On the other hand, the positive contribution indicates that IL is a stabilizer for the glycine residue of glycine peptides. IL exhibits unfavorable interactions with glycine residue.

3.4.2. Transfer Free Energy Contribution of Peptide Backbone Unit from c(GG). The model protein c(GG) was

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Table 2. Apparent Transfer Free Energies ($\Delta g'_{tr}$) of Model Compounds from Water to Aqueous IL Solution at 298.15 K

		$\Delta g'_{tr}$ (J·mol^{-1})		
model compounds	ILs	30% (v/v)	50% (v/v)	70% (v/v)
Gly_2-Gly	DEAA	490.70	709.46	980.08
	DEAS	−864.78	−1699.00	−2652.30
	TEAA	684.54	884.93	1079.53
	TEAS	−883.92	−1806.64	−2920.68
	TEAP	1086.87	1491.11	1922.52
	TMAA	15.23	−14.04	−206.72
Gly_3-Gly_2	DEAA	−1806.84	−2903.09	−4262.66
	DEAS	−991.32	−1634.55	−2033.44
	TEAA	−2424.61	−3967.49	−5657.63
	TEAS	−448.23	−622.04	−806.08
	TEAP	−1622.00	−2547.77	−3384.5
	TMAA	−1011.55	−1665.08	−2542.55
Gly_4-Gly_3	DEAA	603.97	1000.22	1484.55
	DEAS	688.22	1156.00	986.40
	TEAA	2138.95	2786.30	313810
	TEAS	1365.48	1848.63	1929.51
	TEAP	2156.87	2712.48	2639.50
	TMAA	1513.78	1967.47	2033.57
(Gly_3-Gly)/2	DEAA	−658.07	−1096.81	−1641.29
	DEAS	−928.022	−1666.74	−2343.98
	TEAA	−870.03	−1541.28	−2289.05
	TEAS	−666.04	−1214.31	−1843.74
	TEAP	−267.53	−528.45	−730.95
	TMAA	−498.15	−839.56	−1374.64
(Gly_4-Gly_2)/2	DEAA	−601.43	−951.43	−1389.05
	DEAS	−151.54	−239.27	−523.52
	TEAA	−142.82	−590.59	−1259.76
	TEAS	458.62	613.29	561.71
	TEAP	267.43	82.355	−372.49
	TMAA	251.11	151.19	−254.48
(Gly_4-Gly)/3	DEAA	−237.38	−397.79	−599.34
	DEAS	−389.27	−725.82	−1233.85
	TEAA	132.96	−98.75	−479.99
	TEAS	11.127	−193.33	−585.98
	TEAP	540.60	551.85	392.52
	TMAA	172.49	96.11	−238.56

chosen as a model compound for typical interactions found in proteins because it is the cyclic dimer of glycine. Therefore, no end charge effects and hydrogen bonding occur between peptide linkages. The hydrogen-bonded structure makes c(GG) a good model compound for hydrogen bonding which takes place within the peptide backbone of a protein. The close packing of c(GG) molecules in the solid also brings into play van der Waals interactions between the groups.[64] Initially, Gill et al.[64] confirmed that c(GG) has peptide model compound by solubility measurements of c(GG) in aqueous solutions of urea. Later, it has been consecutively developed as a model for the peptide backbone unit in a protein,[65] since it contains two peptide groups in the structure that exposes the peptide unit to the solvent. Consequently, the transfer free energy from water to aqueous electrolyte solutions of this compound is divided by 2 to obtain the contribution of one peptide backbone unit, as shown in Scheme 2, and $\Delta g'_{tr}$ values are collected in Table 3.

As seen from the results of Scheme 2 in Table 3, it is clear that the $\Delta g'_{tr}$ values for the peptide backbone unit (—CH_2C═ONH—) from water to ILs are positive and are observed to increase with increasing IL concentration in aqueous solution.

Scheme 2. Schematic Illustration of Determining the Contribution of the Glycyl Residue of c(GG)

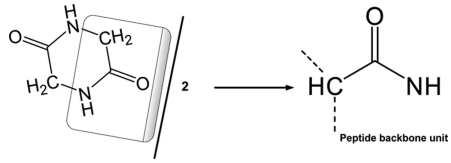


Table 3. Peptide Backbone Unit Contributions of Transfer Free Energy ($\Delta g'_{tr}$) of c(GG) from Water to IL Solution at 25 °C

		$\Delta g'_{tr}$ (J mol^{-1})		
scheme	ILs	30% (v/v)	50% (v/v)	70% (v/v)
c(GG)/2	DEAA	379.80	633.45	944.28
	DEAS	529.78	930.67	1304.88
	TEAA	441.52	724.53	1038.24
	TEAS	661.75	1124.28	1615.71
	TEAP	301.68	518.51	748.71
	TMAA	230.98	431.84	587.69

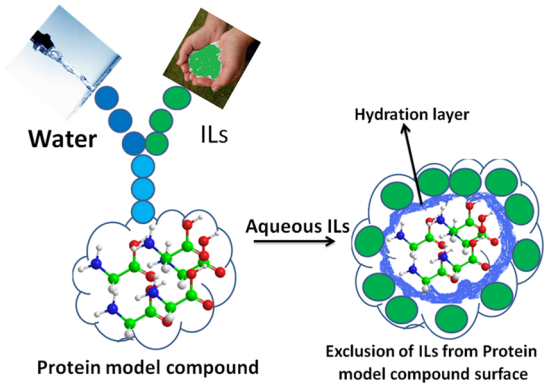
These findings provide an unfavorable transfer free energy of the peptide backbone unit of c(GG) from water to ILs. Within the series of the ILs, TEAS is the strongest protein stabilizer, because high positive $\Delta g'_{tr}$ values (for example, 661.75 J mol^{-1} for 30% (v/v) of TEAS) are obtained while TMAA is the weakest stabilizing IL, since its contribution is very small (for example, 230.98 J mol^{-1} for 30% (v/v) of TMAA). The remaining four ILs have a moderate effect on the stabilizing ability of the peptide backbone unit of c(GG). These results show that the value of $\Delta g'_{tr}$ for the peptide backbone unit is sensitive to the nature of the neighboring groups and is dependent on the ILs. One of the important conclusions drawn from the results of Schemes 1 and 2 is that some of the protecting ILs interact favorably with some of the glycyl residue. This behavior is quite different from the GP contributions, which exhibit unfavorable interactions with protecting ILs. However, the glycyl residue of glycine plays a less significant role in model protein compound stability.

Our solubility, *m*-values, and $\Delta G'_{tr}$ values explicitly demonstrated that ammonium based ILs stabilize the structures of GPs through unfavorable interactions between ILs and the surface of GPs. Virtually, the unfavorable interactions of solvents with proteins account for the increase of protein stability, indicating that solubilites decrease with increasing concentration of ILs, as well as the positive contribution of transfer free energy. Systematic studies by several researchers[60−66] have greatly delineated our understanding of the interplay between polypeptides, water, and cosolvents. These cosolvents can be classified according to their preferential interaction, with respect to water, with the exposed surface of dissolved proteins. Water molecules are preferentially attracted by the surface of protein in solution, and this preferential hydration excludes the cosolvents. Usually, the preferentially excluded cosolvent tends to stabilize the native state of proteins (salting out), without perturbing biomolecule structure and function through unfavorable interactions with the surface of the protein.[8,14] On the other hand, preferentially bound cosolvents favor protein denaturation and solubility increases with the increase in denaturant concentration (salting in).[11,60,61] Virtually, it is linked to the interaction of the cosolvent with water, the sign and magnitude of preferential

interaction with proteins in solution. The different signs and magnitudes reflect differences of interactions among water and proteins or proteins and cosolvents.

Accordingly, unfavorable interactions between GPs and ILs are primarily responsible for the model compound stability. During this period, water interacts more favorably with the surface of the GP. Meanwhile, ILs can be excluded from the GP surface due to the steric repulsion from water molecules (Scheme 3). Subsequently, the backbone of the protein is unable to interact with the organized water molecules around ILs (hydrated). In other words, ILs interact unfavorably with protein surfaces that assist in forming the hydration layer around the protein, further stabilizing it.

Scheme 3. Representation of Model Compounds in the Presence of ILs[a]



[a]IL is excluded from the surface of model compounds due to steric repulsions from the hydration layer (blue color); obviously unfavorable interactions are occurring between IL and the model compound surface.

4. CONCLUSION

We have generated a series of ammonium ILs to quantify the biocompatible effect on protein model compounds. Further, in this work, we have depicted the influence of six different ILs on the stability of five protein model compounds and their functional groups through the transfer free energy measurements at 25 °C under atmospheric pressure. The contribution of the peptide backbone unit is systematically predicted from the $\Delta G'_{tr}$ measurements of GPs from water to IL solutions. This detailed information supports the conclusion that ILs can stabilize the GP structures. On the basis of the analysis of $\Delta g'_{tr}$ values for glycyl residue from glycine compounds, we have observed both negative and positive values. The $\Delta g'_{tr}$ contribution is unfavorable or favorable, depending on the structural arrangements of GPs as well as ILs. In addition, the $\Delta g'_{tr}$ values of the peptide backbone unit of c(GG) from water to ILs are positive and are observed to increase with increasing IL concentration in solution. These findings show that ILs have unfavorable interactions with this residue and appear to have an excellent strategy toward stabilization of c(GG). Our findings conclude that the peptide backbone unit is significantly responsible for the protein folding/unfolding. It is quantified by the *m*-values that ILs are preferentially excluded from the protein model compound surface.

Variation of the alkyl chain at the substituted ammonium cations led to alteration in the efficacy of ILs as stabilizers. Further, we have concluded that the stabilization ability of ILs varies from biomolecule to biomolecule; IL to IL as well as the structural arrangement of materials. From the obtained results, ILs have proved to be effective potential cosolvents and are a good alternative to organic solvents for the biomolecules. In addition, ILs may stabilize the structures that are usually not observed in unfolding processes. In any case, the possibility to tune in such structures opens intriguing and fascinating possibilities for further mechanistic studies of protein folding/unfolding. To utilize the potential of ILs, it is necessary to further explore these studies in biological applications. The obtained results clearly demonstrate that the "tunability" of ILs (changing cation/anion combination and modifying the cation with various substituents) could help to change their biological activity, structure, and stability. Considering the huge applications of both biomolecules and ILs, the present work will trigger new sciences and technologies for physical chemistry and biophysical chemistry, but of course, more extensive screening and further studies into the mechanism of action of ILs in the biological environment are required to develop a full understanding which could lead to multiple biological applications.

■ ASSOCIATED CONTENT

Supporting Information

Tables showing GP solubilities in Water or in Aqueous ILs and Densities at Solubility Limits at 25 °C and Apparent Transfer Free Energies ($\Delta G'_{tr}$) of GPs from Water to Aqueous IL Solution at 25 °C and at Atmospheric Pressure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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