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Counteracting effects of trimethylamine N-oxide and betaine on the interactions of urea with zwitterionic glycine peptides

Pannur Venkatesu[a,b], Ho-mu Lin[a], Ming-Jer Lee[a,*]

[a] Department of Chemical Engineering, National Taiwan University of Science and Technology, 43 Keelung Road, Section 4, Taipei 106-07, Taiwan
[b] Department of Chemistry, University of Delhi, Delhi 110007, India

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ABSTRACT

To compare the counteracting effects of methylamines trimethylamine N-oxide (TMAO) and betaine on the actions of urea, we have determined the apparent transfer free energies ($\Delta G'_{tr}$) of zwitterionic glycine peptides: glycine (Gly), diglycine (Gly_2), triglycine (Gly_3), and tetraglycine (Gly_4) from water to methylamine and urea, and also the blends of methylamine and urea at a 1:2 ratio as well as various urea concentrations (0.5–8 M) in the presence of 1 M methylamine. The $\Delta G'_{tr}$ values of the blends of methylamine with urea revealed that the methylamine strongly counteracted the urea actions on glycine peptides. However, the methylamine partially counteracted the deleterious effects of urea on the Gly_4 in the cases of higher urea concentrations (4–8 M) in the presence of 1 M methylamine. The experimental results were further used to estimate the transfer free energies ($\Delta g'_{tr}$) of the peptide backbone unit ($-CH_2C{=}ONH-$) contributions.

1. Introduction

The field of protein folding and stability has been a critically important area of research for years, and remains today one of the great unsolved mysteries [1]. A polar molecule of urea, one of the most typical protein denaturants, experimentally [2–5] destabilizes biological macromolecules, altering their structure and function. Molecular dynamics simulations [6–8] show that urea acts indirectly by altering the water structure and consequently the solvation of the denatured proteins and also forms hydrogen bonds directly with the proteins. The outcomes of these results are explicitly expected to be deleterious of the proteins. Many organisms produce and accumulate small organic molecules, which comprise the bulk of the osmotically active solutes, termed as osmolytes (or osmoprotectants) [9,10] to counteract the effects of environmental stresses, such as dehydration, temperature and pH variations, freezing and high salinity. Osmolytes are generally categorized into three chemical classes, namely, polyols, amino acid derivatives and methylamines [10,11]. These three groups are compatible osmolytes, which stabilize proteins *in vitro* without substantial changes in protein structure and function [12]. On the other hand, the third group of osmolytes is referred to counteracting osmolytes, which are reversing the perturbations of protein structure caused by urea [13].

Counteracting or compensatory osmolytes, which are trimethylamine N-oxide (TMAO) and betaine (glycine betaine), appear to overcome the deleterious urea effects, and permit normal cellular function of proteins [10,14–19]. Moreover, many significant computational simulations [1,20,21] were devoted to understand the counteracting action of TMAO against the urea denaturing effects on proteins. Obviously, a good deal of effort has been directed towards the counteraction of TMAO against the perturbing effects of urea on proteins [14–17] effectively at the molar ratio 1:2 of TMAO:urea. However, no conclusive results have been explored systematically that the counteracting effects of TMAO (1 M), in the presence of higher urea concentrations (4–8 M) on perturbing effects of urea on proteins. The origin of counteracting effects of TMAO yet remains to be understood and it remains a subject of active research. Apparently, the studies on betaine attenuation of the deleterious effects of urea on proteins are very limited [16,17]. Nevertheless, numerous issues remain to be resolved on counteracting properties of methylamine, in the presence of higher urea concentrations, on opposing to the denaturing effects of urea on proteins.

Recently, we have compared the denaturing effects of urea as well as guanidine hydrochloride (GdnHCl) on the model compounds of cyclic dipeptides (CDs), those are counteracted by TMAO [19]. We found that TMAO strongly counteracted the urea deleterious actions on CDs while TMAO partially counteracted GdnHCl actions on CDs, since GdnHCl is a more effective denaturant than

* Corresponding author. Tel.: +886 2 2737 6626; fax: +886 2 2737 6644.
E-mail addresses: Venkatesup@hotmail.com (P. Venkatesu), mjl@ch.ntust.edu.tw (M.-J. Lee).

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urea. However, a comparison of the counteracting effects of TMAO and betaine on reversing the perturbations caused by urea on the proteins has received less attention. In the light of these considerations as mentioned above and in the view of their importance, it is of interest to compare the counteracting effects of methylamines, such as TMAO and betaine, against the urea actions on proteins. For these reasons, we measured the apparent transfer free energies ($\Delta G'_{tr}$) for glycine peptides from water to various concentrations of TMAO, betaine, urea, separately and aqueous mixtures containing the molar ratio of 1:2 of methylamine:urea and varying the urea concentrations (from 0.5 to 8 M) in the presence of 1 M TMAO or betaine via solubility measurements. The selected glycine peptides are glycine (Gly), diglycine (Gly_2), triglycine (Gly_3), and tetraglycine (Gly_4).

One aim of this work is to elucidate the attenuation effects of methylamines on urea actions on the peptide backbone unit contribution of transfer free energy ($\Delta g'_{tr}$). Additionally, the experimental results of $\Delta G'_{tr}$ allow us to investigate the individual contributions of the transfer free energy ($\Delta g'_{tr}$) for peptide backbone unit (or the glycyl residue (–CH_2C=ONH–)) contribution in the aqueous solutions containing TMAO or betaine, urea and in their mixture solutions.

2. Materials and methods

2.1. Materials

Gly (>99% of purity) was obtained from Acros Organics (USA). Gly_2 (>99.5% of purity), Gly_3 (>99% of purity) and Gly_4 (>99% of purity) were purchased from Sigma Chemical Co. Urea was supplied by Acros Organics (USA). TMAO and betaine were purchased form Sigma chemical Co., USA. All these purchased materials were used as received. High purity water used for preparing the aqueous solutions was treated by NANO pure-Ultra pure water system. The purified water can be distilled and deionized with resistance of 18.3 MΩ. All mixture samples were prepared gravimetrically.

2.2. Methods

The solubility of glycine peptides in water, aqueous methylamines, urea and their mixtures was obtained from the density (ρ) measurements, which are similar to that of Nozaki and Tanford [22–24]. The detailed procedure used in this work has been delineated in our earlier articles [11,19,25]. The densities of the solutions were measured with the aid of a high precision vibrating tube

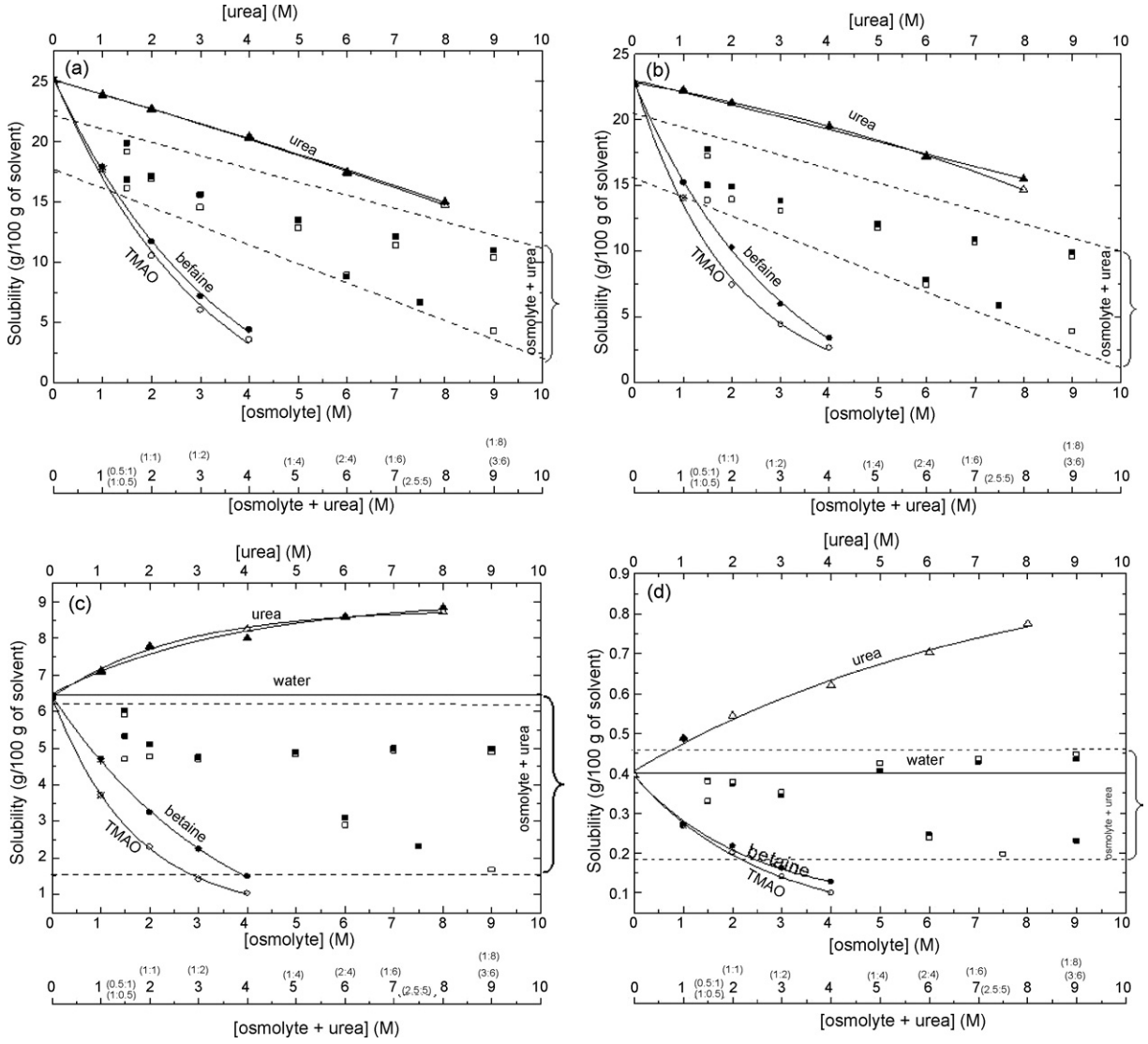


Fig. 1. Solubility limits for glycine peptides of (a) Gly; (b) Gly_2; (c) Gly_3; and (d) Gly_4 in aqueous and aqueous solutions of TMAO (○), betaine (●), urea (Δ), TMAO + urea (▲) and betaine + urea (□). Solid lines show only smoothness of the solubility data points. The dashed lines show the region of methylamine + urea.

digital densitometer (Model 4500, Anton Paar, Austria), with an uncertainty of $\pm 5 \times 10^{-5}$ g cm^{-3}. The temperature was controlled to within ±0.02 °C. The uncertainty of the solubility limit is lower than ±0.8%. The densitometer was calibrated with air and degassed distilled water.

3. Results

In the present study, the solubility limits (S_{AA}, grams of AA/100 g of solvent) of the glycine peptides in water and in the solvent mixtures along with the densities (ρ_{AA}) of the solutions are presented in Supplementary data as a Table and graphically illustrated in Fig. 1. From Supplementary data of Table one can clearly see that the obtained solubility limits of glycines in water are in good agreement with the literature values [22–24,26] at 25 °C.

3.1. Transfer free energy (ΔG_{tr}) of zwitterionic glycine peptides from water to aqueous osmolyte or urea solutions and their mixtures

The solubility data were used to determine the ΔG_{tr} values for the gylcine peptides from water to the aqueous solutions containing TMAO, betaine, urea and various ratios of TMAO:urea and betaine:urea at 25 °C under atmospheric pressure. The detailed description of obtaining ΔG_{tr} has been reported in our earlier work [11,25]. 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 [27]. Assuming that the solid phase is pure compound i, the fugacity equality becomes

$$f_{i(\text{pure solid})} = \hat{f}_{i(\text{solute } i \text{ in liquid solution})} \quad (1)$$

or

$$f_{i(\text{pure solid})} = x_i \gamma_i f_i^o \quad (2)$$

where x_i is the solubility of the 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^o is the standard-state fugacity to which γ_i refers. As a consequence, the ΔG_{tr} value of amino acids (AA) from water to the aqueous osmolyte or urea solutions and in their mixtures, ΔG_{tr}, can be calculated from the following equation:

$$\Delta G_{tr} = \bar{G}^{\infty}_{AA,ws} - \bar{G}^{\infty}_{AA,w} = \mu^{\infty}_{AA,ws} - \mu^{\infty}_{AA,w} = RT \ln\left(\frac{f^{\infty}_{AA,ws}}{f^{\infty}_{AA,w}}\right) = RT \ln\left(\frac{x_{AA,w}\gamma^{*}_{AA,w}}{x_{AA,ws}\gamma^{*}_{AA,ws}}\right) = RT \ln\left(\frac{x_{AA,w}}{x_{AA,ws}}\right) + RT \ln\left(\frac{\gamma^{*}_{AA,w}}{\gamma_{AA,ws}}\right) \quad (3)$$

where the subscript w refers to the aqueous and ws to the aqueous TMAO, betaine, urea solution or various ratios of osmolyte to urea. Eq. (3) can also be expressed in terms of molar concentration,

$$\Delta G_{tr} = RT \ln\left(\frac{C_{AA,w}}{C_{AA,ws}}\right) + RT \ln\left(\frac{\gamma^{\#}_{AA,w}}{\gamma^{\#}_{AA,ws}}\right) \quad (4)$$

Note that Cohn and Edsall [28] and Tanford [29] used mole fraction scale, while Robinson and Jencks [30,31] and Bolen and coworkers [32,33] used the molarity scale for determining transfer free energies. On the basis of a statistical standard thermodynamic treatment, Ben-Naim [34] has strongly suggested in the favor of molarity scale for obtaining transfer free energies. Therefore, we are also used molarity scale for predicting the transfer free energies.

Virtually, many researchers [11,19,23,24,31–35] have ignored the activity coefficient term on the right-hand side of Eq. (4) for determining transfer free energies of various solutions, because rigorously obtaining the activity coefficients of proteins in multicomponent liquid mixtures from phase equilibrium data without using solution theory models is extremely problematic and difficult. Nozaki and Tanford [22,36] have made some efforts to estimate activity coefficient contribution in different solvent systems and this practice was not continued in their further studies [23,24,36]. Eventually, they have noted that 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 term is negligible and its effect is not much greater than the experimental uncertainty [22–24]. Considering the majority of researchers, who have evaluated transfer free energies in a variety of aqueous multicomponent systems, we opt to ignore the activity coefficient term on the right hand side of Eq. (4). 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 of glycine peptides in the presence of TMAO or betaine, urea and various combinations of methylamine with urea are also included in Supplementary data of Table as well as graphically displayed in Fig. 2. This transfer free energy represents the change in free energy of each glycine peptide upon transferring from water (0 M) to an aqueous solution at a specific concentration.

3.2. The contribution of peptide backbone unit for transfer free energy ($\Delta g'_{tr}$)

To estimate the $\Delta g'_{tr}$ values for the glycyl residue, we have subtracted the corresponding values of glycine peptides, as presented in Scheme 1, which depicts in two types of mathematical constructs. Amongst them, the simple subtractional constructs (denoted as SSC) consist of subtracting the $\Delta G'_{tr}$ values of two glycines, such as Gly_2 and Gly; Gly_3 and Gly_2; Gly_4 and Gly_3. On the other hand, the composite constructs (denoted as CC) contain of subtracting the $\Delta G'_{tr}$ values of two glycines that differ in chain length by more constructs of one peptide unit, such as Gly_4 and Gly, then dividing the difference by three the number of remaining peptide units, [(Gly_4 – Gly)/3]. Similarly for other two different chain length peptide units, like [(Gly_4 – Gly_2)/2] and [(Gly_3 – Gly)/2]. The calculations can be performed for all investigated systems and the results of these constituent peptide backbone unit contributions of $\Delta g'_{tr}$ at 25 °C are collected in Table 1. All of 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 aqueous solutions with reference to the difference of their definitions as well as their interactions with different solvents.

4. Discussion

As seen from the experimental results in Fig. 2, the solubilities of the glycine peptides are significantly affected by the addition of methylamines or urea. It is systematically clear from this figure that the solubilities of four investigated glycines decrease monotonically with increasing the concentrations of osmolyte in aqueous solutions. The magnitude of the solubilities decrease depends on the nature of osmolytes and generally follows the physical interface of glycines. Interestingly, we observed different phenomena for urea effects on the solubility of glycine peptides. The solubilities of the simplest Gly and Gly_2 in urea solution decrease linearly with increasing the urea concentrations as also shown in Fig. 1(a) and (b). This abrupt change in solubility behavior of these simple glycines in urea entirely reversed the behavior of urea nature. A similar trend was observed by Nozaki and Tanford [22] that the solubilities of the simplest Gly and Gly_2 in urea solution decrease with increasing the urea concentrations. An explanation of this effect is that Gly is the simplest constituent of peptides, and amide (–CONH–)

groups are absent. Virtually, the extent of interactions of urea with protein surfaces led that urea is accumulated only at polar groups as well as amide surface and is considered to act breaking protein hydrogen bonds, and undoubtedly interact with peptide groups in unfolded proteins by hydrogen bondings [37,38]. The solubility of Gly_2 in aqueous urea solutions indicates that the less peptide group (only one peptide) of Gly_2 is unable to accommodate with urea, thereby we observed the solubilities decrease with increasing urea concentration. However, this behavior of simple glycines becomes sharply reversed with increasing the peptide groups in higher glycines (Gly_3 and Gly_4). In the presence of urea causes a significant enhancement of solubilities of Gly_3 and Gly_4 and these solubilities increase with increasing the urea concentrations as also shown in Fig. 1(c) and (d). These results reveal that urea strongly and favorably interacts with peptide groups (forming hydrogen bonds) of higher glycines.

Fig. 1 also compares the effects of TMAO or betaine in the presence of urea on the zwitterionic glycine peptides. The results in Fig. 1(a) explicitly show that TMAO lowers the solubilities of Gly in urea. For example, the solubility (S_{AA}) of Gly in 1 M TMAO is 17.65 and that in 1 M urea is 23.89. Note that TMAO as combined with urea at 1:1 ratio, the observed solubility is 16.93, considerable lower than the values in the presence of individual 1 M urea. Therefore, it reveals that TMAO can strongly offset the actions of urea on Gly at the ratio of 1:1. Furthermore, increasing the urea concentrations (in the range of 2–8 M urea) in the presence of 1 M TMAO, as well as at a 1:2 ratio of TMAO and urea, clearly show that TMAO counteracts the urea actions on Gly, since the solubilities of the Gly in the combination of TMAO + urea are lower than that in control (water). Furthermore, Fig. 1(a) shows that the similar type of behavior was observed in the offset effects of betaine against the urea actions on Gly. Similarly, TMAO or betaine also decreases the urea actions of solubilities on Gly_2 (Fig. 1(b)).

As also can be seen from Fig. 1(c), the urea actions on Gly_3, in all instances, is greatly compensated and counteracted by TMAO or betaine. The present results with the solubilities of Gly_3 in the combination 1 M TMAO (or 1 M betaine):urea (from 1 to 8 M) is surprising because counteraction is apparent even at high urea concentrations. Over the entire concentration range, the solubilities in the aqueous solutions of TMAO (or betaine) plus urea are no greater than that in water. Different phenomena were observed from the mixtures containing Gly_4, TMAO or betaine (1 M) and urea (from 1 to 8 M). The solubilities of Gly_4 are lower than that in water as the concentrations of urea not greater than 2 M in the presence of

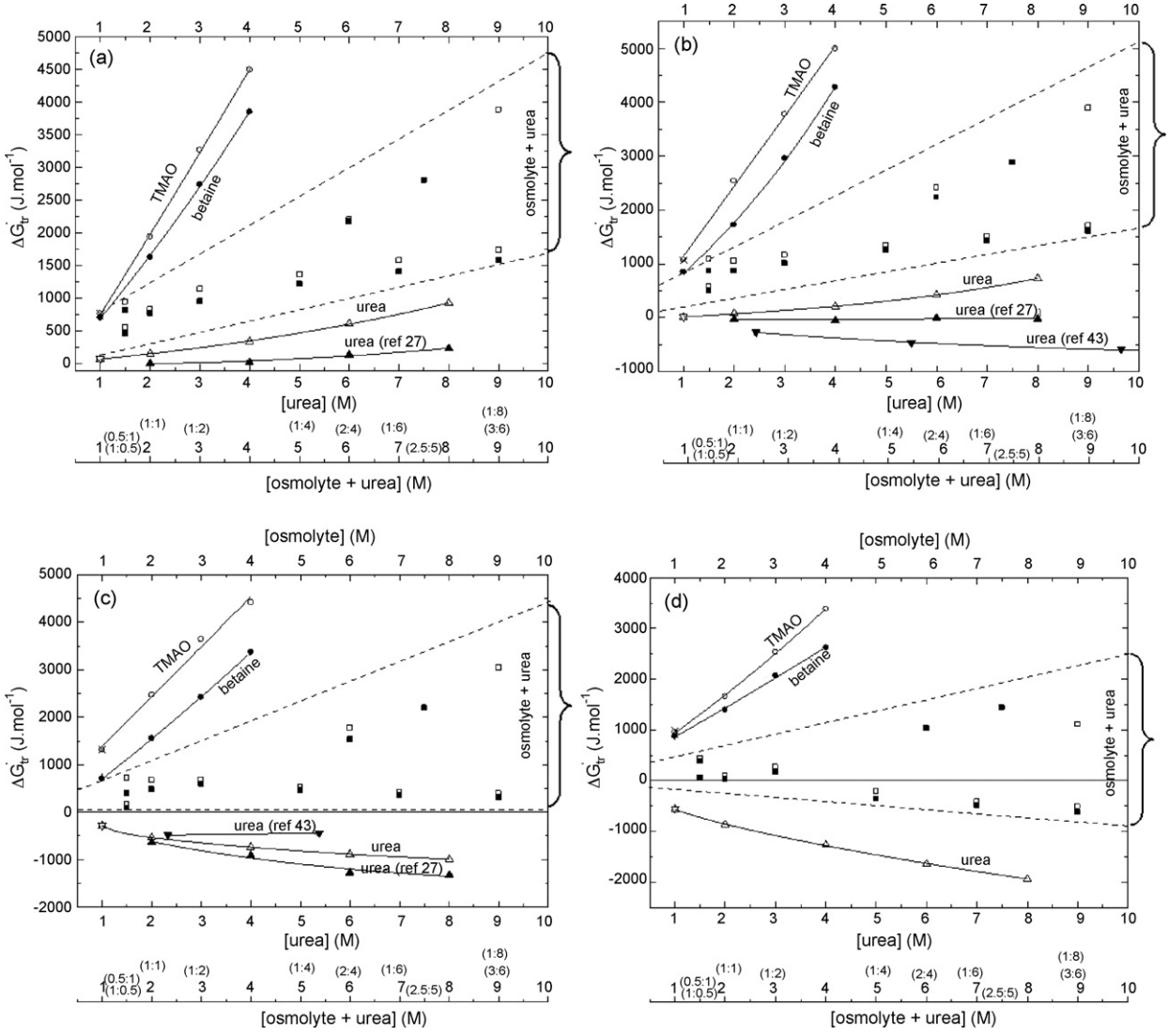
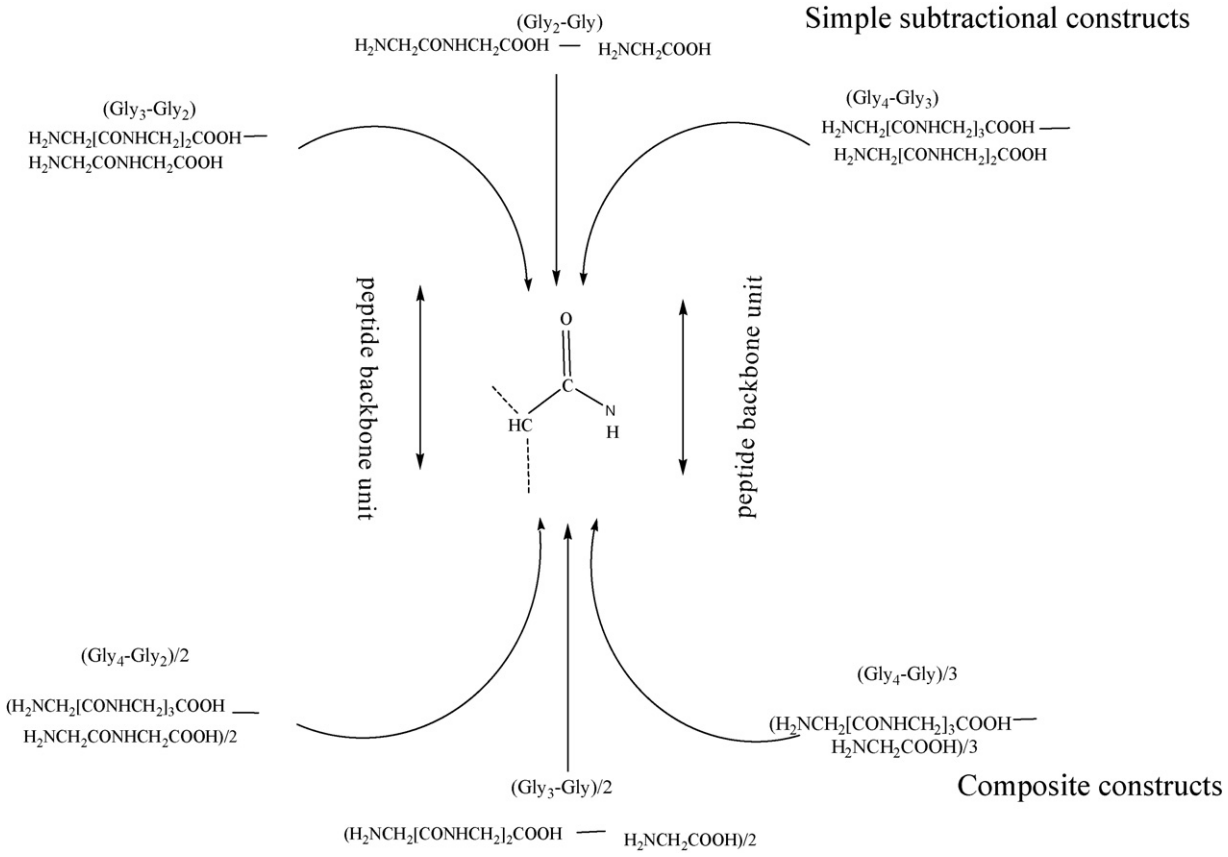


Fig. 2. Effect of TMAO or betaine counteraction against the perturbation of urea actions on zwitterionic glycine peptides. The apparent transfer free energies ($\Delta G'_{tr}$) of glycine peptides of (a) Gly; (b) Gly_2; (c) Gly_3; and (d) Gly_4 in aqueous solutions of TMAO (○), betaine (●), urea (△), TMAO + urea (▲) and betaine + urea (□). Solid lines show only smoothness of the $\Delta G'_{tr}$ data points. The dashed lines show the region of methylamine + urea.



Scheme 1. Schematic illustration of the contribution of peptide backbone unit of glycine peptides.

1 M TMAO or betaine and also in the ratio of 1 methylamine:2 urea, but, those are greater than in water at higher concentrations of urea (from 4 to 8 M) (Fig. 1(d)). Apparently, methylamine somewhat fails to prevent the strong favorable interactions between urea and Gly_4, at higher urea concentrations, since Gly_4 contains more number of peptide groups. Therefore TMAO or betaine partially counteracts the effects caused by urea on Gly_4.

4.1. The contribution of $\Delta G'_{tr}$ of zwitterionic glycine peptides from water to aqueous osmolyte or urea solutions and their mixtures

Transfer free energy is the best suited property for identifying the favorability or unfavorability of transferring proteins from water to other solvent media [11,22,32]. Virtually, protein stability, structure and function are critically depending on the solvent environments. The protecting osmolytes raise positive values of the free energy of the unfolded state, favoring the protein stability while denaturants lower the negative free energy of the unfolded state indicating that the proteins are at destabilized state [11,39]. On the other hand, methylamine class of osmolytes counteracts the urea actions on proteins by unfavorable interactions [13,19]. In order to obtain the compatibility and the counteracting effects of TMAO or betaine, we investigated the $\Delta G'_{tr}$ of the glycine peptides from water to the aqueous solutions containing TMAO or betaine, urea and various combinations of methylamine with urea.

The open and closed circles in Fig. 2 show the effects of TMAO and betaine on glycine peptide molecules, respectively. As depicted the results in Fig. 2, the $\Delta G'_{tr}$ values are positive for each glycine peptide with TMAO or betaine, and these positive values increase with increasing the concentrations of TMAO or betaine. In our previous studies [11,19], we also found that the similar trend of the $\Delta G'_{tr}$ on cyclic dipeptides varies linearly with osmolyte concentration. The results explicitly indicate that TMAO or betaine interacts unfavorably with the surfaces of glycine peptide and these osmolytes stabilize the peptides, while these do not interfere with the functional activity of glycines. As discussed elsewhere [11], the osmolytes increase the stability of the model compounds and also tend to decrease their solubilities. Such osmolytes are excluded from the protein surface where their concentrations near the peptide are lower than those in the bulk solution. Osmolyte enhances water structure and forms the hydration layer with water molecules. Apparently, the peptide bond of protein is less able to interact with hydrated water around osmolyte and, therefore, there is negative binding between osmolyte and the protein surface. During this period, water interacts more favorably with the surface of the protein. Meanwhile, osmolyte can be excluded from the protein surface due to the steric repulsion from water molecules. Interestingly, our results are corroborated with a simple statistical mechanics backbone solvation model [39] and experimental studies [37,40,41], in which the protecting osmolytes raise the free energy of the unfolded state, favoring the folded population. A close observation of Fig. 2 reveals that TMAO is more effective than betaine in stabilizing effects on glycine peptides.

The open triangles in Fig. 2 show that our results of the urea effects on glycine peptides. Virtually, urea, in contrast to osmolyte, interacts favorably with protein surfaces. However, our results in Fig. 2(a) and (b) show that the values of $\Delta G'_{tr}$ are positive for the systems of Gly or Gly_2 with urea and the positive values increase linearly and slightly with increasing the urea concentration. Surprisingly, the results are implying that urea interacts unfavorably with the simple glycines, which are sharply reversed with our previous study in which urea interacts favorably with the model compounds of cyclic dipeptides [19]. The traditional explanations

Table 1
Contributions of peptide backbone unit (glycyl residues) transfer free energy ($\Delta g'_{tr}$) from water to aqueous solutions of TMAO, betaine, urea separately and in their mixtures TMAO or betaine + urea) at 25 °C.

Solvent	$\Delta g'_{tr}$ (J mol^{-1})					
	$Gly_2 - Gly$	$Gly_3 - Gly_2$	$Gly_4 - Gly_3$	$(Gly_3 - Gly)/2$	$(Gly_4 - Gly_2)/2$	$(Gly_4 - Gly)/3$
1 M TMAO	307.40	236.74	−3507.55	272.07	−60.41	62.20
2 M TMAO	599.53	−75.25	−810.06	262.14	−442.65	−95.26
3 M TMAO	515.33	−139.90	−1098.19	187.731	−619.03	−240.91
4 M TMAO	504.74	−579.60	−1030.81	−37.45	−805.23	−368.57
1 M betaine	143.24	−154.90	181.22	−5.82	13.17	56.53
2 M betaine	91.31	−171.10	−158.07	−39.88	−164.56	−79.27
3 M betaine	220.37	−539.90	−351.10	−159.76	−445.50	−223.54
4 M betaine	433.96	−915.20	−743.10	−240.60	−729.14	−408.10
1 M urea	−61.83	−300.20	−274.64	−181.01	−287.42	−212.22
2 M urea	−75.79	−616.01	−332.21	−345.90	−474.11	−341.34
4 M urea	−133.10	−943.02	−531.31	−538.05	−737.17	−535.80
6 M urea	−193.80	−1312.08	−751.67	−752.94	−1031.90	−752.52
8 M urea	−198.90	−1721.83	−944.60	−960.36	−1333.20	−955.11
0.5 M TMAO + 1 M urea	23.99	−420.40	−101.25	−198.21	−260.83	−165.89
1 M TMAO + 0.5 M urea	137.07	−361.60	−281.37	−112.27	−321.49	−168.63
1 M TMAO + 1M urea	224.07	−381.36	−576.82	−78.64	−479.09	−244.70
1 M TMAO + 2 M urea	27.64	−491.79	−409.25	−232.08	−450.52	−291.13
1 M TMAO + 4 M urea	−16.84	−711.17	−743.76	−414.01	−777.46	−523.92
1 M TMAO + 6 M urea	−66.46	−1085.14	−741.57	−575.80	−963.35	−664.39
1 M TMAO + 8 M urea	−29.79	−1325.60	−901.46	−677.70	−1113.50	−752.28
2 M TMAO + 4 M urea	215.86	−651.63	−734.63	−217.88	−693.13	−390.13
3 M TMAO + 6 M urea	11.73	−751.13	−1932.02	−419.70	−1391.60	−923.80
0.5 M betaine + 1 M urea	35.27	−413.91	−33.01	−189.32	−223.46	−137.21
1 M betaine + 0.5 M urea	45.70	−481.83	−5.36	−218.07	−243.59	−147.16
1 M betaine + 1M urea	100.42	−402.68	−438.80	−151.13	−420.74	−247.02
1 M betaine + 2 M urea	56.57	−410.86	−433.15	−177.15	−422.00	−262.48
1 M betaine + 4 M urea	42.59	−798.35	−726.73	−377.88	−712.54	−527.50
1 M betaine + 6 M urea	27.42	−1076.54	−749.12	−524.56	−962.83	−632.75
1 M betaine + 8 M urea	26.29	−1292.26	−934.79	−632.98	−1113.50	−733.59
2 M betaine + 4 M urea	70.57	−709.19	−507.17	−319.31	−608.18	−381.93
2.5 M betaine + 5 M urea	82.23	−686.59	−761.49	−302.18	−724.04	−455.28

of this effect are that Gly, which is monomeric amino acid, has no amide surface, thereby urea cannot interact with Gly and is not possible to form hydrogen bonds with Gly. As mentioned before, urea interacts favorably with protein surface led that urea is accumulated only at polar groups as well as amide surface and is considered to act breaking protein hydrogen bonds, and undoubtedly interacts with peptide groups in unfolded proteins [37,38]. Since, lack of peptide groups in Gly and less peptide group in Gly_2, urea is unable to break the hydrogen bonds of these simple glycines and thereby interacts unfavorably with these glycines without perturbing the structures of these two glycines.

On the other hand, the urea effects on the rest of the higher glycines, Fig. 2(c) and (d) shows that the $\Delta G'_{tr}$ values are negative for the Gly_3 and Gly_4 in aqueous urea solutions. The overall results of urea effects on the series of glycine peptides (Gly, Gly_2, Gly_3 and Gly_4), $\Delta G'_{tr}$ values (75.04, 13.21, −286.99 and −561.62 J mol^{-1}, respectively) become increasingly negative with increasing number of peptide groups. Similar conclusions have been obtained by Cannon et al. [8] that the transfer of glycine peptides from water to urea solutions. As seen from the results in Fig. 2(c) and (d) shows that the negative $\Delta G'_{tr}$ values for Gly_3 or Gly_4 increase significantly with increasing urea concentrations. Apparently, the negative contributions reveal that urea ruptures the hydrogen bonds of higher glycines and preferentially makes new bonds with higher gylcines. The urea actions on Gly_3 and Gly_4, these binding interactions overcome the stabilizing of excluded osmolyte effects, causing the protein denatured state. Preliminary explanations of this effect focused on their obvious potential for hydrogen bonding between the surfaces of higher glycines and urea. The denaturants are considered to act by breaking proteins hydrogen bonds and interact preferentially with the protein surface, thus appearing to be bounded, and the protein is noted to be preferentially binding. Recently, the experimental studies on water accessible surface areas of Cannon et al. [8] and the computer molecular dynamic simulations [6,42] have concluded that urea directly binds to the protein, in particular to its peptide groups, thereby promoting unfolding protein. The difference between preferentially binding and preferentially hydration (excluding effect) is shown schematically in Fig. 3.

Fig. 2 compares the change in $\Delta G'_{tr}$ values as a function of methylamine or urea. It can be seen that TMAO or betaine rapidly enhances the $\Delta G'_{tr}$ values in all four investigated glycine peptides while urea slightly increases the $\Delta G'_{tr}$ values with simple glycine peptides of Gly and Gly_2 and lowers the $\Delta G'_{tr}$ values in the case of higher glycines of Gly_3 and Gly_4. Fig. 2(a) and (b) and Supplementary data Table depict that $\Delta G'_{tr}$ values of TMAO and urea at a molar ratio of 1:1, 1:2 and the urea concentrations varying in the presence of 1 M TMAO (i.e. TMAO plus urea mixture rapidly increases the $\Delta G'_{tr}$ values) have almost the same contribution that the $\Delta G'_{tr}$ values of TMAO alone actions on Gly and Gly_2. For example, Supplementary data Table shows that the values of $\Delta G'_{tr}$ for 1 M TMAO with Gly and Gly_2 systems are 766.97 and 1074.37 J mol^{-1}, respectively, and those for 1 M urea systems are 75.04 and 13.21 J mol^{-1}, respectively. It is interesting to note that TMAO as combined with urea at a 1:1 ratio ($\Delta G'_{tr}$ = 830.83 and 1054.90 J mol^{-1}) does not significantly change the individual effects of 1 M TMAO. Similarly, we also observed the offset effects of TMAO against the urea actions on simple glycine peptides at remaining ratios of TMAO with urea. Betaine combined with urea, in all instances, was also found to rapidly promote the $\Delta G'_{tr}$ values with the Gly and Gly_2 systems. Thus our results strongly indicate that the contribution of methylamine combined with urea is thermodynamically the same contribution as that for the methylamine alone in the systems of simplest amino acids of glycines, which are almost absent or less of peptide groups. These findings reveal that methylamine plus urea mixture substantially increases the positive

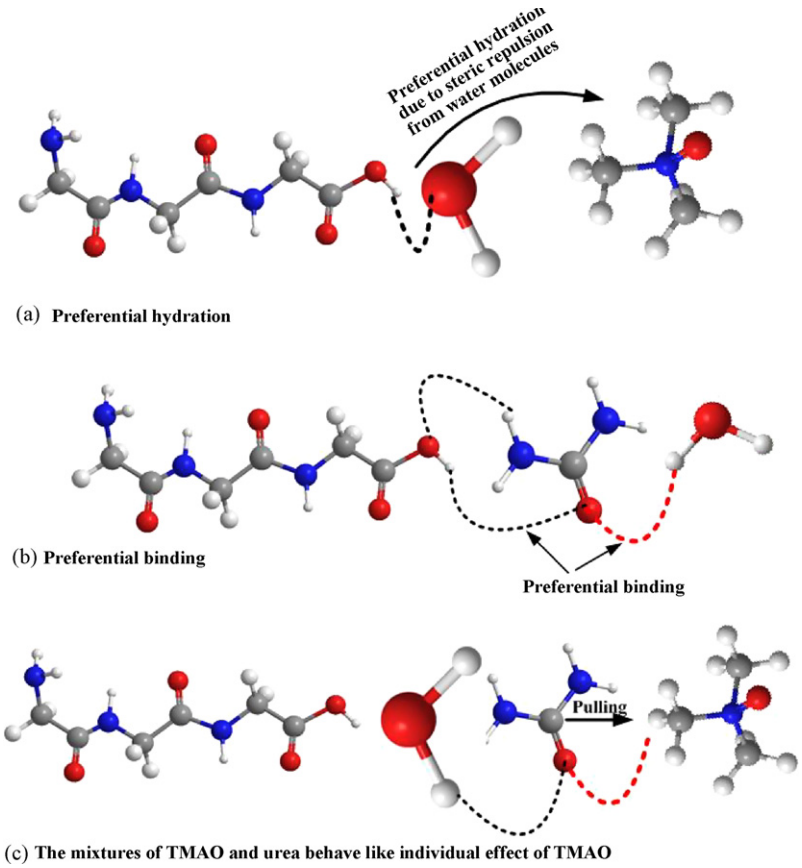


Fig. 3. Schematic depiction of Gly_3 (a) preferential hydration and (b) preferential binding in the presence of TMAO and urea, respectively. Moreover, (c) the mixture of TMAO and urea behaves like individual effect of TMAO. Betaine also behaves like the effects of TMAO.

$\Delta G'_{tr}$ values and was found to stabilize the simple peptides without significantly perturbing the structure and function of these amino acids, since the $\Delta G'_{tr}$ values are under control. It is widely argued that the counteracting ability of the methylamine arises from the unfavorable interactions between methylamine + urea and protein, in which the combination of methylamine + urea is preferentially excluded from the vicinity surroundings of protein [14,19,21].

As shown in Fig. 2(c) and (d), the positive $\Delta G'_{tr}$ values of Gly_3 and Gly_4 in the presence of TMAO or betaine are progressively increased, whereas urea increases the negative $\Delta G'_{tr}$ values. It can be seen that TMAO or betaine successfully competes the perturbing urea actions on Gly_3 in all cases, and enhances the positive $\Delta G'_{tr}$ values in combination of TMAO or betaine with urea at all molar ratios. Methylamine effect becomes progressively larger with increased additions of urea, directly demonstrating that methylamine counteracts the urea effects on Gly_3. These findings are consistent with our previous conclusions [19] that TMAO strongly reversed the deleterious effects of urea on cyclic dipeptides even at high urea concentrations.

The results in Fig. 2(d) demonstrate that Gly_4 is denatured in the presence of urea and this effect is partially counteracted by TMAO or betaine. From this figure, it is evident that, as TMAO was combined with urea at 1:1 ratio as well as 1:2 ratios, the activity of Gly_4 still unaltered, since we observed positive $\Delta G'_{tr}$ values and this positive contribution elucidated that TMAO can offset the damaging effects of urea on Gly_4 at these ratios. On the other hand, it should be noted that TMAO attenuating effect was partially apparent in Gly_4 at higher concentrations of urea (ranging from 4 to 8 M) in the presence of 1 M TMAO (see Fig. 2(d)). Under these experimental conditions, the negative $\Delta G'_{tr}$ values were observed, indicating that the favorable interactions exist between the surfaces of Gly_4 and TMAO + urea. These unexpected negative $\Delta G'_{tr}$ values are caused by urea and the lack of TMAO counteraction to protect the Gly_4 against the actions of urea. This observation revealed that TMAO partially counteracted the perturbing structures of the Gly_4 by urea at higher concentrations in the presence of 1 M TMAO. When the urea concentrations increased in 1 M methylamine, urea can essentially break the largely amide groups of Gly_4 and thereby the methylamine cannot prevent this urea actions on Gly_4 at higher concentrations of urea. Analogously, TMAO partially counteracted the deleterious effects of GdnHCl, which is another effectively classical chemical denaturant, on cyclic dipeptides [19]. As would be expected from these studies, the counteracting effects are strongly dependent on the concentration ratios of methylamine and denaturant as well as the nature of proteins.

There have also been simulations studies [20,21] of aqueous solutions that included TMAO, urea, and proteins, which might explain how TMAO counteracts against the urea actions on proteins. Accordingly, Bennion and Daggett [21] observed that TMAO enhanced water–water hydrogen bonding both in TMAO–water mixtures and in urea–TMAO–water mixtures. In addition, TMAO also strengthened water–water and water–urea interactions [43,44], and led to a decrease in urea–protein hydrogen bonding. In such a situation, TMAO limits the urea denaturing effects. Bennion and Daggett [21] clearly depicted these findings that urea was observed to be sandwiched between water molecules and that formed the hydration layer of the TMAO methyl groups. This enhancement of solvent structure in hydration shell prevented the initial attack of the protein by water and subsequently urea, further, it protecting the protein against the urea actions. The schematic illustration of TMAO counteraction of denaturants on the Gly_3 was also included in Fig. 3. For the sake of clarity presentation we do not

present the rest of three glycine peptides interactions with TMAO or betaine and their mixtures.

The results reported here explicitly indicate that TMAO and betaine have comparable effects on counteracting the urea actions on glycine peptides. The counteracting effects of TMAO against the urea actions on glycine peptides are similar to those seen with betaine. However, TMAO that actually attenuated the effects caused by urea on peptides seems more effective than betaine. Ortiz-Costa et al. [17] and Samuelsson et al. [45] have also noted that TMAO proved to be more effective than betaine towards urea-induced inactivations. On the other hand, this conclusion has sharply reversed the studies of Tseng and Graves [46], in which betaine counteracted effects of urea on tau-induced polymerization of microtubules better than TMAO. The results indicate that these two methylamines have comparable effects on counteracting the reversed actions of urea on proteins. In other words, Holthauzen and Bolen [47] have noted that the mixture of sarcosine, which is another methylamine, and urea does not alter one another's efficacy at high concentrations, suggesting that the number of osmolyte interactions sites on the protein is large and the binding constants are quite small. Consequently, the site occupancies are low enough in the number that the sarcosine and urea neither compete nor cooperate in interacting with the protein. These findings indicate that the methylamine counteraction ability against the urea deleterious effects on proteins depends on the methylamine–protein pair. Recently, Singh et al. [48] concluded that molar concentration of a methylamine required to offset the denaturing effect of urea at a given concentration is different for different proteins.

4.2. The contribution of peptide backbone unit for apparent transfer free energy ($\Delta g'_{tr}$)

Thermodynamic properties for a given model compound of proteins may be estimated from the knowledge of its molecular structure via group contribution approach, that is assumed to be independent of neighboring functional groups [11,19,49,50]. In order to evaluate the peptide backbone unit (glycyl residue) contribution we used SSC and CC methods. As seen the results in Table 1, the transfer free energy ($\Delta g'_{tr}$) contributions of glycine residue from water to TMAO or betaine are negative and also increase with increasing the osmolyte concentration, except for the $\Delta g'_{tr}$ of ($Gly_2 - Gly$) with osmolyte system, $Gly_3 - Gly_2$ and ($Gly_3 - Gly$)/2 of 1 M TMAO and few simple subtractional constructs of 1 M betaine. It is interesting to note that the $\Delta g'_{tr}$ contributions of glycyl residue in TMAO and betaine are negative, indicating that glycyl residue interacts favorably with either TMAO or betaine and interacts unfavorably with osmolyte in the case of simple subtractional constructs of ($Gly_2 - Gly$). Our models differ drastically and often vary nonlinearly with osmolyte or urea concentrations. These discrepancies are due to the different definitions of transfer free energy of the peptide backbone unit as well as their interactions with different solvents. Besides, different values were observed for the glycine residue contribution, depending on the molecule into which the glycine group is inserted. This may be due to either the inadequacies of the mathematical constructs or the chain length not being long enough to eliminate the effects of the charged end groups. Moreover, Cohn and Edsall [28] distinctly enunciated that the obtaining $\Delta g'_{tr}$ values for glycyl residue from glycine series have large difference due to the long-range electrostatic attraction of the formal charges on the ends of the molecules. This peptide backbone unit contribution of zwitterionic glycine peptides with osmolytes is sharply reversed from our earlier peptide backbone unit contribution of cyclo(Gly–Gly) with osmolytes, in which unfavorable interactions were observed between peptide backbone unit of cyclo(Gly–Gly) with osmolytes [11,19]. Apparently, these types of interactions are described as providing an increase in structural order of the solvent systems and the structural arrangements and position of the peptide backbone unit in the proteins.

The results in Table 1 reveal that the $\Delta g'_{tr}$ values are negative for peptide backbone unit contribution of zwitterionic glycine peptides in urea. These findings provide favorable interactions between urea and the glycyl residue, in all constructs. Interestingly, this glycyl residue contribution of zwitterionic glycine peptides with urea is quite consistent from our earlier glycyl residue contribution of cyclo(Gly–Gly) with urea, in which favorable interactions were observed between peptide backbone unit of cyclo(Gly–Gly) with urea [19].

The $\Delta g'_{tr}$ results in Table 1 depict that the simultaneous presence of TMAO and urea causes net unfavorable interactions with glycyl residue, at the molar ratio of 1:1 as well as 1:2, since their $\Delta g'_{tr}$ are positive and these ratios provide optimal counteraction of TMAO by urea action on glycyl residue of $Gly_2 - Gly$. However, the values of $\Delta g'_{tr}$ are negative at higher molar ratios of urea (4–8 M) to TMAO (1 M), indicating that unfavorable interactions between the glycyl residue with TMAO ($Gly_2 - Gly$) slightly fail to offset the favorable interactions of the glycyl residue with urea ($Gly_2 - Gly$) at higher concentrations of urea. On the other hand, betaine might compete with urea action on glycyl residue of $Gly_2 - Gly$ in entire concentration ratios studied, since we observed positive $\Delta g'_{tr}$ values. It is clear that TMAO has shown to offset the deleterious effects of urea on glycyl residue of $Gly_2 - Gly$ at a 1:1 as well as 1:2 of TMAO:urea and betaine overcomes the urea effects on this glycyl residue in all cases of $Gly_2 - Gly$. Taken together, TMAO partially counteracts the deleterious effects of urea on glycyl residue of ($Gly_2 - Gly$), whereas betaine overwhelmingly counteracts the urea actions on glycyl residue of ($Gly_2 - Gly$) over the entire experimental range.

The $\Delta g'_{tr}$ values in Table 1 are negative (except $Gly_2 - Gly$) for osmolytes, urea and their combinations, revealing that glycyl residue contributes favorably to unfolding in TMAO or betaine, urea and the blends of TMAO or betaine–urea, in most cases. In other words, only in a few cases for the $Gly_2 - Gly$ model we obtained positive values, and thus this model is least representative of the protein backbone since Gly_2 and Gly are both highly soluble in the solvents. The rest of the other models show TMAO or betaine and urea, enhancing each others favorable interactions with glycyl group, thereby we obtained $\Delta g'_{tr}$ negative values. This negative contribution indicates that TMAO or betaine absolutely fails to offset the favorable interactions of the glycyl residues with urea, in all cases. On the other hand, the glycyl residue contribution of glycine peptides with the combination of TMAO and urea is entirely reversed from our earlier glycyl residue contribution of cyclo(Gly–Gly) with the blends of TMAO and urea, in which unfavorable interactions were observed between glycyl residue of cyclo(Gly–Gly) and the blends of TMAO and urea [19], indicating that TMAO strongly counteracted the deleterious urea actions on cyclo(Gly–Gly). The possible explanation is that urea-induced denaturation, involving a large variety of reactions, it is much difficult to imagine TMAO offset effects on side chain interactions and functional groups of proteins with denaturants [15,19]. The reason to explain the effects of TMAO or betaine counteracting towards perturbants urea is not supported by the $\Delta g'_{tr}$ values involving glycyl residues of zwitterionic glycine peptides.

5. Conclusions

In conclusion, the values of transfer free energies have revealed unfavorable interactions between methylamine and zwitterionic glycine peptides, while urea exhibited significantly favorable interactions with glycines. From these results, we found that, TMAO is obviously more effective as a stabilizer than betaine and proved more effective than betaine towards stabilizing the zwitterionic

glycine peptide structures and function. Our findings reveal that TMAO or betaine is able to strongly counteract the destabilizing effects of urea on zwitterionic glycine peptides, except at the higher urea concentrations (from 4 to 8 M) in the presence of 1 M methylamine of Gly_4. However, TMAO or betaine partially attenuates urea action on Gly_4, since urea increases negative $\Delta G'_{tr}$ values with increasing the urea concentrations (from 4 to 8 M) in the presence of 1 M TMAO or betaine, and this caused the lack of unfavorable interactions of methylamine with Gly_4. In fact, TMAO or betaine actually decreases the urea actions on Gly, Gly_2, Gly_3 while TMAO or betaine partially decreases the urea effects on Gly_4, which contains more peptide groups. We have shown in this study that TMAO exerted a more powerful counteracting osmolyte to the perturbing effect of urea than betaine. However, TMAO is less efficient to offset the urea perturbing the glycyl residues of $Gly_2 - Gly$ than betaine. Apparently, methylamine fails to restrict the urea deleterious effects on glycyl residues of simple subtractional (except $Gly_2 - Gly$) as well as composite constructs. Note that osmolyte counteraction ability of urea actions depends on osmolyte–protein pair as well as the denaturant–protein pair. Understanding these conclusions will help greatly in elucidating exactly the ability of TMAO or betaine counteracting effects to the deleterious actions of denaturants on the proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tca.2009.02.017.

References

[1] V. Daggett, Chem. Rev. 106 (2006) 1898–1916.
[2] C.N. Pace, Methods Enzymol. 131 (1986) 266–280.
[3] J.A. Schellman, Biophys. Chem. 96 (2002) 91–101.
[4] S.N. Timasheff, G. Xie, Biophys. Chem. 105 (2003) 421–448.
[5] B. Palecz, J. Am. Chem. Soc. 127 (2005) 17768–17771.
[6] B.J. Bennion, V. Daggett, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 5142–5147.
[7] P.E. Smith, J. Phys. Chem. B 108 (2004) 18716–18724.
[8] J.G. Cannon, C.F. Anderson, M.T. Record Jr., J. Phys. Chem. B 111 (2007) 9675–9685.
[9] P.H. Yancey, G.N. Somero, Biochem. J. 183 (1979) 317–323.
[10] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Science 217 (1982) 1214–1222.
[11] P. Venkatesu, M.J. Lee, H.M. Lin, J. Phys. Chem. B 111 (2007) 9045–9056.
[12] P.H. Yancey, J. Exp. Biol. 208 (2005) 2819–2830.
[13] P.W. Hochachka, G.N. Somero, Biochemical Adaptation, Oxford University Press, New York, 2002.
[14] T.Y. Lin, S.N. Timasheff, Biochemistry 33 (1994) 12695–12701.
[15] I. Baskakov, A. Wang, D.W. Bolen, Biophys. J. 74 (1998) 2666–2673.
[16] M.B. Burg, E.M. Peters, K.M. Bohren, K.H. Gabbay, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 6517–6522.
[17] S. Ortiz-Costa, M.M. Sorenson, M. Sola-Penna, Arch. Biochem. Biophys. 408 (2002) 272–278.
[18] S. Ortiz-Costa, M.M. Sorenson, M. Sola-Penna, FEBS J. 275 (2008) 3388–3396.
[19] P. Venkatesu, M.J. Lee, H.M. Lin, Arch. Biochem. Biophys. 466 (2007) 106–115.
[20] Q. Zou, B.J. Bennion, V. Daggett, K.P. Murphy, J. Am. Chem. Soc. 124 (2002) 1192–1202.
[21] B.J. Bennion, V. Daggett, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 6433–6438.
[22] Y. Nozaki, C. Tanford, J. Biol. Chem. 238 (1963) 4074–4081.
[23] Y. Nozaki, C. Tanford, J. Biol. Chem. 240 (1965) 3568–3573.
[24] Y. Nozaki, C. Tanford, J. Biol. Chem. 245 (1970) 1648–1652.
[25] P. Venkatesu, M.J. Lee, H.M. Lin, Biochem. Eng. J. 32 (2006) 57–170.
[26] M. Auton, D.W. Bolen, Biochemistry 43 (2004) 1329–1342.
[27] K.P. Murphy, Protein Structure, Stability, and Folding, Humana Press, Totowa, New Jersey, 2001.
[28] E.J. Cohn, J.T. Edsall, Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, Reinhold Publishing Corporation, New York, 1943.
[29] C. Tanford, The Hydrophobic Effect: Formation of Micelles and Biological Membranes, John Wiley and Sons, New York, 1973.
[30] D.R. Robinson, W.P. Jencks, J. Biol. Chem. 238 (1963) 1558–1560.
[31] D.R. Robinson, W.P. Jencks, J. Am. Chem. Soc. 87 (1965) 2462–2470.
[32] Y. Liu, D.W. Bolen, Biochemistry 34 (1995) 12884–12891.
[33] A. Wang, D.W. Bolen, Biochemistry 36 (1997) 9101–9108.
[34] A. Ben-Naim, J. Phys. Chem. 82 (1978) 792–803.
[35] H. Talukdar, S. Rudra, K.K. Kundu, Can. J. Chem. 66 (1988) 461–468.
[36] Y. Nozaki, C. Tanford, J. Biol. Chem. 246 (1971) 2211–2217.
[37] J. Hong, M.W. Capp, C.F. Anderson, R.M. Saecker, D.J. Felitsky, M.W. Anderson, M.T. Record Jr., Biochemistry 43 (2004) 14744–14758.
[38] T.E. Creighton, Proteins, Structures and Molecular Properties, W. H. Freeman and Company, New York, 1993.
[39] T.O. Street, D.W. Bolen, G.D. Rose, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 13997–14002.
[40] A.J. Saunders, P.R. Davis-Searles, D.L. Allen, G.J. Pielak, D.A. Erie, Biopolymers 53 (2000) 293–307.
[41] M. Niebuhr, M.H.J. Koch, Biophys. J. 89 (2005) 1978–1983.
[42] A. Caballero-Herrera, K. Nordstrand, K.D. Berndt, L. Nilsson, Biophys. J. 89 (2005) 842–857.
[43] S. Paul, G.N. Patey, J. Phys. Chem. B 111 (2007) 7932–7933.
[44] S. Paul, G.N. Patey, J. Am. Chem. Soc. 129 (2007) 4476–4482.
[45] L.M. Samuelsson, J.J. Bedford, R.A.J. Smith, J.P. Leader, Comp. Biochem. Physiol. Part A 141 (2005) 22–28.
[46] H.C. Tseng, D.J. Graves, Biochem. Biophys. Res. Commun. 250 (1998) 726–730.
[47] L.M.F. Holthauzen, D.W. Bolen, Protein Sci. 16 (2007) 293–298.
[48] L.R. Singh, T. Dar, I. Haque, F. Anjum, A.A. Moosavi-Movahedi, F. Ahmad, Biochim. Biophys. Acta 1774 (2007) 1555–1562.
[49] G.I. Makhatadze, P.L. Privalov, J. Mol. Biol. 213 (1990) 375–384.
[50] R.S. Spolar, J.R. Livingstone, M.T. Record Jr., Biochemistry 31 (1992) 3947–3955.