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Effect of cosolvents in the preferential binding affinity of water in aqueous solutions of amino acids and amides

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ABSTRACT

Effects of two naturally occurring osmolytes, urea and trimethylamine-N-oxide (TMAO) on the solvation structure of hydrophobic moiety of alanine, glycine, *N*-methylacetamide and acetamide are investigated by classical molecular dynamics simulations. Our results are analysed in terms of site-site radial distribution functions (RDF), spatial distribution functions (SDF), number of hydrogen bonds, orientation profile, KB integrals, preferential binding coefficient and hydrogen bond dynamics. RDF and SDF showed presence of an extra hydration shell near the hydrophobic unit when TMAO is present in the solution. This hydration shell mainly consists of broken hydrogen bonds. In urea-water solution, intramolecular association is favoured compared to intermolecular association: which is in contrast to the TMAO-water solution. Alanine, glycine, NMA and acetamide showed preferred interactions with the water molecules in presence of TMAO compared to urea. Urea and TMAO both are found to be excluded from the alanine, glycine, NMA and acetamide surface but presence of urea was slightly favoured at higher distances in case of NMA and acetamide. The strong hydrogen bond between TMAO-water increases the hydrogen bond lifetime of other hydrogen bonds in the system. The preferential binding affinity of water with the protein molecules and strong hydrogen bonds are found to be the key reasons for stability in presence of TMAO.

1. Introduction

Water is one of the simplest molecules, but its counterintuitive behaviours have not been clearly explained [1,2]. Its unravelling properties drives many natural and biological processes. The presence of water as hydration layer around the biomolecules is crucial for their stability and functions in aqueous solutions. Stability of the proteins results due to fine balance between the protein-water interactions and intramolecular interactions of the functional groups. This stability can be profoundly modulated by the addition of cosolvents [3–5].

Mixture of osmolytes that exists in the cells of several organisms, suggests that they have influence on the stability of proteins. Osmolytes such as urea, TMAO effects the stability of biomolecules through direct or indirect mechanism [5]. TMAO, a naturally occurring amphiphilic osmoprotectant, stabilizes the biomolecules whereas urea destabilizes it [6]. At a biologically relevant ratio of 2:1 M of urea-TMAO ternary mixture, the denaturing effects of urea is counteracted by TMAO [6–10]. This ratio is generally found in the tissues of sea creatures in order to maintain the osmotic pressure with the environment [11]. Many studies have proposed two pathways for protein denaturation by urea [12–15]. In the "indirect" process, urea alters the structure of water, acting as a structure breaker which enhances the protein hydration, in contrast; the "direct" mechanism hypothesizes direct interactions with the protein molecule through strong interactions with the side-chains or backbone. Both of these possible pathways contribute towards the denaturation of proteins and are not mutually exclusive. The stabilization of proteins by TMAO is proposed through exclusion of TMAO molecules from the vicinity of protein surfaces or by strengthening the surrounding water structure and hydrogen bond (HB) network [16,17] Stabilization also occurs due to direct interactions with the proteins, affecting their stability and structure [18–21]. Another unique stabilization pathway of TMAO is due to its hydrophilic fragment [22]. All these studies have been done considering only urea-water [13,23–27] or TMAO-water [4,28–34] systems. Very few simulation studies have been done where both the effect of urea, TMAO have been considered to explain the stability of the proteins [14,16,35,36].

Free energy measurements suggest that the protein backbone plays a key role in governing the scale of protein stabilization while the side-chains plays a minor role [37]. The penalty of hydrophobic interactions by transferring a non-polar molecule to water can be reduced or increased based on the model parameters used in the simulation studies [38–40]. It is also suggested that in a ternary mixture of 2:1 M ratio of urea-TMAO, each osmolytes has insignificant effects on each other and their interactions with the protein molecule are independent of the other's presence [8]. Counteraction of the deleterious effects of urea on protein denaturation by TMAO are through enhancement of water-urea and water-water interactions or through osmolyte-induced conformational changes on protein-water interactions [7,14]. TMAO is

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also found to induce structural modification of water network and water-water interactions [6,12]. Another proposed mechanism is that TMAO tends to enhance the hydrophobic attraction among nonpolar groups leading to stabilization of the folded states [28,35]. However, a conclusive pathway that accounts for the stabilization ability of TMAO and also its counteraction effects on urea remains elusive [38] and it is highly dependent on the model parameters [4,38].

The objective of this present work is to explore the molecular pathway by which proteins gets stabilized in the presence of TMAO and have opposing effects in presence of urea. Therefore, we consider three different types of solutions, namely aqueous alanine/glycine/NMA/acetamide, alanine/glycine/NMA/acetamide in urea solution, TMAO solution and ternary mixture of 2:1 M ratio of urea-TMAO. A protein molecule is basically composed of four subunits: an amine group, carboxylic group, hydrophobic group and an amide linkage. Therefore, it will be interesting to note the effect of these osmolytes on these sub-groups to explain the stabilization and destabilization of proteins. Since, proteins are big molecules, presence of more than one factor such as intermolecular hydrogen bonds, disulphide bonds, ion pairs etc., can make difference in showing their effect in protein stabilization. In view of this we considered simple amino acids like Alanine, glycine and simplest amides like *N*-methyl acetamide (NMA), acetamide; to consider the effect on the basic entities; i.e. amine group, carboxylic group, hydrophobic group and the amide linkage. We have performed molecular dynamics simulations using CHARMM36 FF force field; which is used generally in the simulation of proteins to study these effects. It can be noted here that the studies of TMAO as cosolvents is mainly done with Kast [41], Neitz [32], Gracia [31] and Shea [42] models and very few studies have done in CHARMM forcefield [43,44]. As it is evident from the literature survey, the behaviour of TMAO, urea towards the protein backbone is highly model dependent. Therefore, it would be interesting to see the behaviour of these osmolytes in different force field, CHARMM36 FF which is commonly used in studying protein dynamics and see whether it is possible to explain their opposing effects.

It is said that urea mainly interacts with the protein backbone and TMAO is excluded from the vicinity of protein due to entropy effect. We would like to see whether such preferential solvation between urea and TMAO exists and contributes in the stabilization. Further, in our previous work, we have seen that there is an enhancement in the protein stability in presence of co-solvent TMAO due to formation of an extra hydration shell near hydrophobic unit of glycine [45]. This extra hydration shell is also found in case of LiCl which explains the anomalous behaviour of lithium salts in salting out of proteins [46]. It would be interesting to find out whether such effect is also visible in case of alanine, NMA and acetamide. Alanine and NMA consist of two hydrophobic moieties which are present in very different neighbouring groups. Therefore, it would be curious to know whether these hydrophobic groups have contributions in imparting stability to the proteins. The results are further compared with respect to glycine and acetamide solutions. Achieving a molecular level of understanding about the influence of osmolytes, TMAO and urea on amine group, carboxylic group, hydrophobic group and amide linkage and calculation of the forces that stabilizes and destabilizes a biomolecule are the main objective of the present work. We have carried out the simulations for two different water models to find out the qualitative relevance of our conclusion.

The rest of this article is categorized into following three parts. Computational details and methodology are described in Section 2, followed by results and discussions in Section 3 and in Section 4, we summarize and conclude our results.

2. Model and simulation details

In order to gain detailed insights on the protein stability in presence of the osmolytes like TMAO, urea, we have performed classical MD simulations of alanine, glycine, *N*-methyl acetamide (NMA) and acetamide in pure water as well as in aqueous binary and ternary osmolyte solutions of urea and TMAO. Alanine, glycine, NMA, acetamide, Urea, TMAO and water molecules are characterized by multi-site interaction models. The expression for interaction between two atomic sites in these models are given as

$$u\left(r_{ij}\right) = 4\varepsilon_{ij}\left[\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{6}\right] + \frac{q_i q_j}{r_{ij}} \quad (1)$$

where, $r_{ij}$is the inter-atomic distance between molecular sites i and j, $q_{ij}$is the charge of the site i. The LJ parameters $\varepsilon_{ij}$and $\sigma_{ij}$are obtained by using combination rules $\sigma_{ij} = (\sigma_i + \sigma_j)/2$and $\varepsilon_{ij} = \sqrt{\varepsilon_i \varepsilon_j}$, where ε_i and $\sigma_i$are the well-depth and LJ diameter parameters for i^{th} atom. The force field parameters for Alanine, Urea and TMAO were taken from CHARMM36 FF [47]. SPC/E and SPC potential models were considered for water [48,49]. The potential parameters for alanine, glycine, NMA, acetamide, osmolytes and water molecules are summarized in Table 1.

The simulations were performed with a total of 1024 molecules in the simulation box consisting of water, zwitterionic alanine/glycine/NMA/acetamide and osmolytes at 298 K. In Table 2, the compositions of aqueous alanine, NMA and osmolyte mixtures of different concentrations are tabulated. We have run the simulations with same composition for glycine and acetamide and details are given in supplementary table 1. Atomistic simulations were carried out with GRO-

Table 1
The Lennard-Jones parameters and charges used in models for Alanine, glycine, NMA, Acetamide, Urea, TMAO and water. *e* represents the elementary charge.

Name	Atom	$\sigma(A^0)$	$\varepsilon(kJ/mol)$	Charge (*e*)
Alanine	N	3.29	0.8360	−0.30
	H	0.40	0.1924	0.33
	C_α	3.56	0.1338	0.21
	H_α	2.35	0.0920	0.10
	C_β	3.63	0.3263	−0.27
	H_β	2.38	0.1004	0.09
	C	3.56	0.2928	0.34
	O	3.02	0.5020	−0.67
Glycine	C	3.56	0.2928	0.34
	C_α	3.58	0.2343	0.13
	O	3.02	0.5020	−0.67
	N	3.29	0.8368	−0.30
	H	0.40	0.1924	0.33
	H_α	2.38	0.1171	0.09
NMA	CH_3 (C)	3.65	0.3263	−0.27
	C	3.56	0.4602	0.51
	O	3.02	0.5020	−0.51
	N	3.29	0.8368	−0.47
	CH_3 (N)	3.65	0.3263	−0.11
	H (N)	0.40	0.1924	0.31
	H	2.38	0.1000	0.09
Acetamide	C	0.365	0.3260	−0.27
	CH_3 (C)	0.356	0.4602	0.55
	N	0.329	0.8360	−0.62
	H (N)	0.040	0.1924	0.32
	O	0.302	0.5020	−0.55
	H $(CH)_3$	0.238	0.100	0.09
Urea	C	3.56	0.2928	0.60
	O	3.02	0.5020	−0.58
	N	3.29	0.8368	−0.69
	H	0.40	0.1924	0.34
TMAO	N	3.29	0.8368	−0.83
	O	3.11	0.5020	−0.37
	C	3.94	0.3221	−0.35
	H	1.24	0.1924	0.25
Water (SPC/E)	O	3.16	0.6502	−0.8476
	H	–	–	0.4328
Water (SPC)	O	3.16	0.6501	−0.82
	H	–	–	0.41

Table 2
$N_{alanine/NMA}$, N_{urea}, N_{TMAO}, N_{water} represents the corresponding number of alanine/NMA, Urea, TMAO and water molecules in the simulation box.

System	$N_{alanine/NMA}$	N_{urea}	N_{TMAO}	N_{water}
(1) AW	15	0	0	1009
(2) AUW (3 M)	15	66	0	943
(3) ATW (3 M)	15	0	66	943
(4) AUTW (6:3 M)	15	132	66	811
(5) NMAW	15	0	0	1009
(6) NMAUW (3 M)	15	66	0	943
(7) NMATW (3 M)	15	0	66	943
(8) NMAUTW (6:3 M)	15	132	66	811
(9) AUW (2 M)	15	44	0	965
(10) ATW (2 M)	15	0	44	965
(11) AUTW (4:2 M)	15	88	44	877
(12) AUW (1 M)	15	22	0	987
(13) ATW (1 M)	15	0	22	987
(14) AUTW (2:1 M)	15	44	22	943

MACS (v2018.4) MD simulation package [50,51]. Lennard-Jones electrostatic interactions were treated with the particle mesh ewald (PME) summation method [52,53] with the nonbonded interaction space cut-off of 1.2 nm. Leapfrog algorithm was employed to integrate the equations of motions with integrating time step of 10^{-15} s (1 fs) beside minimum image conventions [54] and periodic boundary conditions (PBC) applied in all spatial directions. Parrinello-Rahman barostat (τ_P = 2.0 ps) [55] and Velocity-rescale thermostat (τ_T = 0.1 ps) [55] were employed to keep the pressure and temperature constant respectively. LINCS algorithm was employed to keep the bond lengths constrained [56].

Different numbers of osmolyte molecules with random orientations were placed in the starting configuration for all the systems rendering different concentrations. At first, each system was subjected to equilibration for 10 ns in the NVT ensemble. Subsequently, NPT ensemble was run for another 10 ns in order to attain an appropriate box length corresponding to 1 atm pressure. Finally, each simulation was run for further 50 ns using NPT ensemble [54] and the results are reported.

3. Results and discussions

3.1. Radial distribution functions

The influence of osmolytes on the local structural properties of aqueous alanine solution are characterized by various intermolecular alanine-water, osmolyte-water, alanine-osmolyte pair correlation functions. The hydration pattern of water molecules in presence of osmolytes around the alanine molecules are calculated from the intermolecular N_a-O_w, C-O_w, C_α-O_w, C_β-O_w, O_a-O_w and O_w-O_w radial distribution functions. In Fig. 1, the RDF's of systems 1–4 are shown and the results for SPC/E water and systems 9–14 are shown in supplementary information (Figs. 1, 2 and 3 respectively). In case of N_a-O_w (Fig. 1(a)), the peak height is found to be highest for urea solution and least for TMAO, indicating that more tetrahedral water molecules are bonded to the first coordination shell of N-terminal of alanine in presence of urea, but the second coordination shell is found to be more well defined for TMAO and ternary mixture solutions compared to aqueous alanine-water and urea-water mixtures. This means collapse of the secondary solvation shell near the amine group of alanine in pure alanine-water and in presence of urea. The structural arrangement of water molecules near carbonyl carbon is shown in Fig. 1(b). The peak height is found to be more in case of mixed urea-TMAO mixture which decreases in the order: mixed Urea-TMAO > TMAO > Urea. In case of hydrophobic carbon (C_α-O_w), shown in Fig. 1(c), the first peak height of the ternary mixture and urea are found to be more than aqueous alanine solution and TMAO-water solutions which changes its trend in the second peak. TMAO-water solutions and ternary mixture is found to have bigger and broader second peaks compared to the urea-water solutions suggesting greater hydration of the hydrophobic moiety and less tetrahedral water. The first peak of water molecules near C_α in presence of urea can be attributed due to the presence of more solvation shell near the amine group. The structural analysis of water molecules in this region can be seen in the subsequent sections. The positions of maxima of the first peak of C_β-O_w (Fig. 1(d)) is found to be same for urea-water, TMAO-water and mixed urea-TMAO solutions. However, the peak is found to be bigger and broader for ternary mixture and TMAO-water. The minima is slightly shifted towards higher distances.

Fig. 1(e) shows the radial distribution function of the carbonyl oxygens with oxygen of water (O_a-O_w). The increase in the peak height of the first peak and shallow minima for ternary mixtures indicates rise in water density around carbonyl carbon compare to other cosolvents. Further, on careful observation of O_w-O_w RDF (Fig. 1(f)), it is found that the second solvation shell is more well defined for TMAO and ternary mixture compared to aqueous alanine solution and urea-water solution. Similar graphs were obtained for systems 9–14 and glycine which are given in supplementary information.

Next, we plan to see the solvation structure of NMA to see the distribution of the water molecules around the amide linkage and its contribution towards the solvation structure. In Fig. 2, we have plotted the intermolecular radial distribution functions of nitrogen-water (N-O_w), carbonyl carbon-water (C-O_w), methyl carbon attached to C-water (Me(C)-O_w), methyl carbon attached to N-water (Me(N)-O_w). The change in the water structure is mainly seen near the -NH group and the carbonyl group. The first and second solvation shells of N-O_w and C–O_w are visibly more in case of ternary mixture and TMAO compared to urea-water and aqueous alanine. This contributes in the solvation shell structure of the water molecules around the hydrophobic groups. It can be seen here that the peak height and the peak mimina for the carbonyl carbon is evidently smaller and deeper in presence of urea. We see a small hump near the minima of the first peak of C_α-O_w, the hump size decreases from TMAO > ternary mixture > urea. The effect is less seen in case of the other hydrophobic group due to the increase in proximity from the carbonyl group. Such effect is also reported for other amino acid like glycine [45].

We find similar results for acetamide solutions and are shown in the supplementary information. Therefore, it is quite evident from our simulation results that it is the neighbouring polar groups i.e., the carboxylic and the amine group which actually make changes in the distribution of water near the amino acid in presence of different cosolvents. It would be curious to know the spatial distribution of the hydration shell near the alanine and NMA molecules.

3.2. Spatial density plots

The spatial distribution functions (SDF's) of oxygen molecules around the central alanine molecule and NMA in presence of different osmolytes were calculated with the TRAVIS software package [57]. The calculated SDF's of urea-water and TMAO-water for same isovalues are depicted in Fig. 3. The water densities around the alanine molecule can be distributed in three main regions namely, near the amine group (Region I), carbonyl group (Region II) and near the hydrophobic unit (Region III).

It is clearly visible that the water density near the hydrophobic region (C_α) of alanine i.e., Region III is significantly more in case of TMAO than urea. This protective hydration shell near the hydrophobic unit in case of TMAO correlates well with the second solvation shell of C_α-O_w (Fig. 1(c)) which is very unique.

In NMA, we have two hydrophobic units, one near the carbonyl carbon (Region I) and the other near the amine group (Region II). Both the hydrophobic group is found to have more water density in presence of TMAO, which confirms the fact that in presence of TMAO, alanine and NMA molecules are more surrounded by the water molecules in comparison to urea. Now it will be interesting to see whether this extra solvation shell can be related to the stability of the proteins. In the subsequent sections we plan to note down the structural changes in these hydration shells.

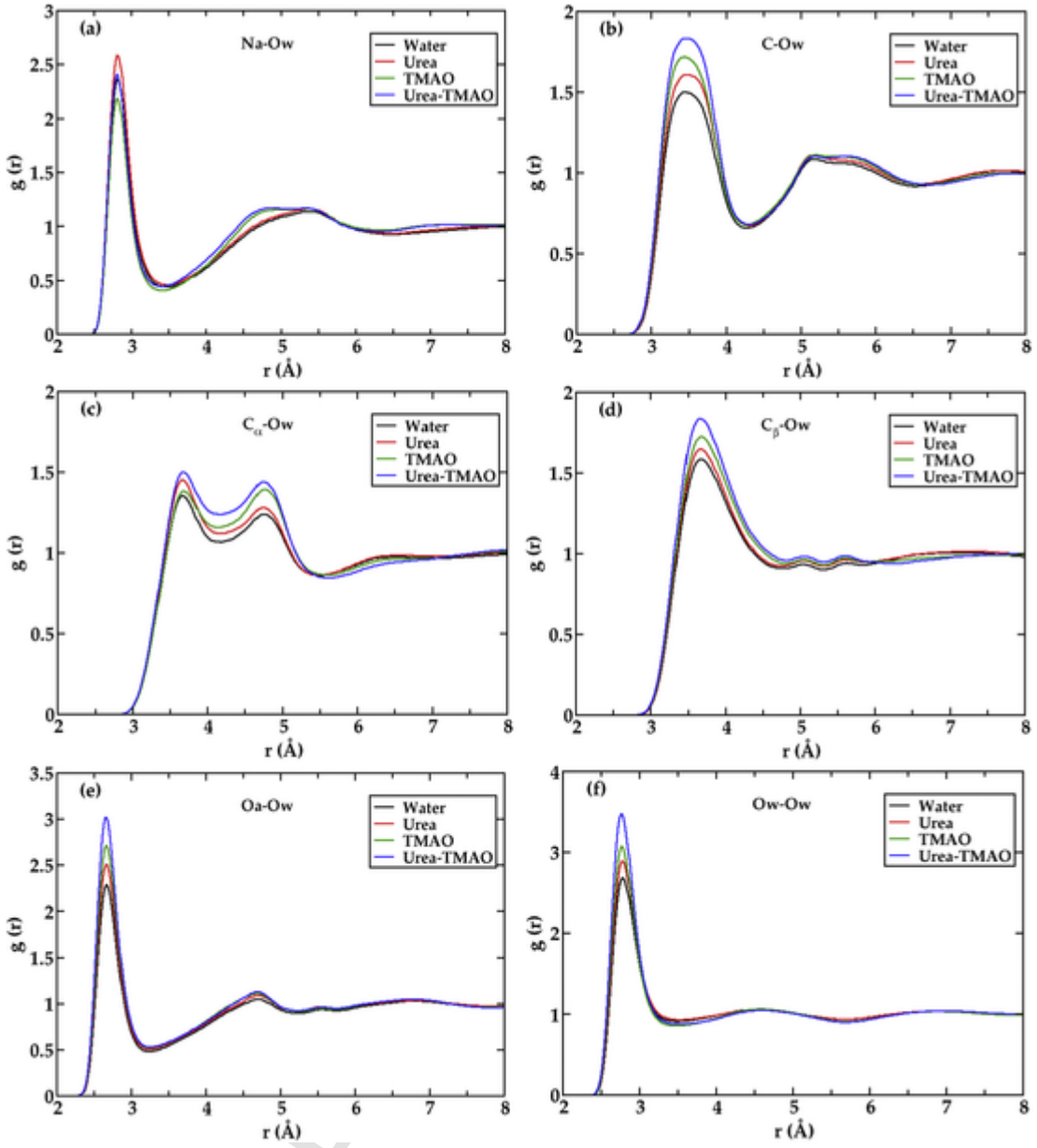

Fig. 1. Site-site radial distribution functions g (r) of aqueous alanine of different atoms (a) N_a-O_w, (b) C-O_w, (c) C_α-O_w (d) C_β-O_w, (e) O_a-O_w and (f) O_w-O_w in different osmolytes.

3.3. Number of hydrogen-bonded water molecules

It has been already seen that the solvation structure near the alanine and NMA molecules are different in presence of different cosolvents. To gain further insights into the structure of water molecules near the interface of alanine, we have plotted the fraction (f_n) of oxygen atoms of water molecules that engage in *n* number of water-water hydrogen bonds in Fig. 4. The distance criteria considered to be hydrogen bonded between two inter oxygen atoms is 3.25 Å. We select only the interfacial water molecules which are present within a distance of 6.0 Å from amine nitrogen, 5.6 Å from the C_α, 6.1 Å from C_β and 6.6 Å from carbonyl carbon of alanine in the calculations (decided from the RDF).

It is seen that in all the cases the probability of occurrence of lower coordinated (i.e., one or two coordinated) water molecules are found to be more compared to three and four coordinated water. The effect of addition of osmolytes on the water-water hydrogen bond number is significant compared to aqueous alanine. The fraction of lower coordinated water molecules (f1) increases with addition of osmolytes, whereas higher coordinated water molecules (f3, f4 and f5) show the opposite trend suggesting that water looses some of its identical nearest neighbours near the solute surface. Similar trend was found for NMA (Supplementary Fig. 4). The comparatively large enhancement of one coordinated water molecules and the reduction of three, four and five coordinated water molecules can be related to the number of osmolytes/alanine/NMA that has been accommodated in the cavities of water molecules. The osmolytes/alanine/NMA preferably replaces water molecules resulting in lesser number of hydrogen bonded water molecules. This effect is more in presence of mixed urea-TMAO solutions which promotes more broken type of hydrogen bonds, then TMAO > Urea > pure alanine water. The fraction of two coordinated water molecules however, does not show any dependence on the number of osmolyte molecules. The trend is found to be similar near the N-terminal, C-terminal and the C_α carbon.

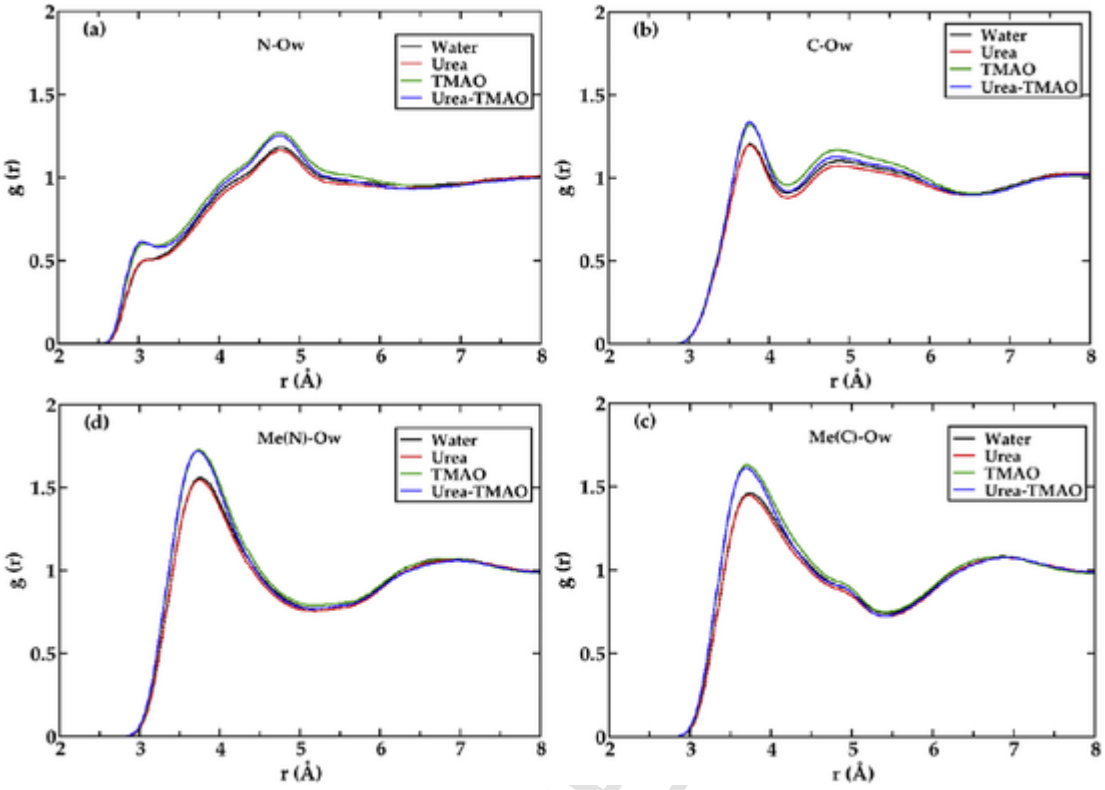

Fig. 2. Site-site radial distribution functions of aqueous NMA of different atoms (a) N-O_w (b) C-O_w (c) Me(C)-O_w (d) Me(N)-O_w in presence of different osmolytes.

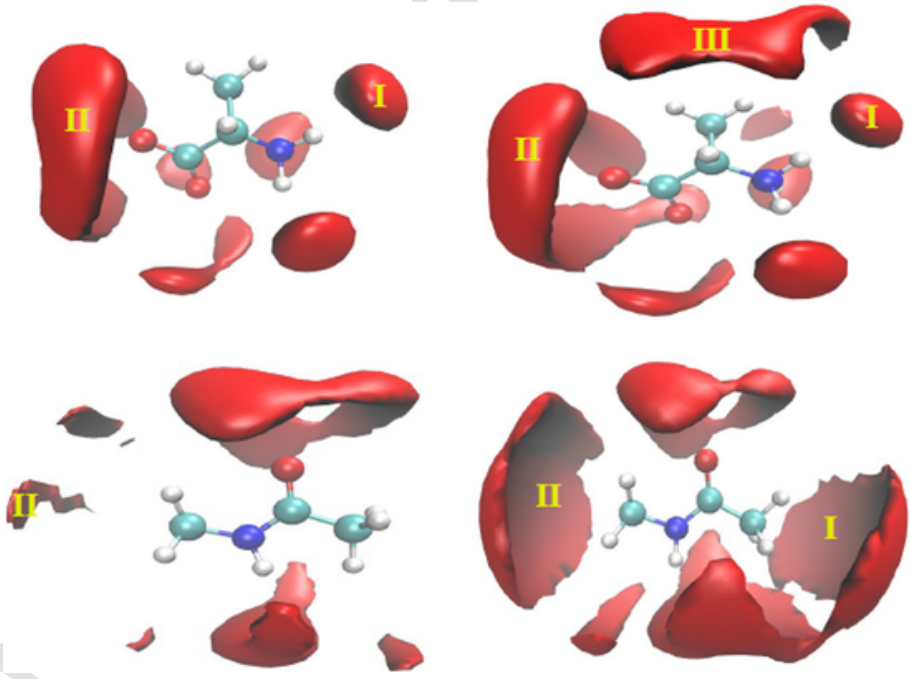

Fig. 3. SDF of water oxygen around aqueous alanine (top) and NMA (bottom) in presence of osmolytes urea (left) and TMAO (right).

3.4. Orientation profile

To elucidate the tetrahedrality structure of water molecules we calculated the angular distribution function θ_{OOO} of the interfacial water molecules near the interface of alanine. In Fig. 5, we have plotted the probability distribution $P(\theta_{OOO})$ of the interfacial water molecules near the hydrophobic C_α, C_β and the hydrophilic carbonyl carbon.

Water molecules up to a distance of 5.6 Å from C_α, 6.1 Å from C_β and 6.6 Å from carbonyl carbon are considered as interfacial molecules for calculations. In all the cases, it is seen that there is a small peak near 50° and a broad distribution of angles near 104.5°. It is well known that the ideal angle for tetrahedral water is 104.5°. The peaks are found to shift slightly towards the higher angles in case of TMAO and mixed urea-TMAO solutions compared to urea solution and alanine water system. This can be related with the number of hydrogen

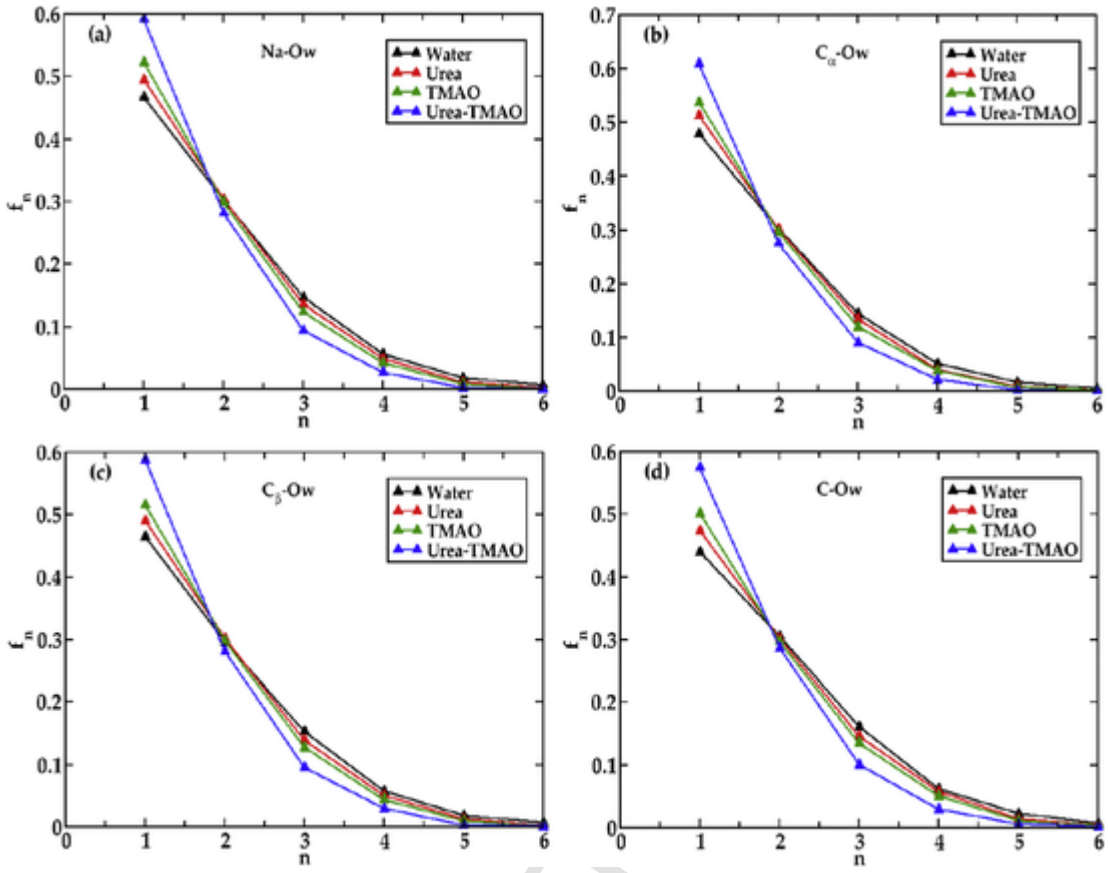

Fig. 4. The fraction of water molecules having n number of hydrogen bonds within the distances (a) 6.0 Å from amine nitrogen (b) 5.6 Å from C_α (c) 6.1 Å from C_β and (d) 6.6 Å from carbonyl carbon of alanine-osmolyte solutions.

bond distribution. TMAO and mixed Urea-TMAO solutions showed more fraction of broken dimer and trimer water molecules than tetrahedral water. The shift of broad peak towards 120° is due to breaking of tetrahedral network of water at higher distances. The effect is however found to be prominent in case of the interfacial molecules near C_α. The calculated probability distribution P (θ_{OOO}) of water molecules present near the hump region of the C_α carbon for all the cosolvents, i.e., in the distance range of 4.2–5.6 Å, corresponding to the shoulder of the first peak and rise of the second peak is shown in Fig. 6(a). The rise in the second peak seen in the RDF of C_α-O_w in presence of TMAO and in mixed urea-TMAO solutions mainly consists of more broken hydrogen bonds which contributes towards the higher angles. The results for NMA (Supplementary Fig. 5) in different cosolvents follow the same trend as seen in case of alanine.

3.5. Potentials of mean forces

The solvent free energy is one of the important factors determining the thermodynamics of a biological systems. The Potentials Mean Force (PMF's) between alanine-water and NMA-water were computed with the help of pair correlation functions, g(r), using the relation:

$$W(r) = -k_B T \ln g(r) \tag{2}$$

where *r* is the inter-atomic separations, k_B is the Boltzmann constant and T is the temperature.

In Fig. 6(a), the potential of mean force (PMF) is plotted as a continuous function of distance between hydrophobic carbon (C_α) of alanine and the water molecules. Similarly, in Fig. 6(b) and (c), we show the PMF of the N-terminal and C-terminal of NMA with the water molecules. It can be seen that in all the cases, urea is found to have less stabilization energy compared to the ternary mixture and TMAO. This can be related due to the presence of extra hydration shell noticed near the C_α of alanine and also near the hydrophic groups of the NMA; indicating that the hydrophobic hydration has some contribution towards the stability of the biomolecules.

3.6. Kirkwood-buff integrals

To have an overview of the solvation structure of the protein (amino-acid and amides) molecule with the cosolvents and water, we calculated the Kirkwood-Buff Integrals (KBI) [58,59] between water-water, water-osmolytes and protein-osmolytes. In this process, the structure of the solution can be related to the thermodynamic properties by using the pair correlation functions. The physical significance of the KBI's can be viewed as a measure of mutual affinities between the interacting molecular species in a solution. This will give us information about the interactions between the protein-water, water-water and water-osmolytes. The KB integrals between solution components can be expressed using the equation:

$$G_{\alpha\beta} = 4\pi \int_0^\infty r^2 \left[g_{\alpha\beta}(r) - 1 \right] dr \tag{3}$$

where, g(r) is the radial distribution function, *r* is the inter-atomic separations. A higher value of G_{ij} indicates an overall stronger interatomic attraction between the species *i* and *j* (either direct or mediated by other components). In Fig. 7, we have shown the KB integrals with the correction factor applied in the tail region [60,61] for water-water, water-osmolyte and for osmolyte-osmolyte interactions by taking centre of mass as a function of interatomic distance for systems 1–8.

From the graph, (Fig. 7(a)) it is clear that in the solution water-water association is more in case of ternary mixture. More water-water association is also found in presence of urea in comparison to TMAO. Fur-

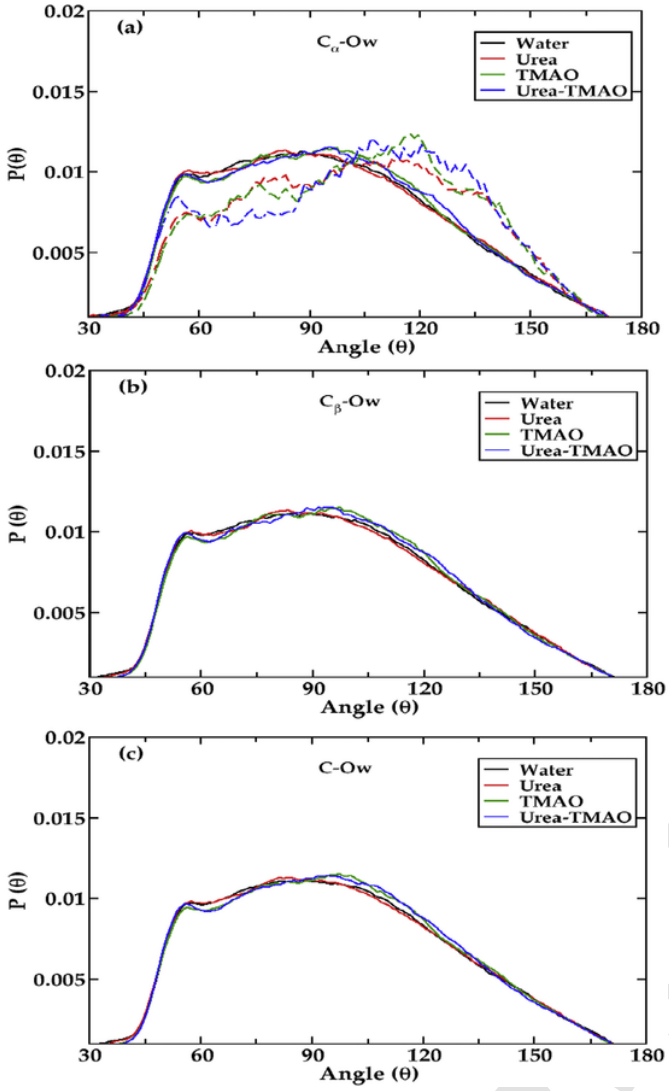

Fig. 5. Normalized probability distribution of <O-O-O angle of oxygen atoms of water molecules which are within the distance (a) 5.6 Å from C_α (b) 6.1 Å from C_β and (c) 6.6 Å from carbonyl carbon for systems 1–4. The dotted lines represent the region between 4.0 and 5.6 Å from C_α.

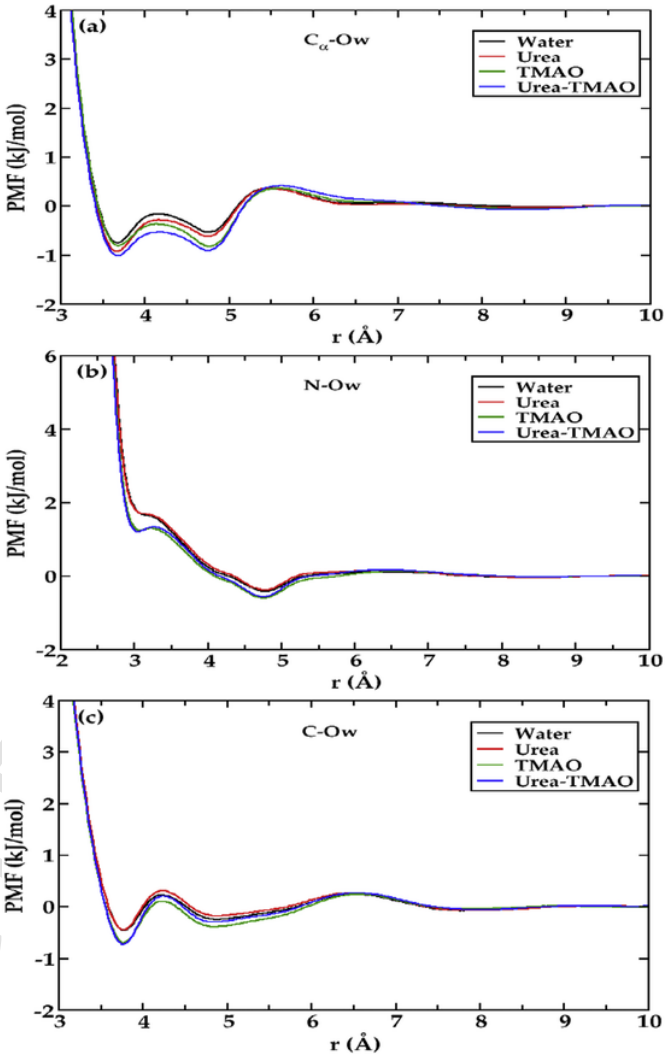

Fig. 6. PMF depicting the free energies of interaction between (a) hydrophobic carbon C_α of alanine, (b) nitrogen of NMA and (c) carbonyl carbon of NMA with the water oxygen sites as a continuous function of distance.

ther, it can be seen that water-TMAO association is more favoured over water-urea association (Fig. 6(b) and from Fig. 6(c)), it is clear that urea-urea like to associate more in comparison to TMAO-TMAO. This suggests that in urea-water-alanine system, urea like to aggregate with urea and water with water than urea-water combination. In presence of TMAO, we find water-TMAO association is more favourable than water-water, which is again more favourable than TMAO-TMAO association. Similar trend is observed for the ternary mixture. Therefore, it can be commented here that the water molecules interact with TMAO more favourably than urea molecules. Also, water likes to interact with the water molecules in comparison to TMAO-TMAO interactions in a TMAO-water system.

The stability of proteins being modulated in presence of osmolytes can be quantified via preferential solvation of water or the osmolytes to the protein resulting due to the competition between protein-osmolyte and protein-water interactions. These interactions measure the net excess or deficit of species around a particle in a solution. In order to calculate these, we have the estimated preferential binding coefficient [62] $\nu_{\alpha\beta}$ between the solute-water, solute-urea and solute-TMAO. If solvent water is denoted by subscript 1, solute by 2 and osmolyte by 3, then ν_{21} preferential binding of water to protein, is given by.

$$\nu_{21} = \rho_1 \left(G_{21} - G_{23}\right) \tag{4}$$

ν_{23} preferential binding of osmolytes to protein molecule, is given by.

$$\nu_{23} = \rho_3 \left(G_{23} - G_{21}\right) \tag{5}$$

where ρ_3 denotes the osmolyte and ρ_1 denotes the water number densities.

Fig. 8(a) and (b) depicts the $\nu_{\alpha\beta}$ values for urea, TMAO and water around alanine and NMA respectively as a function of distance. The preferential binding behaviour can be detected from the value $\nu_{\alpha\beta} > 1$ and preferential exclusion of the species can be detected from $\nu_{\alpha\beta} < 1$ region. It can be easily seen that the $\nu_{\alpha\beta}$ values for alanine-water is more compared to alanine-osmolytes. This suggests that water is preferentially favoured over other solvents near alanine surface. Further, it can be noted that both TMAO and urea are excluded from the alanine surface. The $\nu_{\alpha\beta}$ values for alanine-TMAO is slightly lesser than that of alanine-urea. The $\nu_{\alpha\beta}$ values for alanine-water in presence of TMAO is found to be more compared to urea. This implies that water-alanine interaction is more favoured in presence of TMAO solutions compared to that of urea solutions and both urea and TMAO are excluded from the alanine surface. Similar observation was noticed for aqueous solu-

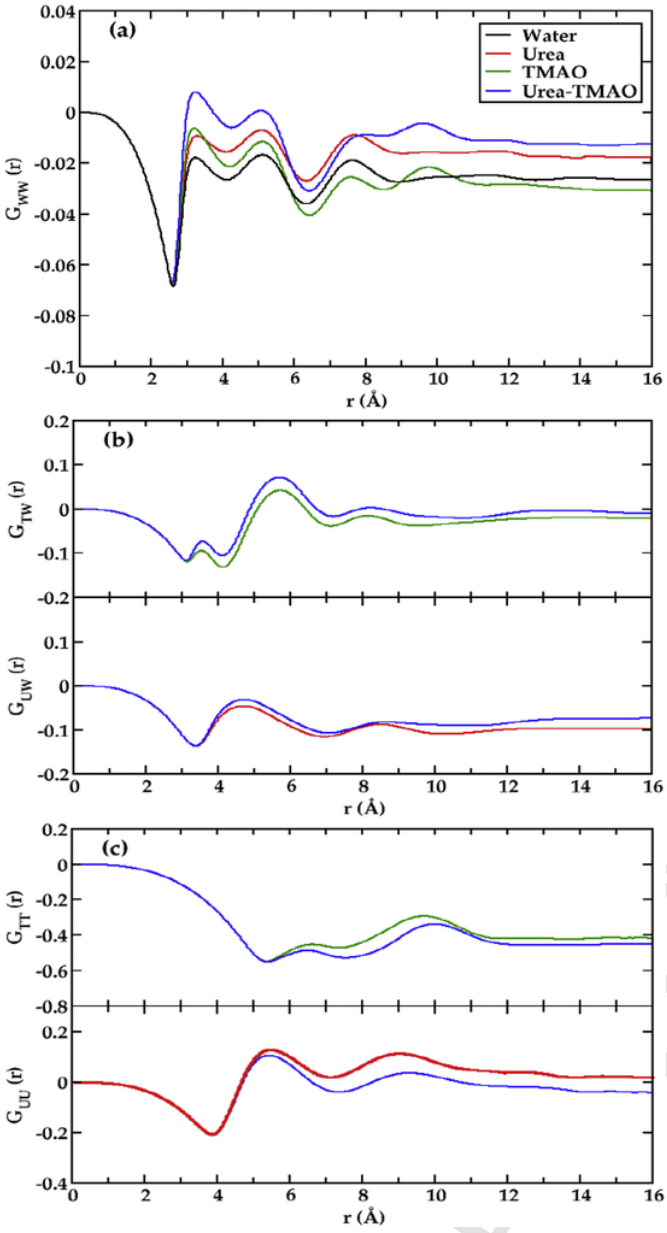

Fig. 7. Kirkwood-Buff running integrals $G_{ij}(r)$ for (a) water-water (b) osmolyte-water and (c) osmolyte-osmolyte interactions for systems 1–4.

tions of glycine (Fig. 8(c) and (d)). The scenario is however different in case of urea-water solution of NMA/acetamide. For NMA and acetamide, we find preferential binding affinity for water is more in comparison to urea at lower r values. However, at larger r values > 4.35 Å for NMA and r values > 4.5 Å for acetamide, water is excluded from amide surface and presence of urea is favoured. For TMAO-water system, water is favoured over the osmolyte near the amide surface which is similar to alanine case. It has been already noticed in the RDF and SDF results that in presence of urea, we find less water molecules near the protein surface compared to TMAO solution. In case of alanine, the preference of water over urea in urea-water system, may be due to the location of the polar groups (-NH, –COO) near the end of the backbone which is present in the middle in case of amides. As a result, hydration of water is favoured up to a small distance (r < 4.35 Å) in case of NMA. This gives space to the urea molecule to approach the amides favourably compared to the amino acids. The polar groups help in the hydration of proteins.

Therefore, the $G_{\alpha\beta}$ values of the solution and the $v_{\alpha\beta}$ values of the protein interface suggests that TMAO which is a stabilizing agent encourages protein hydration which is lacking in case of urea-water system. Also, there is an increment in the intermolecular (TMAO-water) interaction in presence of TMAO. In case of urea, we see favourable intramolecular (urea-urea, water-water) association. It would be interesting to check the strength of the hydrogen bond lifetime in these solutions to further confirm the fact.

It can be commented here that, we observed very little preferential exclusion of TMAO from the protein surface compared to urea near the amide surface. In case of bigger proteins, consisting of more protein backbones and amide linkages this effect may be more visible. In case of ternary mixture of urea-TMAO-water, we observe the same trend while comparing TMAO and water.

3.7. Hydrogen bond dynamics

As evident in the previous section, water has an important role in the solvation structure of the protein molecule; it will be interesting to study the hydrogen bond dynamics of water molecules in different co-solvent systems. We define the distance between hydrogen bond donor and acceptor pairs to be hydrogen bonded if the interatomic distance is <2.5 Å. The average continuous lifetimes of hydrogen bond population are calculated as [63–65].

$$S_{HB}(t) = \frac{\langle h(t_0)\, h(t)\rangle}{\langle h(t_0)^2\rangle} \tag{6}$$

where $\langle \ldots \rangle$ denotes the average over all pairs of a given type. The population parameter $h(t) = 1$ if a particular hydrogen bond between two molecules exists from time $t = 0$ to $t = t$ or zero otherwise. The hydrogen bond lifetime of water-water (O_w-H_w), alanine-water (O_w-H_{na} and O_a-H_w), urea-water (O_u-H_w), TMAO-water (O_t-H_w) and urea-TMAO (O_t-H_u), NMA-water and Acetamide-water are given in Table 3.

It can be seen that addition of osmolytes increases the hydrogen bond lifetime of the system. The hydrogen bond lifetime is found to be more in presence of ternary urea-TMAO mixtures for all the cases followed by TMAO, urea, aqueous alanine/acetamide/NMA solutions. In general, SPC/E water model exhibits higher hydrogen bond lifetimes compared to SPC water model. We found higher lifetimes between carbonyl oxygen and water when compared to amine-water interactions in all the cases indicating stronger interaction of the water molecules with the carbonyl group as compared to amine group.

It is clearly evident from Table 3, that addition of TMAO increases the hydrogen bond lifetime of the solutions. The stabilization of water hydrogen bonding network by TMAO has pivotal role in counteracting the denaturing effects of urea. The higher lifetime of TMAO-water (O_t-H_w) can be attributed due to the significant partial charges located on the oxygen atom [66]. The TMAO-water (3.87 ps) hydrogen bond lifetime in ternary urea-TMAO mixture is found to be three times higher than water-water (1.03 ps) and water-urea (1.13 ps) hydrogen bond lifetime. The hydrogen bond lifetime of water-water and urea-water increases in the ternary mixture compare to normal urea-water system [4]. This strengthening of the hydrogen bond in the water structure in presence of TMAO can be related to the stability imparted to the protein molecules.

4. Summary and conclusions

In this study, we have examined the effects of TMAO, urea and their ternary mixture on the solvation structure of amino acids (alanine, glycine) and amides (acetamide, NMA) to explain the stability aspect of TMAO and denaturing effect of urea. Structural properties were studied in terms of RDF, SDF, number of hydrogen bonds, <O-O-O angle distributions and Kirkwood-Buff Integrals. It is found that in presence of TMAO, amino acids and amides are solvated by water more in comparison to the aqueous solution and urea solution. Specially, near the hydrophobic groups, an extra hydration shell is observed. This hydration shell results mainly from the strong hydration shell of the neighbour-

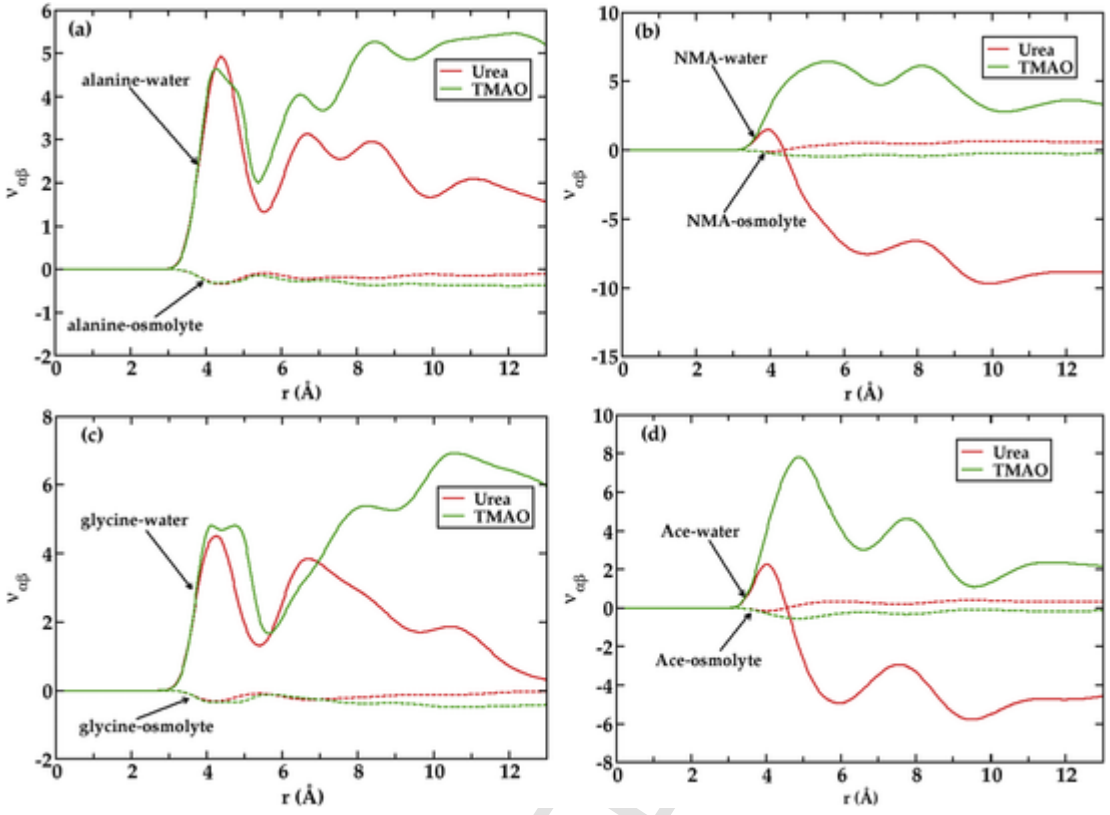

Fig. 8. Preferential binding coefficients $\nu_{\alpha\beta}$ according to Eq. (4) for (a) alanine-water, alanine-osmolytes (b) NMA-water, NMA-osmolytes (c) glycine-water, glycine-osmolytes and (d) acetamide-water, acetamide-osmolytes in 3 M aqueous solutions of urea and TMAO as a function of distance.

Table 3
The lifetime (τ_{HB}) of hydrogen bonds (in ps) formed by Alanine, NMA, Acetamide with water and osmolytes for SPC/E and SPC water models for systems 1–8. [a] The lifetime for SPC/E water models are given in the parenthesis.

Species	A-W	A-U-W	A-T-W	A-U-T-W
O_w-H_w	0.60 (0.89)	0.65 (0.94)	0.93 (1.42)	1.03 (1.60)
O_w-H_{na}	0.98 (1.19)	1.08 (1.31)	1.36 (1.76)	1.66 (1.80)
O_a-H_w	1.40 (1.70)	1.57 (1.99)	1.86 (2.50)	2.23 (2.97)
O_u-H_w	–	0.72 (0.89)	–	1.13 (1.43)
O_t-H_w	–	–	3.61 (5.13)	3.87 (5.72)
O_t-H_u	–	–	–	0.92 (0.91)
O_w-U_H	–	0.23 (0.25)	–	0.30 (0.32)
Species	NMA-W	NMA-U-W	NMA-T-W	NMA-U-T-W
O_{NMA}-H_w	0.579	0.611	0.815	0.876
O_w-$H_{N\text{-}NMA}$	0.307	0.323	0.363	0.390
Species	Ace-W	Ace-U-W	Ace-T-W	Ace-U-T-W
O_{Ace}-H_w	0.564	0.624	0.790	0.870
O_w-$H_{N\text{-}Ace}$	0.20	0.221	0.260	0.282

[a] In all the cases the first species is the acceptor and the donor as the second species.

ing polar groups i.e. the carbonyl carbon and the amine group of the biomolecule. The water molecules near the amino acids and amides interface mainly consists of broken hydrogen bonds which were found to increase on addition of the osmolytes. This broken hydrogen bonds are mainly composed of dimer and trimer water molecules. This is also reflected in the <O-O-O angle distribution. The <O-O-O angle distribution shifts towards the higher angle due to the loss of tetrahedrality of the water molecules near the C_α carbon. Potentials of mean force showed that presence of these water molecules actually imparts stability to amino acids and amides.

To shed light on the solvation structure of the solution, we further examined the KB Integrals and preferential binding affinity, $\nu_{\alpha\beta}$ of the protein (amino acids and amides) with water and co-solvents. In urea solution, the urea-urea and water-water association is found to be more favoured than urea-water association; whereas in case of TMAO solutions, the water-water and TMAO-TMAO association is less favoured than water-TMAO interactions. Similar trend was found for ternary mixture of urea-TMAO. It is also found that near the interface of amino acids and amides, water is preferentially favoured over the presence of other cosolvents. For amides, we found this trend up to smaller region of $r < 4.35$–4.5 Å. Preferential exclusion of both TMAO and urea are found from amino-acid surface, however in case of amides, we found TMAO is excluded slightly more in comparison to urea. This can be explained on the basis of the placement of the polar groups. Amides are found to be less solvated with water in comparison to amino acids in urea-water system which helps the urea molecule to come closer to amides compared to amino acids.

The hydrogen bond strength near the surface of amino acids and amides are increased on addition of osmolytes. Addition of TMAO was found to increase the hydrogen bond lifetime of the system. The TMAO-water (O_t-H_w) bond was found to be the strongest bond due to the polar oxygen of TMAO. The water-TMAO interaction actually increases the corresponding hydrogen bond life time of water-water and water-urea life time in a ternary solution compared to normal water-urea and aqueous water-biomolecule system.

In conclusion, TMAO is found to impart stability to the biomolecules by increasing the hydration shell and strengthening the hydrogen bond network of the solution. In urea solution, this protective hydration shell is missing and less interaction between the solvent molecules and the protein is found. The extra hydration shell in presence of TMAO is strikingly observed more near the hydrophobic group of the biomolecules. Further, TMAO-water has the highest hydrogen bond lifetime which also increases the hydrogen bond lifetime of water-water and water-urea in case of ternary mixture. This could be the possi-

ble reasons for the stability of proteins in presence of TMAO and also in ternary mixture.

Notes

The Authors declare no competing financial interest.

Declaration of competing interest

The Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2019.112375.

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