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Protein thermal stabilization in aqueous solutions of osmolytes

Piotr Bruździak[1]✉, Aneta Panuszko[1], Muriel Jourdan[2] and Janusz Stangret[1]

[1]Department of Physical Chemistry, Chemical Faculty, Gdansk University of Technology, Gdańsk, Poland; [2]Département de Chimie Moléculaire, CNRS UMR5250, ICMG FR2607, Université de Grenoble, 570 Grenoble Cedex 9, France

Proteins' thermal stabilization is a significant problem in various biomedical, biotechnological, and technological applications. We investigated thermal stability of hen egg white lysozyme in aqueous solutions of the following stabilizing osmolytes: Glycine (GLY), N-methylglycine (NMG), *N,N*-dimethylglycine (DMG), *N,N,N*-trimethylglycine (TMG), and trimethyl-*N*-oxide (TMAO). Results of CD-UV spectroscopic investigation were compared with FTIR hydration studies' results. Selected osmolytes increased lysozyme's thermal stability in the following order: Gly>NMG>TMAO≈DMG>TMG. Theoretical calculations (DFT) showed clearly that osmolytes' amino group protons and water molecules interacting with them played a distinctive role in protein thermal stabilization. The results brought us a step closer to the exact mechanism of protein stabilization by osmolytes.

Key words: water structure, protein stability, circular dichroism, FT-IR spectroscopy, DFT calculations

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INTRODUCTION

Osmolytes are small organic compounds accumulated in living cells in harsh environmental condition (Yancey, 2005; Yancey *et al*., 2004). Osmolytes' classification is difficult because of wide range of their chemical origin and composition. They possess an extra feature – they can modify macromolecules' stability. This influence is usually nonspecific, i.e. stabilizing properties are universal in a given group of macromolecules. However, a good protein stabilizer can also be a strong nucleic acid denaturant (e.g. Glycine Betaine) or can change its stabilizing/destabilizing abilities in a different protonation state (Singh *et al.,* 2011, Denning *et al.,* 2013). Osmolytes may influence the energetic barrier between native and intermediate states during protein denaturation, and delay or promote unfavorable aggregation processes (Seeliger *et al.,* 2013; Wei *et al.,* 2013). The exact stabilization or destabilization driving force is still under debate. Many theories were proposed to explain the basics of osmolytes' influence on protein stability (Auton *et al.,* 2011; Mondal *et al.,* 2013; Damodaran, 2012; Bennion & Daggett, 2003). However, none of them explained it sufficiently, although the insight into the subject of protein stability and stabilization mechanism is of the great importance from the practical and scientific points of view.

Protein thermal stability is a superposition of thermodynamic stability (characterized by ΔG of the denaturation process) and kinetic stability (dependent on the barrier Gibbs' function $\Delta G^{\ddagger}$ between native and denatured states). Water molecules affect proteins' structure in solution both through hydrophobic effect and by participation in favorable interactions at the protein surface. Osmolytes' presence can interfere in both of these phenomena. Mostly accepted hypothesis concerning the basis of osmolytes' stabilizing and destabilizing effects are focused on their influence on a biomolecule's denatured state. There is a general consensus that osmolytes interact favorably with protein's unfolded form through direct hydrogen bonds. This interaction causes the enthalpic stabilization of the unfolded state because the number of peptide groups available to this kind of osmolyte is higher than in the native form of a protein.

In the case of stabilizing osmolytes (e.g. aminoacids and their derivatives) it is stated that they are preferentially excluded from the protein surface. Therefore, the presence of these osmolytes should destabilize the unfolded state through the decrease of effective volume available to random coil (as the effect of conformational entropy decrease). In this kind of considerations, stabilizing and destabilizing osmolytes' influence on protein's native state is usually ignored.

Our results (Panuszko *et al.,* 2009; Bruździak *et al.,* 2013; Panuszko *et al.,* 2012) made us lean toward the hypothesis that osmolytes' influence on proteins is determined by their ability to modify structural and dynamic properties of water in protein hydration shell. Stabilization/destabilization of protein native state may have an influence on ΔG of denaturation process, as well as on the transition state barrier height of the unfolding process.

Our previous studies concerning hen egg white lysozyme's denaturation (Panuszko *et al.,* 2009; Bruździak *et al.,* 2013; Panuszko *et al.,* 2012) allowed us to suspect that no direct interactions occur between lysozyme and osmolytes, and that water properties are the most important factors affecting the stability of protein: (1) lysozyme's secondary structure does not change significantly in the presence of many osmolytes studied so far (Panuszko *et al.,* 2009; Bruździak *et al.,* 2013); (2) a clear difference exists between the influence of some stabilizing and destabilizing osmolytes on organization of water molecules surrounding them (Panuszko *et al.,* 2012); (3) energetic and structural properties of water affected by stabilizing osmolytes are significantly similar to the properties of water affected by lysozyme in its native state (Bruździak *et al.,* 2013); (4) strong interrelationships exists between water properties in osmolytes' hydration spheres and their influence on lysozyme thermal stability

✉e-mail: piotr.bruzdziak@pg.gda.pl

Abbreviations: CD-UV, circular dichroism spectroscopy; DFT, density functional theory; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; NMG, *N*-methylglycine; DMG, *N,N*-dimethylglycine; TMAO, trimethyl-*N*-oxide; TMG, *N,N,N*-trimethylglycine

(Bruździak *et al.,* 2013). In all these cases water properties characterized by the distance distribution function (obtained from infrared spectral data) between oxygen–oxygen atoms of water molecules which are influenced by osmolytes or by lysozyme.

We used circular dichroism in the UV region (CD-UV) and performed DFT calculations of aqueous clusters of the solutes using the polarized continuum model (PCM). Osmolytes selected for this paper are generally considered as protein stabilizers: Glycine, *N*-methylglycin (NMG, Sarcosine), *N,N*-dimethylglycine (DMG), *N,N,N*-trimethylglycine (TMG, Betaine), and trimethyl-*N*-oxide (TMAO). TMAO is considered as one of the strongest stabilizers in nature, and participate in the protection of renal cells of mammals from deleterious influence of urea (Jackson-Atogi *et al.,* 2013; Sarma & Paul, 2013), or stabilization of the cells of deep see organisms against high pressure (Sarma & Paul, 2013; Sarma & Paul, 2012). Glycine, NMG, DMG, and TMG (Betaine) are generally considered as moderate stabilizers (Santoro *et al.,* 1992; Wang *et al.,* 2013; Guinn *et al.,* 2011; Caldas *et al.,* 1999).

MATERIAL AND METHODS

HEW lysozyme (Fluka) was dissolved in deionized water (about 3 g/15 mL) and dialyzed against water (24 h/4ºC, with membrane cut-off 10 kDa). The dialyzed solution was then lyophilized (10 mm Hg/–60ºC/48 h). The lysozyme native secondary structure was restored after re-solvation in water (determined with FT–IR spectroscopy). All osmolytes: Glycine (Sigma), NMG (Aldrich), DMG (Sigma), TMG (Sigma), TMAO (Fluka) were used as supplied. All the osmolytes solutions were prepared in 20 mM phosphate buffer, pH 6.5. These solutions were used to dissolve appropriate amounts of purified protein to a final concentration of 0.4 mg/mL (0.028 mol/dm^3).

All spectra and denaturation curves were recorded using Jasco J-810 CD-UV spectrometer, equipped with Peltier temperature controller. For each sample a spectrum at 25ºC before denaturation was measured. All spectra were recorded with 0.5 nm resolution, and a bandwidth of 4 nm, and three repeats were recorded and averaged. The temperature scanning speed was set to 0.5ºC/min. The spectra were baseline corrected by solvent subtraction. In almost every sample osmolytes severely deteriorated the quality of the spectra in the low wavenumber region. Thus, we were unable to measure full CD-UV spectra of lysozyme in the presence of osmolytes, and all spectra were recorded between 275–220 nm.

Denaturation curves were measured from 65ºC to 85ºC, with a 0.1ºC temperature step. The delay before recording the signal was set up to 60 s. In each case the wavelength of denaturation curve measurement was set up to 225 nm. Only in the case of two solutions of TMG (1.00 and 1.50 mol/dm^3) denaturation curves were measured at 230 nm because of noisy data at 225 nm. This did not affect the melting temperature, because the denaturation of lysozyme is a co-operative process, as determined previously in our DSC studies (Bruździak *et al.*, 2013). Denaturation temperatures of lysozyme in the presence of osmolytes were determined using the maximum of the first derivative of denaturation curves (Savitsky-Golay algorithm with 5ºC window and third-order polynomial). The maximum of the derivative is a more straightforward method of T_m determination and does not require a model fitting step. To compare osmolytes' influence on lysozyme thermal stability we proposed to use a $(dT_m/dC)_{C\to 0}$ parameter, which is the derivative of the curve at the zero molar concentration showing the denaturation temperature evolution as the osmolyte concentration function (Bruździak *et al.*, 2013). The propagation of $(dT_m/dC)_{C\to 0}$ values errors was estimated on the basis of N error (±0.5, determined by Panuszko *et al.*, 2011) and the intercept errors of the fitted quadratic curves from Fig. 1.

DFT calculations were performed using GAUSSIAN 03 package (Frish *et al.*, 2003). The optimized geometries and energies of the non-hydrated and hydrated structures of osmolytes were calculated using density functional theory (DFT) with B3LYP hybrid functional using the 6–311++G(d,p) basis set (Krishnan *et al.*, 1980). The polarizable continuum model (PCM) formalism of the self-consistent reaction field theory (SCRF) was used to study systems in the liquid phase (Cossi *et al.*, 2002; Mennucci & Tomasi, 1997; Tomasi *et al.*, 1999). Initial structures of all studied systems showed C1 symmetry and no symmetry constraints were imposed during geometry optimizations. Water molecules were placed near an appropriate osmolyte molecule atom so that the intermolecular distance between hydrogen of water molecule and osmolyte's oxygen atom or between oxygen atom of water molecule and osmolyte's amino proton had length of about 1.45 Å to force hydrogen bond formation. Additionally, individual spheres were placed on each of the osmolyte hydrogen atoms (explicit hydrogens) to assure the continuity of the PCM cavity. The zero point energy contribution to the vibrational energy was taken into account. Covalent contributions to the hydrogen–bond energy for the complexes were calculated as the differences between the total energies of the complexes (in the water continuum) and the energies of isolated monomers (in the water continuum), and have been corrected for the basis set superposition error (BSSE) using the standard counterpoise method (Boys & Bernardi,1970). The covalent energy effect is weak for weak H-bonds, but it increases distinctly as the H-bond energy increases (Grabowski *et al.*, 2006). HyperChem 8 software was used at the stage of visualization of computed results.

RESULTS AND DISCUSSION

Osmolytes influence on lysozyme thermal stability

All of the data concerning osmolytes' stabilizing effect on the lysozyme denaturation temperature are presented in Table 1. The dependencies of lysozyme's melting temperature vs. osmolyte's concentration are shown in Fig. 1. Glycine appears to be the most effective stabilizer among all other selected osmolytes in CD-UV experimental conditions. The $(dT_m/dC)_{C\to 0}$ parameter calculated for this compound is very high (8.2 K·dm^3/mol) (Table 2). Its mono- and dimethyl derivatives (NMG and DMG) influence the lysozyme melting temperature in similar way, with lower $(dT_m/dC)_{C\to 0}$ parameters: 6.6 and 5.5 K·dm^3/mol, respectively. In this group of compounds TMG is the less effective stabilizer, with $(dT_m/dC)_{C\to 0}$ parameter equal to 2.7 K·dm^3/mol. Surprisingly, TMAO, which is considered as one of the most effective protein stabilizers, exhibits $(dT_m/dC)_{C\to 0}$ parameter (5.7 K·dm^3/mol) lower than glycine, its methyl derivatve, and close to dimethyl derivatives.

Melting temperatures measured in the present study by CD-UV are lower by 2 to 3°C than previously reported ones obtained by the DSC method and corresponding to a higher protein concentration (Bruździak *et*

Table 1. Parameters of denaturation of lysozyme in the presence of stabilizing osmolytes.

GLY		NMG		DMG	
C[a]	T_m[b]	C[a]	T_m[b]	C[a]	T_m[b]
mol·dm^{-3}	°C	mol·dm^{-3}	°C	mol·dm^{-3}	°C
0.50	75.8±0.6	0.49	75.1±0.3	0.49	74.8±0.4
1.00	78.7±0.5	0.97	77.8±0.3	0.97	77.4±0.5
1.50	80.7±0.6	1.45	80.1±0.3	1.46	80.2±0.5
TMG		TMAO		Lysozyme	
C[a]	T_m[b]	C[a]	T_m[b]	C[a]	T_m[b]
mol·dm^{-3}	°C	mol·dm^{-3}	°C	mol·dm^{-3}	°C
0.50	73.1±0.5	0.43	74.1±0.5	0.00	72.1±0.4
1.00	74.4±0.4	0.87	76.5±0.5		
1.50	74.9±0.4	1.31	77.5±0.5		

[a]Osmolyte concentration. [b]Melting temperature (maximum of the first derivative of the denaturation curve, uncertainties result from the melting curve noisiness).

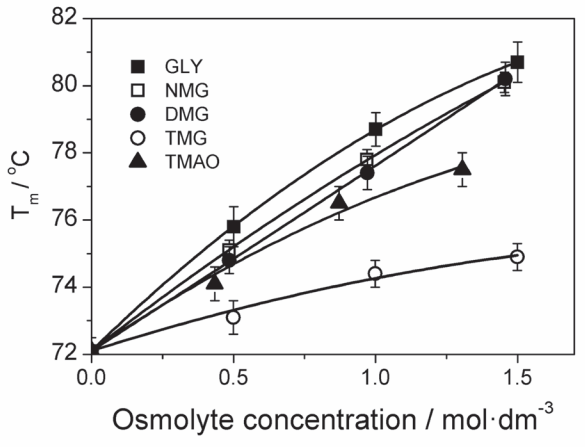

Figure 1. The dependencies of lysozyme's denaturation temperature (the maximum of the first derivative of CD-UV melting curves) versus osmolytes concentration.

al., 2013). The higher protein concentration might have promoted protein self-stabilization and thus increase the observed melting temperature. Thus, the order of stabilizers obtained from the CD–UV melting curves is as follows: Gly>NMG>TMAO≈DMG>TMG. This stabilizers' order correlates quite well with the number of water molecules (*N*) affected by these compounds that was previously determined by FTIR studies (see Table 2), except TMAO which affects a large number of water molecules (*N*=8.5). However, after recalculation of the $(dT_m/dC)_{C\to 0}$ parameter per one affected water molecule (i.e. to obtain the contribution of a single affected water molecule in the T_m increase – $(dT_m/dC)_{C\to 0}/N$ parameter, see Table 2) one can see that osmolytes can be divided into two subgroups: Gly-NMG-DMG (possessing amino protons), and TMG-TMAO (possessing no amino protons). On the other hand, it can be seen that CD-UV and previous DSC results are similar, and both correlate well with the previously obtained most probable intermolecular oxygen–oxygen (R_{OO}^{o}) distance of water molecules affected by osmolyte (Panuszko *et al.*, 2009; Bruździak *et al.*, 2013; Panuszko *et al.*, 2011). This observation proves that the interdependence of water properties in osmolytes' hydration spheres and their influence on lysozyme thermal stability exists.

Table 2. The influence of stabilizing osmolytes on lysozyme thermal stability in respect to their influence on water molecules

	N[a]	dT_m/dC	$(dT_m/dC)/N$
		K·mol^{-1}·dm^3	K·mol^{-1}·dm^3
GLY	5.8±0.5	8.2±1.2	1.4±0.3
NMG	5.0±0.5	6.6±0.7	1.3±0.3
DMG	4.8±0.5	5.5±1.0	1.1±0.3
TMG	4.0±0.5	2.7±1.0	0.7±0.3
TMAO	8.5±0.5	5.7±1.3	0.7±0.2

[a]The number of water molecules affected by osmolytes.

Amino protons promote osmolyte's stabilizing property

The DFT calculations of stabilizing osmolytes in the aqueous solution (PCM model) gives an insight into the observed results and correlations between CD-UV, and FTIR data. The PCM model was used because only in the PCM model their stable form is the zwitterion (as in real solution). The procedure used in this case takes into account only the covalent contribution of hydrogen bond energies. Nonspecific interactions like dipole–dipole, dipole-induced dipole, and dispersive forces, are hidden in the values of water cluster energies, separated central molecule (i.e. separated osmolyte molecule), and separated water molecule in the PCM model. Direct interactions between water molecules and different hydrophilic centers of osmolytes were analyzed. We examined complexes in which osmolytes' hydrophilic centers formed an utmost number of direct hydrogen bonds. All considered structures are presented in Fig. 2. On the basis of the difference between the water cluster energy value and the sum of separated water molecules energies we calculated the hydrogen bond energies' covalent contributions. Next, the obtained value was divided by the water molecule number in each analyzed structure. It is believed that the obtained value corresponds to the hydrogen bond energy's covalent contribution in respect to one water molecule. These results are presented in Table 3, while Fig. 3 shows the average energetic characteristics of hydrogen bonds covalent contribution (in

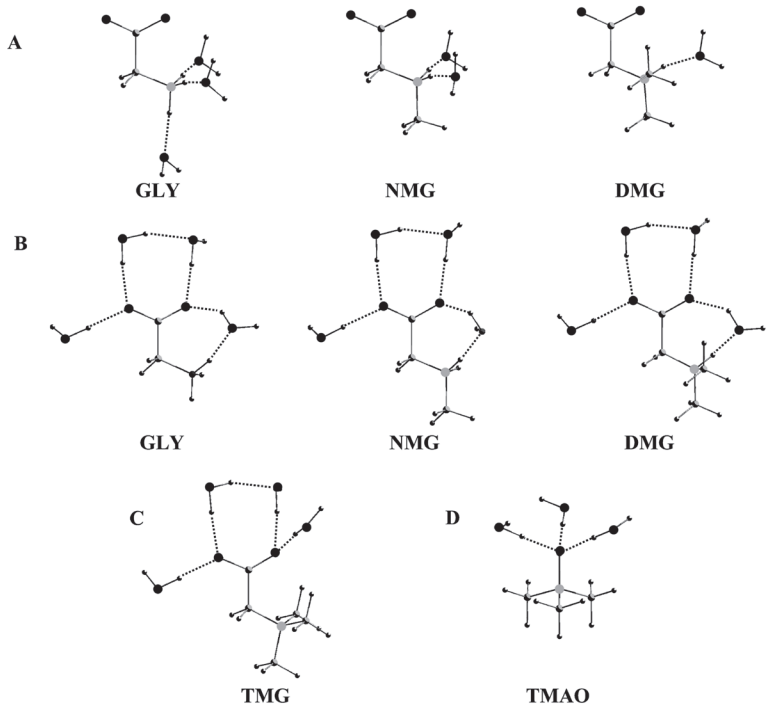

Figure 2. Hydrogen bonds formed between hydrophilic groups of osmolytes and water molecules.
Larger black spheres denote oxygen atoms; larger grey spheres denote nitrogen atoms; small gray spheres denote hydrogen atoms. The hydrogen bonds are indicated with dashed lines. Water complexes for glycine are taken from Bruździak *et al.,* 2013.

respect to one water molecule) between water molecules and osmolytes' hydrophilic centers.

Gly, NMG, and DMG possess two hydrophilic centers. The covalent contributions of hydrogen bond energies (in respect to one water molecule) indicates that a strong hydrogen bond exists between water molecule and the hydrogen of amino group. It is worth to notice that the interaction between water molecule and the amino group is stronger than between water molecule and carboxylic group. The increase of methyl substituent number in this group of osmolytes is accompanied with the decrease in covalent contributions of hydrogen bond energy between water molecule and amino group hydrogen, and between water molecule and carboxylic group. However, in the part B of Fig. 2 one can see that the simultaneous interactions of water molecule with carboxylic and amino groups has an influence on the covalent contribution of hydrogen bond energy.

In the case of TMG and TMAO (possessing no amino hydrogen) the covalent contribution values of hydrogen bond energy are similar. The interaction between water molecule and TMAO oxygen atom is slightly stronger than the interaction between water molecule and TMG carboxylic group.

The DFT calculations of osmolytes' water clusters drew us to the conclusion that lysozyme's higher thermal stability is assured by an osmolyte molecule possessing a higher number of hydrogen atoms on the amino group. It appears that the strong interaction of water molecules with osmolyte's amino groups determines osmolyte's influence on protein thermal stability. This

Table 3. Total energies, covalent contributions to the hydrogen–bond energies and covalent contributions to the hydrogen-bond energies per one water molecule of osmolyte water clusters calculated at the B3LYP/6–311++G(d,p) level of theory in the PCM model

		E^a	$E_{H\text{-}bond}{}^b$	$E_{H\text{-}bond/nH2O}{}^c$
	H_2O	–76.452346		
	GLY[d]	–284.471558		
A	GLY + $3H_2O$	–513.840305	–17.44	–5.81
B	GLY + $4H_2O$	–590.291549	–13.70	–3.43
	NMG	–323.758991		
A	NMG + $2H_2O$	–476.670056	–7.06	–3.53
B	NMG + $4H_2O$	–629.576805	–7.88	–1.97
	DMG	–363.043537		
A	DMG + H_2O	–439.498062	–0.73	–0.73
B	DMG + $4H_2O$	–668.858263	+0.56	+0.14
	TMG	–402.321585		
C	TMG + $4H_2O$	–708.139341	–8.83	–2.21
	TMAO[d]	–249.597040		
D	TMAO + $3H_2O$	–478.961047	–8.83	–2.94

[a]Total energy (hartree). [b]Covalent contribution to the hydrogen-bond energy calculated as the difference between the energy of a cluster in the water continuum and the sum of the energies of separated molecules in the water continuum (kJ·mol^{-1}). [c]Covalent contribution to the hydrogen-bond energy per one water molecule (kJ·mol^{-1}). [d]Data are taken from Ref. 26 and Ref. 25, respectively. All the covalent contributions to the hydrogen–bond energy were corrected for the basis set superposition error (BSSE), using counterpoise procedure. The letters A, B, C and D refer to respective labels in Fig. 2.

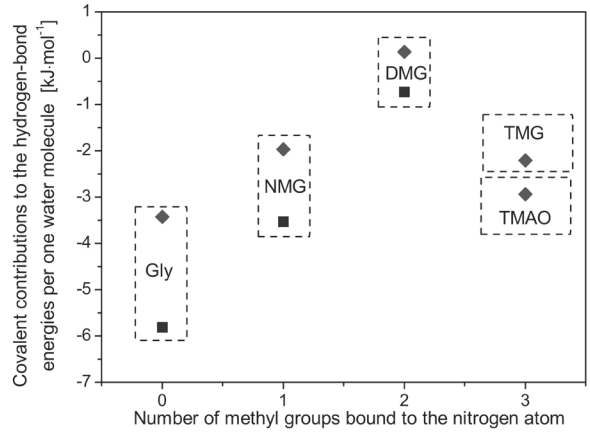

Figure 3. Energetic characteristics of covalent share of hydrogen bonds (in respect to one water molecule) between water molecules and hydrophilic centers of osmolytes:
square denotes interaction between water molecules and amine group, rhombus denotes interaction between water molecules and carboxylic group (or oxygen atom, in the case of TMAO). All values correspond to complexes presented in Fig. 2.

can be explained by taking into account our previous observation concerning lysozyme hydration (Panuszko *et al.,* 2012). The infrared spectrum of lysozyme hydration water may be treated as a superposition of two absorption contributions: water molecules affected by *N*-methylacetamide (NMA) and by carboxylate anion. We also noticed that water molecules were not involved in the H-bond formation with the amino group of NMA molecule (Panuszko *et al.,* 2008). Therefore, water molecules strongly H-bonded by oxygen atom to the amino group of currently studied osmolytes can form stronger H-bonds (because of the cooperative effect) by hydrogen atoms with above mentioned oxygen centers on the surface of lysozyme. According to our previous experimental FTIR results stabilizing osmolytes do not interact directly with the protein. Thus, we can conclude that it is the osmolyte affected water that influences protein directly, not the osmolyte molecule itself.

Osmolyte's ability to stabilize protein decreases with the number of methyl substituents: Gly>NMG>DMG. TMG and TMAO, which do not possess amino hydrogens, turned out to be weaker stabilizers. In this case, the interaction between water molecule and carboxylic group is the parameter determining their stabilizing ability. Additionally, the higher value of covalent hydrogen bond energy contribution indicates better stabilizing ability (i.e. TMAO is a better stabilizer than TMG). These results are in agreement with CD-UV melting curves, and FTIR data.

CONCLUSIONS

The correlation between CD–UV data and previously published results concerning water structure around osmolyte molecules (*N* parameters) allowed us to divide the investigated group of stabilizing osmolytes into two groups: good stabilizers (Gly, NMG, DMG with $(dT_m/dC)_{C\to 0}/N$ between 1.4 and 1.1) and weak stabilizers (TMG and TMAO with $(dT_m/dC)_{C\to 0}/N \sim 0.7$). These results are also supported and explained by DFT calculations – molecules possessing amino protons are better stabilizers. We suspect that the interaction of water molecules with these protons is responsible for osmolytes' ability to protein thermal stabilization. The ability decreases with the number of methyl substituents on their amino groups, though the decrease is small.

It appears that water molecule interacting with osmolyte's amino proton, which can act as a proton donor in protein–water interactions, possesses structural and energetic characteristics allowing to interact with proteins in a favorable manner. In the case of osmolytes possessing no amino protons, stabilizing properties of an osmolyte increase with the strength of water–osmolyte interaction (through the oxygen atom).

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