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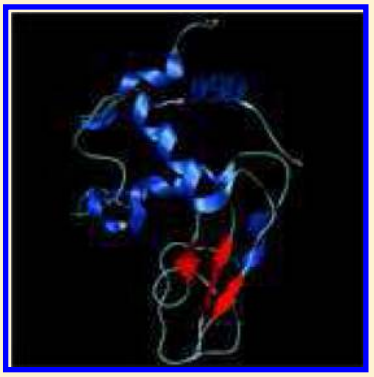
Influence of Osmolytes on Protein and Water Structure: A Step To Understanding the Mechanism of Protein Stabilization

Piotr Bruździak,* Aneta Panuszko, and Janusz Stangret

Department of Physical Chemistry, Chemical Faculty, Gdańsk University of Technology, Narutowicza 11/12, 80-233 Gdańsk, Poland

Supporting Information

ABSTRACT: Results concerning the thermostability of hen egg white lysozyme in aqueous solutions with stabilizing osmolytes, trimethylamine-*N*-oxide (TMAO), glycine (Gly), and its *N*-methyl derivatives, *N*-methylglycine (NMG), *N,N*-dimethylglycine (DMG), and *N,N,N*-trimethylglycine (betaine, TMG), have been presented. The combination of spectroscopic (IR) and calorimetric (DSC) data allowed us to establish a link between osmolytes' influence on water structure and their ability to thermally stabilize protein molecule. Structural and energetic characteristics of stabilizing osmolytes' and lysozyme's hydration water appear to be very similar. The osmolytes increase lysozyme stabilization in the order bulk water < TMAO < TMG < Gly < DMG < NMG, which is consistent with the order corresponding to the value of the most probable oxygen−oxygen distance of water molecules affected by osmolytes in their surrounding. Obtained results verified the hypothesis concerning the role of water molecules in protein stabilization, explained the osmophobic effect, and finally helped to bring us nearer to the exact mechanism of protein stabilization by osmolytes.



I. INTRODUCTION

Osmolytes are small organic compounds produced by living organisms mainly in harsh environmental conditions.[1−3] Huge interest in this group of chemical compounds can be attributed to their ability to modify the structure and stability of macromolecules.[4] However, the mechanism of the stabilizing influence is still under debate. The explanation of the unusual properties of osmolytes has significant meaning for stabilization/destabilization phenomenon but also lead to an approach to the core of the macromolecule folding problem. The ability of osmolytes to modulate the thermostability of a given protein or DNA can have an immense influence on many branches of modern biotechnology.[5−11]

The unresolved problem inspired many researchers to investigate the basics of the interaction mechanism in water solutions of osmolytes,[12−26] binary systems of two osmolytes,[27−31] and solutions of osmolytes and macromolecules. A few theories, concerning the exact mechanism of interactions between osmolytes and macromolecules, have been stated so far. Yet, none of them fully explains the observed effects. One of these theories assumes that direct interaction between the polypeptide backbone and the osmolyte molecule plays a crucial role in structural stabilization.[32−37] However, it is also stated that there is no direct interaction, and the observed effect is due to structural modification of water caused by osmolytes.[1,29−31,38−40] Thus, thermal stabilization of macromolecules seems to be a resultant effect of osmolyte−water−macromolecule interactions.[41−45]

In this paper we present results of studies concerning the thermostability of hen egg white lysozyme in the presence of stabilizing osmolytes: trimethylamine-*N*-oxide (TMAO), glycine (Gly), and its *N*-methyl derivatives, *N*-methylglycine (NMG), *N,N*-dimethylglycine (DMG), and *N,N,N*-trimethylglycine (betaine, TMG). We try to correlate results of direct measurements of protein structure and stability (FTIR and DSC studies, respectively) with recently reported spectroscopic results[17,46] concerning water structure in solutions containing osmolytes as well as lysozyme. A variety of experimental and computational techniques gives us deep insight into the problem of osmolytes influence and leads to more precise and reliable conclusions; first, a strong argument that water molecules are the most important factor modifying protein stability.

Vibrational spectroscopy is a perfect tool not only to observe the secondary structure of macromolecules[47−55] but also to investigate intermolecular interactions in water solutions.[15,17,19,56−58] To avoid experimental and mainly the interpretative problems connected with spectra of H_2O, we use isotopic dilution techniques of HDO in H_2O.[56,59−62] Differential scanning calorimetry (DSC) allowed us to determine the influence of osmolytes on the thermal stability of lysozyme, mainly the denaturation temperature.

II. MATERIALS AND METHODS

II.1. Sample Preparation. All osmolytes and buffer components were used as supplied. HEW lysozyme purchased from Fluka was dissolved in deionized water (ca. 3 g/15 mL) and dialyzed against pure water for 24 h at 4 °C. Purified

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Table 1. Parameters of HDO Bands of Water Affected by Glycine and Its *N*-Methylated Derivatives (taken from ref 46), by TMAO (taken from ref 16), by Bulk Water, and the Respective Intermolecular Oxygen−Oxygen Distances

solute	N^a	ν^o_{OD},[b] cm^{-1}	ν^g_{OD},[c] cm^{-1}	$R_{OO}{}^o$,[d] Å	$R_{OO}{}^{\&}$,[e] Å
bulk water		2509 ± 2	2505 ± 2	2.828 ± 0.003	2.843 ± 0.003
Gly	5.8 ± 0.5	2509 ± 2	2496 ± 2	2.793 ± 0.003	2.833 ± 0.003
NMG	5.0 ± 0.5	2457 ± 2	2476 ± 2	2.767 ± 0.003	2.813 ± 0.003
DMG	4.8 ± 0.5	2515 ± 2	2488 ± 2	2.785 ± 0.003	2.826 ± 0.003
TMG	4.0 ± 0.5	2522 ± 2	2492 ± 2	2.800 ± 0.003	2.828 ± 0.003
TMAO	8.5 ± 0.5	2495 ± 2	2470 ± 2	2.810 ± 0.003	2.805 ± 0.003

[a]Affected number, which means the number of moles of water affected by 1 mole of solute. [b]Band position at maximum. [c]Band position at gravity center. [d]Most probable O···O distance. [e]Mean O···O distance. R_{OO} errors have been estimated on the basis of the HDO band position errors.

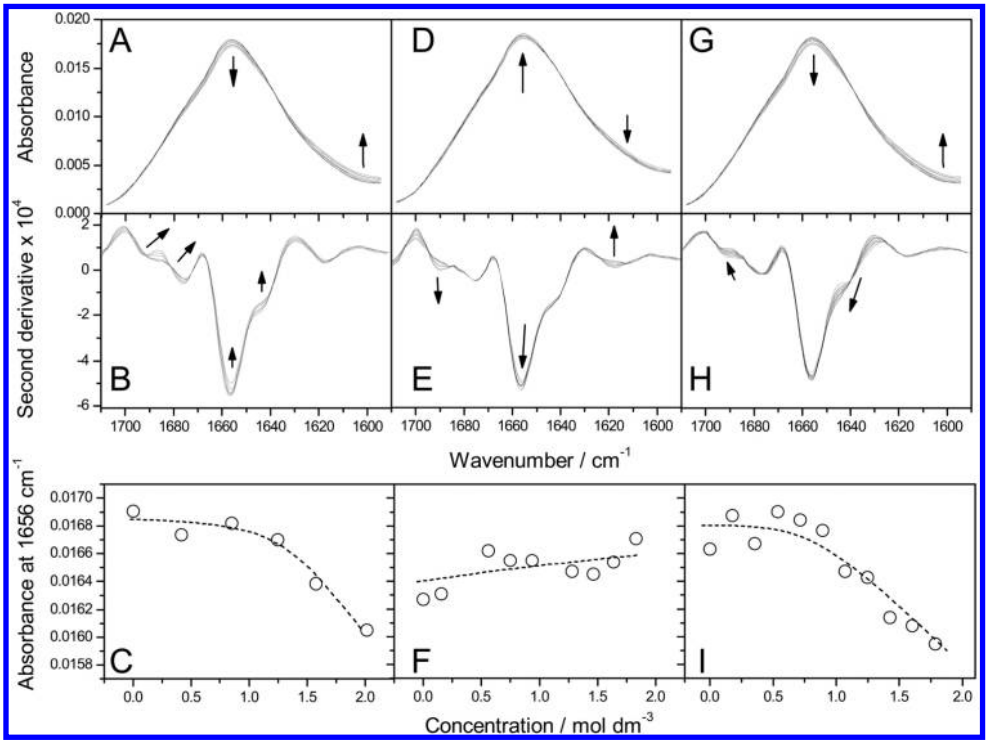

Figure 1. Influence of Gly (A−C), NMG (D−F), and DMG (G−I) on the secondary structure of lysozyme. Arrows indicate the direction of changes in the band shape of FTIR spectra (upper graphs, in the region of the amide I band) and their second derivatives (middle graphs). Influence of Gly, NMG, and DMG on the intensity of the FTIR spectra maximum (1656 cm^{-1}) of amide I band of lysozyme (lower graphs).

protein was then lyophilized at a pressure of 10 mmHg and a condenser temperature of −60 °C for 40 h. Dried protein samples were suspended before experiments in an appropriate buffer to a final concentration of 200 mg·mL^{-1}. Initial solutions of osmolytes were prepared in 20 mM phosphate buffer, pH 6.5. Five solutions of each osmolyte were prepared in the range of 0−4 mol·dm^{-3}. These solutions were used to dissolve appropriate weighed amounts of pure lysozyme and obtain a protein concentration of 1.6 or 200 mg·mL^{-1} for DSC and FTIR measurements, respectively. A high concentration of protein was necessary to obtain a satisfactory signal-to-noise ratio during attenuated total reflection ATR-FTIR measurements.

II.2. FTIR Spectra Measurements of Secondary Structure of Lysozyme. ATR-FTIR spectra of all solutions of osmolytes and protein were recorded on a Nicolet 8700 spectrometer (Thermo Electron Co.) using a GoldenGate ATR accessory (Specac) equipped with a single-reflection diamond crystal. The temperature during measurements was kept at 25 ± 0.1 °C using an electronic temperature controller (Specac). For each spectrum, 256 scans were collected with a resolution of 4 cm^{-1}. The spectrometer's EverGlo source was on turbo mode during measurements. The spectrometer was purged with dry nitrogen to diminish water-vapor contamination of the spectra.

All ATR-FTIR spectra were water-vapor subtracted and corrected using an advanced ATR correction algorithm (part of the OMNIC software). The ATR correction algorithm is based on the refractive index of the crystal material, angle of incident ray (45°), and measured refractive index of each solution. Isolation of lysozyme spectra in the presence of osmolytes was performed according to the previously published method.[16] This method allowed us to obtain information concerning a secondary structure of the protein and its changes in the presence of osmolytes.

II.3. FTIR Spectra of Water Affected by Osmolytes. In our previous papers[17,46] we presented detailed analysis of obtained spectral data and results concerning hydration of lysozyme, glycine, and its methyl derivatives: FTIR spectra of HDO (diluted in H_2O) affected by these osmolytes were obtained using the quantitative difference spectra method or chemometric procedures. Details of the isotope dilution

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technique and the method of spectral data analysis were presented and discussed in our previous papers.[56,60−62]

Band shapes of Gly and its methyl derivatives affected HDO spectra obtained in ref 46 are now characterized in Table 1 and additionally transformed into the function of the probability of water oxygen−oxygen distribution distance. The procedure of transformation was described in refs 17, 61, and 63. All data take advantage of the explanation of lysozyme behavior in aqueous solution containing the studied osmolytes.

II.4. Differential Scanning Calorimetry. DSC experiments were performed on the CSC 6300 nano-DSC III calorimeter (CSC Corp.) equipped in a capillary cell (V 0.299 mL) at a temperature range 25−95 °C with a scanning rate of 1 K·min^{-1}. Data was collected using DSCRun software (CSC Corp.) and analyzed with Matlab-based scripts.

III. RESULTS AND DISCUSSION

III.1. FTIR Spectroscopy of Lysozyme. Detailed results concerning spectroscopic studies of osmolyte−lysozyme interactions are presented in the Supporting Information. FTIR results indicate that Gly, its *N*-methyl derivatives, and TMAO in lower concentration do not interact with lysozyme molecule. No strong or non-monotonic changes with increasing concentrations of osmolytes can be observed in the shape of the amide I band of lysozyme (Figures 1 and 2). TMAO influences this band to a larger extent than Gly and its derivatives.[16,46] We are also inclined to link up the relatively strong influence of TMG on lysozyme with the presence of the hydrophobic part of this osmolyte.

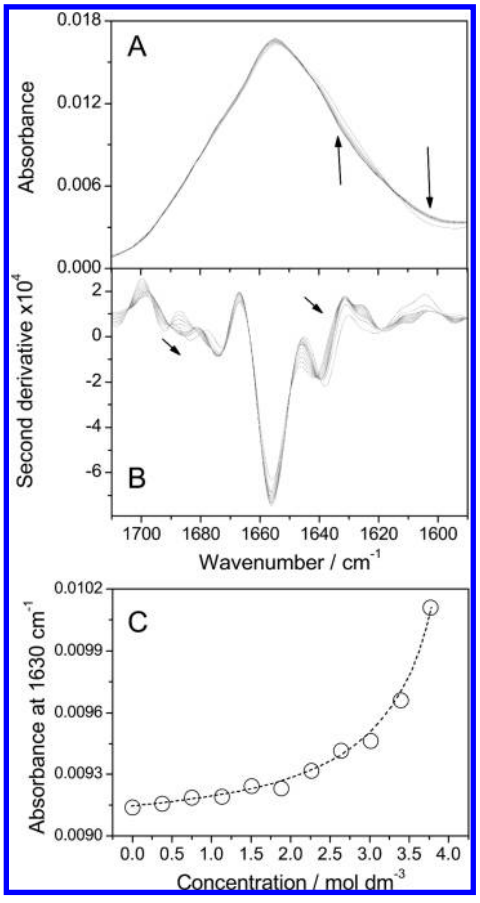

Figure 2. Influence of TMG on the secondary structure of lysozyme. Arrows indicate the direction of changes in the band shape of FTIR spectra (upper graph, in the region of the amide I band) and their second derivatives (middle graph). Lower graph presents the influence of TMG on the intensity of FTIR spectra at 1630 cm^{-1}.

III.2. Lysozyme Denaturation Temperature. All stabilizers increase the melting temperature of the lysozyme, T_m. This temperature is meant as a maximum of the DSC thermogram (see Table 2 and Figures 3 and 4). To compare the influence of osmolytes we propose a parameter dT_m/dc that is a derivative of denaturation temperature versus osmolyte's concentration function corresponding to $c = 0$. This parameter tells how strong the melting temperature change is with the increase of osmolyte concentration by 1 mol·dm^{-3}. The dT_m/dc parameters for all osmolytes are presented in Table 3. Gly, NMG, and DMG cause a similar increase in the melting temperature (ca. 5 K mol^{-1}). The influence of TMG is weaker (2.7 K mol^{-1}) and similar to the influence of TMAO (3.2 K mol^{-1}).

In the Supporting Information we present results concerning the thermokinetic characteristics of lysozyme denaturation in the presence of osmolytes. Possible mechanisms of protein denaturation, influence of osmolytes on heat effects, changes in heat capacity, and reversibility accompanying the melting of lysozyme molecule have been discussed.

III.3. Lysozyme Denaturation Temperature vs Characteristics of Osmolytes' Hydration Water. On the basis of Badger−Bauer's rule,[64] which states that water hydrogen-bond energy changes linearly with the shift in OH (OD) band position, it is possible to define the energetic state of water molecules in hydration spheres of selected osmolytes. The value of the maximum of absorption band position (ν^o) corresponds to the most probable hydrogen-bond energy, and the value of the gravity center band position (ν^g) corresponds to the mean hydrogen-bond energy. Accordingly, we recognized the most probable ($R_{OO}{}^o$) and the mean ($R_{OO}{}^g$) intermolecular oxygen−oxygen distance of water, which are related to the corresponding band positions. According to our previously published results,[16,46] all stabilizing osmolytes shorten interatomic oxygen−oxygen distances of water in their hydration spheres, and in this sense they enhance the water structure. All results are presented in Table 1 and Figure 5A.

It is worth noting that substitution of the first hydrogen of Gly's amine group with a methyl group increases the mean energy of hydrogen bonds of water, and following substitutions reduce this effect. The mean oxygen−oxygen distances of water affected by osmolytes increase in the following order: TMAO < NMG < DMG < TMG < Gly < bulk water. However, the most probable oxygen−oxygen distances increase in the following order: NMG < DMG < Gly < TMG < TMAO < bulk water. The most probable oxygen−oxygen distance correlates well with DSC results and can be used to predict the stabilizing properties of a solute compound.

Thus, it turned out that the increase of protein melting temperature in the presence of stabilizing osmolytes correlates well with the most probable oxygen−oxygen distance of water affected by osmolytes (Figure 5C). Because the osmolytes affect water molecules to a different extent, we divided the value of the dT_m/dc parameter by the value of N (obtained from FTIR spectra of water from osmolyte aqueous solution), which tells how many water molecules are affected by one molecule of an osmolyte. The resultant $(dT_m/dc)/N$ parameter describes the influence of 1 mol of affected water on the thermal stability

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Table 2. Parameters Characterizing Denaturation of Lysozyme in the Presence of Osmolytes

	c,[a] mol·dm^{-3}	ΔH_{cal},[b] kJ·mol^{-1}	ΔH_{vH},[c] kJ·mol^{-1}	$\Delta H_{vH}/\Delta H_{cal}$	ΔS_{cal},[d] kJ·mol^{-1}·K^{-1}	ΔC_{p},[e] kJ·mol^{-1}·K^{-1}	T_m,[f] °C	ΔH_{rescn},[g] kJ·mol^{-1}
lysozyme (reference values)		570	457	0.8	1.64	5.1	74.9	52
glycine	0.5137	423	434	1.03	1.21	6.8	77.7	99
	1.0273	446	461	1.03	1.26	8.8	80.4	156
	1.5044	441	418	0.95	1.24	11	82.4	189
N-methylglycine (NMG)	0.4674	471	466	0.99	1.34	4.6	77.5	59
	0.9349	466	489	1.05	1.32	7.6	79.9	88
	1.4917	490	495	1.01	1.37	13	83.2	138
N,*N*-dimethylglycine (DMG)	0.522	452	463	1.02	1.29	4.3	77.5	51
	1.0441	396	372	0.94	1.12	5.5	79.6	119
	1.4608	492	440	0.9	1.38	9.5	82.3	220
betaine	0.3049	442	346	0.78	1.27	5.1	75.1	44
	1.0053	450	512	1.14	1.28	4.8	77.6	29.4
	1.3292	475	432	0.91	1.35	6.2	77.9	14
TMAO	0.4834	582	464	0.78	1.67		76.3	26
	0.9703	478	441	0.92	1.36		77.7	12
	1.4537	468	607	1.3	1.32		79.8	5

[a]Osmolyte's concentration. [b]Calorimetric enthalpy of denaturation. [c]van 't Hoff's enthalpy. [d]Calorimetric entropy. [e]Change of heat capacity of protein at melting temperature. [f]Melting temperature. [g]Calorimetric enthalpy in rescan (a repeated scan of sample after one cycle of heating and cooling). [h]Reversibility of denaturation measured as a ratio of ΔH_{rescn} to ΔH_{cal}.

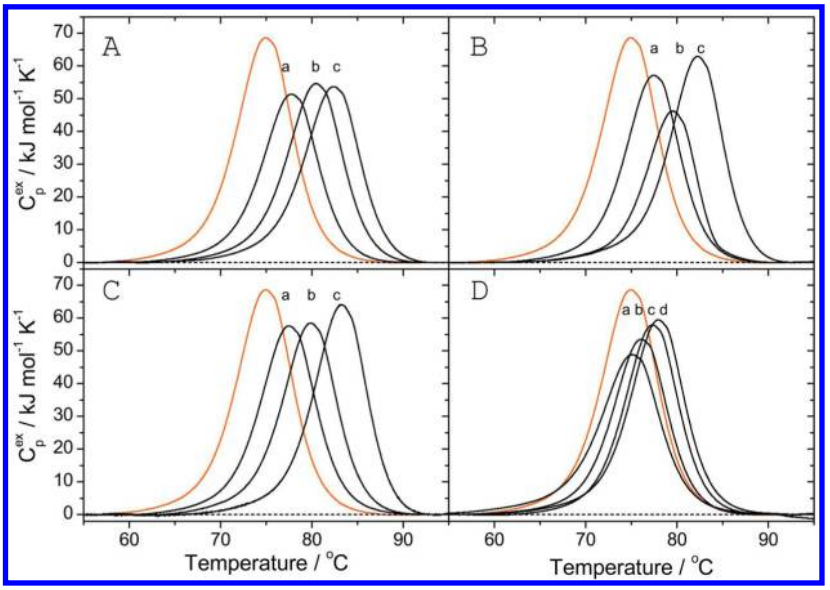

Figure 3. Thermograms corresponding to denaturation of lysozyme in the presence of stabilizing osmolytes: (A) Gly, (B) NMG, (C) DMG, (D) TMG. Red line indicates a thermogram corresponding to denaturation of lysozyme in pure phosphate buffer. Small letters indicate increasing concentration of an osmolyte.

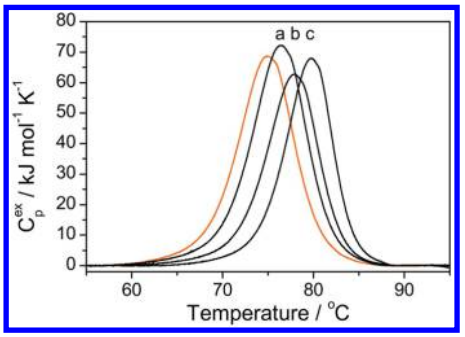

Figure 4. Thermograms corresponding to denaturation of lysozyme in the presence of TMAO. Red line indicates a thermogram corresponding to denaturation of lysozyme in pure phosphate buffer. Small letters indicate increasing concentration of an osmolyte.

Table 3. Change of Lysozyme'S Melting Temperature in the Presence of Stabilizing Osmolytes: dT_m/dc Parameter

	dT_m/dc K·dm^3·mol^{-1}
Gly	5.0
NMG	5.5
DMG	4.9
TMG	2.7
TMAO	3.2

of lysozyme. A corrected correlation is presented in Figure 5D. It corresponds directly to the most probable hydrogen-bond energy. Thus, water molecules from the osmolyte hydration sphere representing the most numerous population of H-bond energy state determine the thermal stability of the protein.

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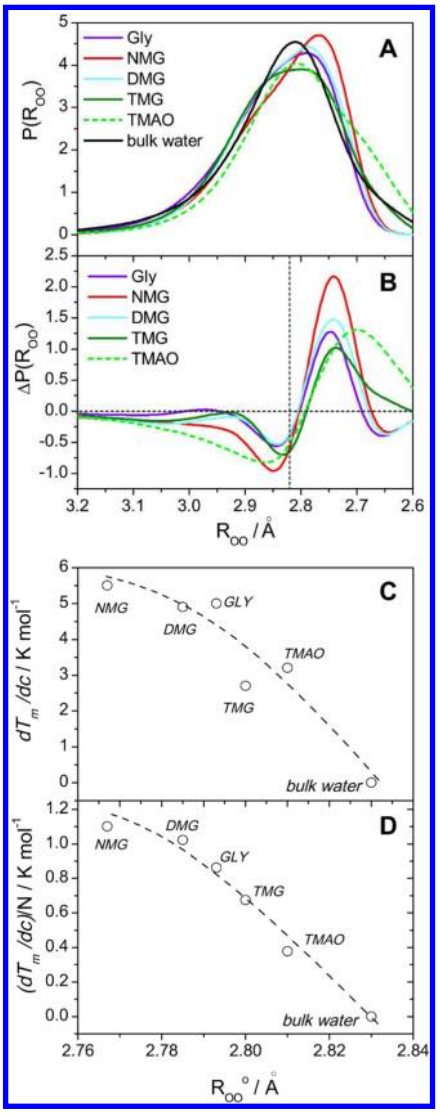

Figure 5. (A) Function of interatomic oxygen−oxygen distance distribution of water affected by osmolytes and of bulk water. (B) Differences between the intermolecular oxygen−oxygen distance distribution of osmolyte-affected water and bulk water. (C) Correlation of dT_m/dc vs R_{OO}° parameter. (D) dT_m/dc parameter corrected for one molecule of affected water vs R_{OO}°.

The above-mentioned finding explains the relatively weak peptide-stabilizing ability of TMAO. Our observation is in opposition to the general opinion about this osmolyte, which has been built up mainly on the basis of the property of counteracting the peptide-destabilizing effect of urea in the urea−TMAO systems. This is however another question. According to our previous investigation, the presence of TMAO strongly shortens water H-bonds, which can be directly correlated with the mean oxygen−oxygen distances of water, and in this sense it is the strongest enhancer of water structure among selected osmolytes.[16,46] However, the data from Table 1 and Figure 5A−D show that this strong enhancement of the water hydrogen bond (i.e., a shortening in interatomic oxygen−oxygen distances) concerns a relatively small population of affected water molecules. It influences mainly the mean ν^{g}_{OD} and R_{OO}^{g} values, while the most probable values (ν°_{OD} and R_{OO}°) are less affected in comparison to other studied stabilizers. Since TMAO does not cause the most probable oxygen−oxygen distances of water to shorten distinctly, it is not distinguished by stabilizing properties in the presented group of osmolytes.

For illustrative purposes, differences between intermolecular oxygen−oxygen distribution functions of water affected in the hydration spheres of osmolytes and the same function corresponding to the bulk water were calculated (Figure 5B). These differences were later averaged, and the average difference for all selected stabilizing osmolytes was obtained (see Figure 6). It should be mentioned that the corresponding

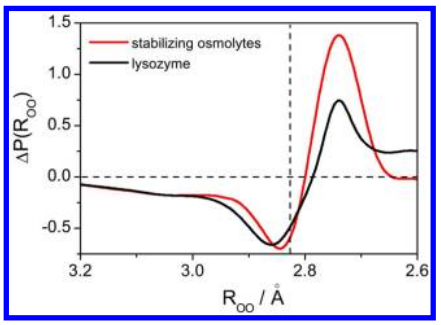

Figure 6. Difference between the intermolecular oxygen−oxygen distance distribution of lysozyme-affected water and bulk water (taken from ref 17) and the averaged difference corresponding to all stabilizing osmolytes from Figure 5B.

average difference plot for destabilizing osmolytes, alkyl derivatives of urea, is distinctly different (the article in preparation). It was interesting to compare the average difference for stabilizing osmolytes with the difference corresponding to water affected in the hydration sphere of lysozyme molecule.[17] We noticed that water molecules within hydration spheres of stabilizing osmolytes and lysozyme exhibit a very similar intermolecular distance distribution. The distances are shortened, and water H bonds are expected to be enhanced. Therefore, introduction of stabilizing osmolyte to an aqueous solution of protein enhances the water structure, which promotes tighter protein folding. On the other hand, the similarity of hydration spheres of a stabilizing osmolyte and protein does not promote direct contact of the osmolyte and peptide. This explains the frequently mentioned hypothesis of stabilizing osmolytes' exclusion from the surface of a natively folded protein and gives molecular justification of the osmophobic effect.

IV. CONCLUSIONS

FTIR studies of protein secondary structure in the presence of osmolytes confirm the hypothesis that stabilizing osmolytes generally do not interact directly with a protein, at least in low and moderate concentration.

An increase in the number of hydrophobic groups in Gly's derivatives does not play a key role in the thermal stabilization mechanism of lysozyme. Studied osmolytes increase their stabilization in the order bulk water < TMAO < TMG < Gly < DMG < NMG, which is consistent with the order corresponding to the value of the most probable oxygen−oxygen distance of water molecules affected by osmolytes in their hydration sphere. Thus, the specificity of osmolyte hydration has a great influence on protein stability in water solution.

All selected stabilizing osmolytes enhance water structure in their surroundings, which promotes tighter protein folding in

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aqueous systems. The intermolecular oxygen−oxygen distance distribution, and hence the distribution of the H-bond energy, of water in the hydration spheres of osmolytes and lysozyme appear to be very similar, which explains preferential hydration of the protein and gives molecular justification for the osmophobic effect.

The combination of spectroscopic and calorimetric data, concerning water and protein structure and protein thermal stability, allowed us to establish a link between osmolytes' influence on water structure and their ability to thermally stabilize protein. The results verified the hypothesis concerning the role of water molecules in protein stabilization and brought us closer to finding the mechanism of protein stabilization in the presence of osmolytes.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: piotr.bruzdziak@pg.gda.pl.

Notes

The authors declare no competing financial interest.

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