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*Research article*

# Exploring the role of preferential solvation in the stability of globular proteins through the study of ovalbumin interaction with organic additives

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**Abstract:** The impact of denaturing and stabilizing osmolytes on protein conformational dynamics has been extensively explored due to the significant contribution of protein solvation to the stability, function, malfunction and regulation of globular proteins. We studied the effect of two nonspecific organic molecules, urea, which is a conventional denaturant, and dimethyl sulfoxide (DMSO), which is a multilateral organic solvent, on the stability and conformational dynamics of a non-inhibitory serpin, ovalbumin (OVA). A differential scanning microcalorimetry (DSC) experimental series conducted in the phosphate buffer solutions containing 0–30 % of additives revealed the destabilizing impact of both urea and DMSO in a mild acidic media, manifested in the gradual decrease of thermal unfolding enthalpy and transition temperature. These findings differ from the results observed in our study of the mild alkaline DMSO buffered solutions of OVA, where the moderate stabilization of OVA was observed in presence of 5–10% of DMSO. However, the overall OVA interaction patterns with urea and DMSO are consistent with our previous findings on the stability and conformational flexibility of another model globular protein, α-chymotrypsin, in similar medium conditions. The obtained results could be explained by preferential solvation patterns. Positive preferential solvation of protein by urea in urea/water mixtures mainly weakens the hydrophobic interactions of the protein globule and eventually leads to the disruption of the tertiary structure within the whole range of urea concentrations. Alternatively, under certain experimental conditions in DMSO/water mixtures,

positive preferential solvation by water molecules can be observed. We assume that the switch to the positive preferential solvation by DMSO, which is shown to have a soft maximum around 20–30% DMSO, could be shifted towards lower additive concentrations due to the intrinsic capability of ovalbumin OVA to convert into a heat-stable, yet flexible set of conformations that have increased the surface hydrophobicity, characteristic to molten-globule-like states.

**Keywords:** globular protein; protein stability; thermal unfolding; differential scanning calorimetry; ovalbumin; urea; dimethyl sulfoxide

**Abbreviations:** DSC: differential scanning calorimetry; OVA: ovalbumin; S-OVA: S-ovalbumin; DMSO: dimethyl sulfoxide; α-CT: α-chymotrypsin

## 1. Introduction

One of the major goals of both fundamental and applied biomedical sciences includes the profound understanding of mechanisms governing various proteins' functions. Recent theoretical and experimental studies unveiled that protein function is essentially linked to its structure, stability and conformational flexibility, which, in turn, can be drastically altered by the crowded intracellular environment of proteins. According to the energy landscape theory of protein folding, general reaction coordinates of the multidimensional energy funnel of protein include both coupled and independent conformational and chemical transformations that are essential for protein folding and functional dynamics [1–4]. Thus, advanced knowledge and detailed understanding of protein structure and dynamics could provide new insights into the mechanisms governing protein function, as well as protein regulation, malfunction and degradation.

Our previous published works focused on the disclosure of intrinsic links between the stability, flexibility and function of globular proteins, such as α-chymotrypsin, carbxypeptidase A, cytochrome C, azurin and others, by altering their environment (solvent composition, immobilization etc). One of these works was devoted to understanding the impact of the same organic additives, urea and dimethyl sulfoxide (DMSO), on the conformational dynamics and enzymatic activity of α-chymotrypsin.

The goal of present work is to study the stability and conformational properties of model globular protein, hen egg albumin, or ovalbumin (OVA), from chicken egg whites in the presence of two nonspecific organic additives, urea and DMSO. The main conception of current manuscript emerged from the intention to investigate the peculiar action of DMSO on various model globular proteins. Ovalbumin is a major avian egg white protein with a well-studied structure, characteristic to the serpin superfamily of proteins. It is a glycoprotein consisting of a single 385 amino acid polypeptide chain glycosylated at Asn292 and cross-linked by one disulfide bond between Cys73–Cys120 [5,6]. Additionally, it should be mentioned that OVA has four more Cys residues with free sulfhydryl groups [5,6].

Previously referred to as the serine protease superfamily, serpins are numerous superfamily of proteins found in a vast majority of organisms, including animals, plants, fungi and viruses [7]. Most serpins are protease inhibitors, and are involved in processes such as coagulation (antithrombin), inflammation and immune processes (C1-inhibitor), among others. However, some of them, including ovalbumin, perform a non-inhibitory role, such as hormone transport (thyroxine-binding globulin) [8] and chaperone functions (heat shock serpin 47, myeloid and erythroid nuclear termination stage-

specific protein (MENT)) [9,10].

Although possessing a similar amino acid composition and conformational resemblance, OVA is an atypical member of the serpin superfamily. It does not share an ability to inhibit serine proteases and little is known about its function so far [10,11]. A lack of inhibitory properties in OVA could derive from a difference in the part of the polypeptide chain that is homologous to the reactive center loop of inhibitory serpins and has an α-helical conformation [6]. Nevertheless, it is theorized that the presence of a charged arginine residue within the active center drastically slows the loop insertion into the α-sheet, further preventing any inhibitory function [6,12]. Presumably, OVA mainly has an amino acid storage function, though it could also be involved in the transport and storage of metal ions [13].

Another remarkable feature of OVA is an ability to irreversibly transform into a thermostable conformation, S-ovalbumin (S-OVA), upon storage [14]. The melting temperature of the S-OVA increases by approximately 8 °C compared to that of the native protein [15]. In vivo, the conversion process spontaneously occurs during egg storage as a consequence of a natural increase of pH level. The rate of the conformational transfer is temperature and pH dependent [15]. Remarkably, although heat-stable S-OVA has a more compact structure and is more stable against thermal- or denaturant-induced unfolding [13,14], it has a more hydrophobic surface and increased flexibility [6,12,16] compared to the native, heat-unstable conformer. In addition to egg whites, heat-stable ovalbumin is found in egg yolks, then is transported into the amniotic fluid and subsequently absorbed by the embryonic organs [17]; it seems feasible that S-ovalbumin is required for the normal development of embryos [10,17].

The native ability of serpins to perform significant conformational changes is essential for their function. On the other hand, this ability is the very reason they are susceptible to misfolding and aggregation, leading to physiological disorders associated with serpin deficiencies, fatal accumulation of malfunctioning polymers and amyloid sheets formation [18], that eventually cause cell apoptosis and organ damage [19]. Considering the remarkable conformational flexibility, wide range of important functions and serpin misfolding related diseases [11,20], serpin family proteins have long been considered as a relevant research object of fundamental studies [5–10,21,22], as well as medical research [23,24] and bio-nanotechnology [25,26].

In the present work, we carried out a comparative study of the effect of urea, which is a conventional denaturant, and DMSO, which is a multilateral affecter, to explore the stability and conformational flexibility of OVA. DMSO is a dipolar organic solvent and is widely used in scientific research and medicine [27–29]. DMSO is reported to have multilateral action on protein stability and folding, exhibiting either stabilizing [29,30] or denaturizing [27,31] effects depending on experimental conditions. Additionally, it is used as a molecular chaperon [32,33], inhibitor [34,35] and activator [36]. However, its impact on OVA has not yet been sufficiently investigated.

## 2. Materials and methods

### *2.1. Differential scanning microcalorimetry*

We applied differential scanning microcalorimetry (DSC) to measure the thermodynamic parameters of OVA thermal unfolding in the presence of a wide range of additives concentrations. Calorimetric measurements were carried out using a DASM-4 adiabatic scanning calorimeter (Biopribor, Russia) directly connected to a computer via a PCI-DAS1001 (Measurement Computing

Corporation) interface unit. Further calorimetric data proceeding was carried out using the OriginLab software. Throughout all experiments, the heating and cooling rates were 1 K/min.

DSC is a very powerful experimental method for the investigation of the thermodynamic stability of globular proteins under the influence of various organic additives. It allows for the direct measurement of the biomolecule's enthalpy by plotting the partial heat capacity as a function of temperature. From the recorded DSC thermograms, the melting temperature T (melting peak x-coordinate), transition enthalpy ΔH (melting peak area) and other thermodynamic parameters can be calculated [37,38].

If the protein thermal unfolding (melting) process follows a two state model, the heat capacity of the dissolved protein $C_{p(prot)}$ can be determined at any temperature, according to the following equation [37,38]:

$$\Delta C_{p(app)} = C_{p(prot)} m_p - C_{p(solv)} \Delta m_s \quad (1)$$

where $\Delta C_{p(app)}$ is the deviation of the protein sample's calorimetric curve from the baseline curve, $C_{p(prot)}$ and $C_{p(solv)}$ are the partial heat capacities of the protein and solvent, respectively, $m_p$ is the mass of the dissolved protein, and $\Delta m_s$ is the mass of the replaced solvent. The calorimetric enthalpy $\Delta H_{cal}$ of thermal melting having a single transition temperature, $T_m$, can be calculated using the following equation:

$$\Delta H_{cal} = \int_{T_1}^{T_2} C_p(T) dT \quad (2)$$

where T is the absolute temperature, and $T_1$ and $T_2$ are the temperatures of the initial and end point of the thermal melting peak, respectively.

Since the value of the calorimetric enthalpy $\Delta H_{cal}$ is the area of the melting peak, it does not depend on the absolute value of the $C_{p(prot)}(T)$. Consequently, determination of $\Delta H_{cal}$ does not require the measurement of the absolute values of the partial heat capasities of both the protein and the solvent. Thus, a zero-baseline-correction of the initial calorimetric curves can be made to simplify the data processing and to allow for a direct comparison of the calorimetric enthalpies of the protein in the presence of various mixed solvents.

All the calorimetric melting curves obtained throughout the experimental series (Figure 1) were baseline-corrected to avoid any errors derived from unknown parameters, such as $C_{p(solv)}$ and $\Delta m_s$, and $\Delta H_{cal}$ was calculated using Formula (2). An analysis of the thermograms provides reliable information on the reversibility and cooperativity of the protein unfolding process and allows for the detection of the transition to the molten-globule state, alongside the presence of unfolding intermediates, protein domains or aggregation, if available [37]. Comparison of the thermodynamic parameters of the protein under different environmental conditions (several denaturing/stabilizing additives at variable concentrations etc.) gives new insights into the fundamental aspects of the protein globule stability and flexibility [38,39].

### *2.2. Chemicals and experimental details*

Highly purified albumin from hen egg white (OVA) (M = 42.7 kDa) was purchased from Sigma and used without further purification. DMSO was a product of Lugal (Ukraine) and contained 1%

water as an impurity, which was considered to prepare any DMSO buffered solutions. Urea and all other chemicals were from Reakhim (Russia), of the highest purity available, and used as received. Phosphate buffer components were from Sigma. Doubly distilled water was used throughout all experiments.

The OVA samples for the DSC experiments were prepared by dissolving it in a 0.1 M phosphate buffer solution pH 6.1 at concentrations of 3 mg/ml, containing various concentrations of either urea or DMSO.

Since the addition of DMSO causes a concentration dependent increase in the pH of DMSO-buffer mixtures, the buffer solutions with different DMSO concentrations were prepared by mixing solutions containing zero and maximal (30% DMSO v/v) concentration of DMSO, with the pH values being adjusted separately using concentrated citric acid.

## 3. Results and discussion

The thermal stability of OVA was studied in a mildly acidic media, namely 0.1 M phosphate buffer solutions containing no additive, 5, 10, 20 and 30% of either urea or DMSO. The initial DSC thermograms of the OVA at the heating rate of 1 K/min showed a single cooperative endothermic peak corresponding to a heat-unstable conformer of ovalbumin, while no peaks corresponding to S-ovalbumin were detected. Calorimetric parameters obtained from reference curves containing no additive in the solution were consistent with other published data obtained under similar conditions [12,40]. No protein refolding after denaturation was observed throughout the experimental series.

Figures 1a and 1b show the temperature-dependent changes of the partial heat capacity of the OVA obtained in the presence of various urea and DMSO concentrations. The initial experimental curves were zero-baseline corrected and aligned. DSC experiments revealed a similar behavior of the thermodynamic parameters in the presence of urea and DMSO additives. Namely, in the whole range of buffered additive solutions, OVA exhibited gradual destabilization, manifested in a decrease of both the denaturation temperature, $T_m$, and calorimetric enthalpy $\Delta H_{cal}$ (i.e., the peak position gradually shifted towards lower temperatures and the peak area decreased monotonically with the increase of either additive concentration) (See Table 1 for details). However, it should be noted that within the concentration range between 0–20% of an additive the calorimetric enthalpies of OVA in DMSO solutions exceed that of the urea solutions, while the $\Delta H_{cal}$ values for the solutions containing 30% DMSO dropped lower compared to the 30% urea solution. Urea is known to be a nonspecific denaturant, thereby increasing the flexibility of the protein globule and eventually causing destabilizing effects [39,41,42]; alternatively, since DMSO is a nonspecific affecter, it has been shown to exhibit either a stabilizing or destabilizing action, depending on the experimental conditions [27,29–31,43]. Thus, our results indicate that in the mildly acidic media (pH 6.1), DMSO acts as a conventional denaturant, causing destabilization of the OVA globule in the whole range of DMSO concentrations.

These results are opposite to that acquired in our study of OVA within the mildly alkaline DMSO buffered solutions [44]. The thermodynamic parameters obtained from the thermal melting (unfolding) curves of OVA at pH 8 in the presence of similar DMSO concentrations indicate a notable increase of both, the melting temperature and the enthalpy, for the 5% DMSO solution. Moreover, in the presence of 10% DMSO (pH 8), the calorimetric enthalpy and the transition temperature are comparable to that of the reference solution with no DMSO added (Figure 2 and 3). However, a further increase of DMSO concentrations leads to a gradual decrease of both thermodynamic parameters. However, for the

solutions containing 0–30% of urea, the calorimetric plots depict a gradual destabilization of the protein globule comparable to that of the pH 6.1 series, which means that both the melting temperature $T_m$ and the calorimetric denaturation enthalpy $\Delta H_{cal}$ decreased gradually within the whole range of urea concentrations. Thus, within the applied concentration range of additives, the experimental data indicate a similar denaturizing impact of urea at both an acidic and alkaline pH, and a diverse impact of DMSO at an alkaline pH, differing by the moderate stabilization of OVA in the presence of low concentrations of DMSO.

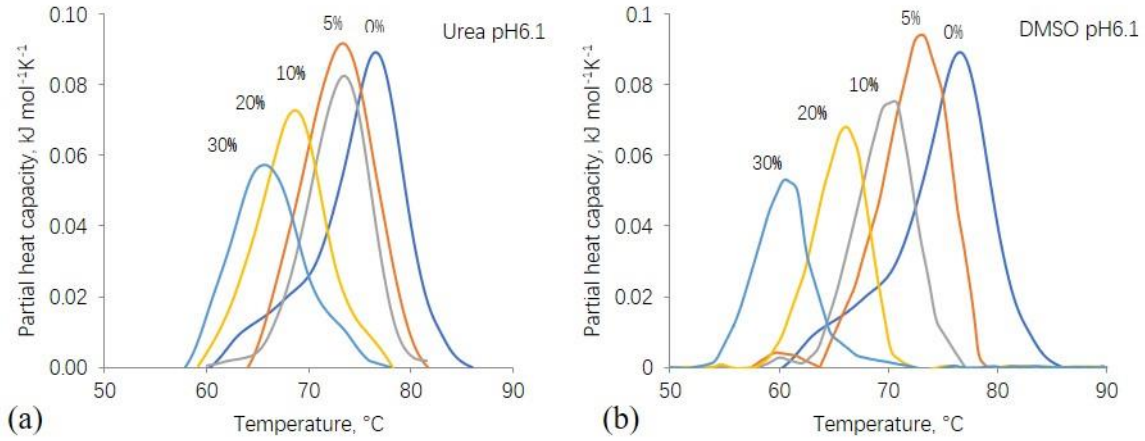


**Figure 1.** DSC curves for the OVA thermal unfolding in the presence of 0–30% urea (a) and DMSO (b) concentrations, 0.1 M phosphate buffer pH 6.1.

**Table 1.** Calorimetric enthalpy and melting temperature for the thermal unfolding of the OVA in the presence of 0–30% additive concentrations, 0.1 M phosphate buffer pH 6.1 and pH 8 [44].

| Additive concentration, % | Urea, pH 6.1 | | DMSO, pH 6.1 | | Urea, pH 8 [44] | | DMSO, pH 8 [44] | |
|---|---|---|---|---|---|---|---|---|
| | $\Delta H_{cal}$ (kJ/Mol) | $T_m$, °C | $\Delta H_{cal}$ (kJ/Mol) | $T_m$, °C | $\Delta H_{cal}$ (kJ/Mol) | $T_m$, °C | $\Delta H_{cal}$ (kJ/Mol) | $T_m$, °C |
| 0 | 287 | 77 | 287 | 77 | 243 | 74 | 243 | 74 |
| 5 | 240 | 74 | 262 | 73 | 204 | 72 | 285 | 75 |
| 10 | 191 | 73 | 219 | 71 | 191 | 70 | 239 | 73 |
| 20 | 163 | 69 | 170 | 66 | 173 | 68 | 174 | 67 |
| 30 | 154 | 65 | 116 | 61 | 169 | 65 | 131 | 62 |

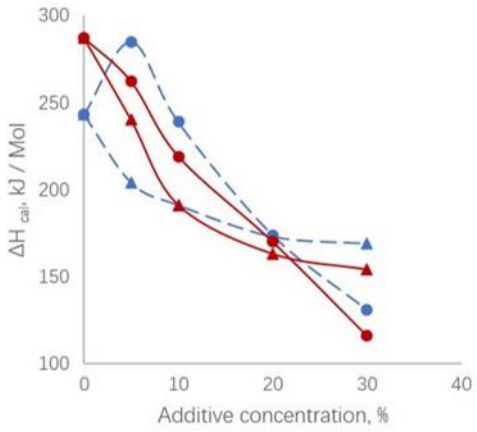


**Figure 2.** Dependencies of calorimetric enthalpies of thermally induced denaturation of ovalbumin on the DMSO concentration at pH 6.1 (red lines) compared with pH 8 (blue lines) [44]. Triangles represent urea and circles–DMSO.

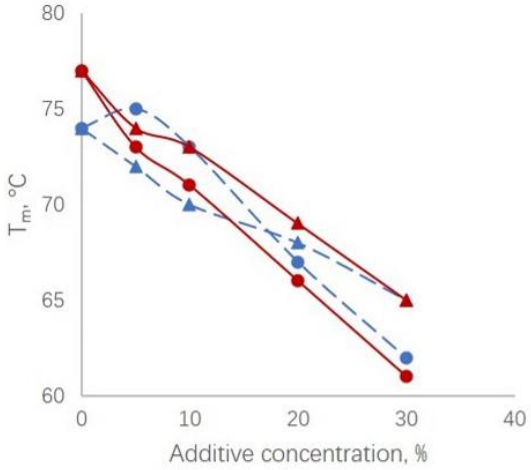


**Figure 3.** Dependencies of transition temperatures of thermally induced denaturation of ovalbumin on the DMSO concentration at pH 6.1 (red lines) compared with pH 8 (blue lines) [44]. Triangles represent urea and circles–DMSO.

The presented results are consistent with our previous findings on the impact of urea [39] and DMSO [43] on the stability and conformational flexibility of another model globular protein, α-chymotrypsin (α-CT). These studies were also carried out in acidic and alkaline phosphate buffer solutions in presence of a wide range of urea (0-6M) and DMSO concentrations (0–70% vol.). Calorimetric parameters obtained for α-chymotrypsin displayed a similar behavior of the protein; namely, in alkaline DMSO solutions (pH 8.1), both the transition temperature $T_m$ and calorimetric enthalpy of thermal unfolding $\Delta H_{cal}$ increase in presence of low DMSO concentrations (5–30% vol.), thereby indicating protein globule stabilization with a soft maximum around 20% DMSO. A further increase of the DMSO concentration was followed by a gradual decrease of both thermodynamic parameters and resulted in the total destabilization of the protein in the presence of 70% DMSO. However, in acidic buffered solutions of DMSO (pH 2.6), as well as in both acidic and alkaline urea

solutions, α-chymotrypsin was shown to undergo monotonous destabilization through the $T_m$ within the whole range of additives concentrations (0–70%), while still retaining an enthalpic stabilization in acidic DMSO solutions [39,43].

It is noteworthy that while global stabilization of α-chymotrypsin exhibited a smooth maximum of protein stability around 20% DMSO, the enzymatic activity of α-chymotrypsin exhibited a monotonic decrease within the whole range of additive concentrations [43]. This effect can be explained by the different distribution of solvent-exposed hydrophobic amino acids within the active site and the surface of α-CT; thus, partially hydrophobic DMSO molecules can potentially cause local destabilization of the protein prior to the global destabilization of the entire globule. On the other hand, the relatively small area of the active site of α-chymotrypsin is characterized by an intrinsic flexibility, which is crucial for the substrate recognition and its further hydrolysis. Consequently, it is not expected to make a quantitatively significant enthalpic contribution into the global stability of the protein globule.

A computational study of Roy et al., which is consistent with a large number of experimental studies [45], has also reported a stabilizing effect of low concentrations of aqueous solutions of DMSO on lysozyme. It has been shown that conformational fluctuations of the protein globule are rather confined around 5–20% DMSO compared to the native state (0% DMSO), resulting in the formation of more compact protein conformations. However, in contrast with the functional kinetic study of chymotrypsin [43], the enzymatic activity of lysozyme was increased in presence of low DMSO concentrations. Unfortunately, in case of OVA, it is not possible to explore local destabilization patterns to compare with that of either the lysozyme or α-CT, because OVA is not capable of inhibiting serine proteases due to minor structural differences within the active site area [6,10–12] and no other active function of OVA is well described within the literature.

According to computational and experimental studies impact of urea and DMSO the on the stability of globular proteins can be explained by preferential solvation patterns [27,39,43–48]. Considering the nonspecific interaction of both additives with a protein globule, the either stabilizing or destabilizing effects occur due to multipoint weak interactions of the additive molecules with the protein amino acid residues and/or interfacial water. The positive preferential solvation of proteins by urea in urea/water mixtures mainly weakens hydrophobic interactions of the protein globule within the whole range of concentrations and eventually leads to the disruption of the protein tertiary structure [41,42,47,48]. However, in DMSO/water mixtures under certain experimental conditions, the positive preferential solvation by water molecules can be observed [45,46]. In the presence of low concentrations of DMSO, the latter can strengthen hydrogen bonds of interfacial water molecules and raise the compactness of the protein globule. This effect is common for a number of organic additives such as glycerol, TMAO and sugars that stabilize globular proteins, mainly via the confinement of water molecules in the protein hydration shell [47]. It is noteworthy that mainly hydrophobic protein-DMSO interactions may provide a chaperone effect and protect some proteins from aggregation depending on the environmental conditions and intrinsic properties of the protein. As shown earlier, DMSO molecules primarily interact with the hydrophobic residues of proteins, which are mainly hidden inside the core of the native protein, but are largely presented on the surface of the molten-globule-like conformations. In fact, our study of the thermal unfolding of α-chymotrypsin revealed a remarkable chaperone effect of DMSO. Although partial refolding of α-CT at pH 2.6 was observed even in the total absence of DMSO additives, the repetitive reversibility of the protein thermal unfolding only occurred in 20% DMSO solutions (pH 2.6). Namely, the refolding ability of α-CT was raised to 75%, as compared to 54% (0% DMSO), thereby displaying a multifold total repeatability

for 24 hours [43].

Although the positive preferential solvation by water (thus, negative preferential solvation by DMSO) does equate to the total distancing of DMSO molecules from the protein interface, the further increase of DMSO concentration (20–30% DMSO or more) leads to the transition of preferential solvation in favor of DMSO, thereby causing a decrease of protein compactness, the reveal of a hydrophobic core and a drastic increase in the number of DMSO interactions with protein hydrophobic groups [45,46,48]. This leads to the destabilization of α-helices and eventually results in the total denaturation of the protein at higher DMSO concentrations [49]. The transition of the preferential solvation of OVA in buffered DMSO solutions is visualized in a rather noticeable drop of enthalpy when compared to a more gradual enthalpy decrease observed in presence of urea solutions at both pH values (Table 1). We assume that the soft maximum of DMSO/water preferential solvation pattern could be shifted towards the lower concentrations of the additive when compared to α-chymotrypsin due to the intrinsic ability of ovalbumin to perform an irreversible conversion to the specific thermo-stable conformer, S-ovalbumin. Despite being more compact compared to the native form, S-OVA form has exceptionally flexible intermediate conformations with increased surface hydrophobicity, which, in general, is characteristic to partially unfolded molten-globule-like states [10–14,16,19,20]. Thus, the destabilization of OVA via direct hydrophobic interactions with DMSO molecules, could start in the presence of lower DMSO concentrations.

According to a comparative computational study of solvation patterns of the conventional denaturant urea with the conventional stabilizer TMAO in water-additive mixtures, in presence of TMAO, an extra hydration shell is observed near hydrophobic groups; alternatively, in urea solutions, the urea-urea and water-water association is found to be more favored than the urea-water association [50]. The addition of TMAO displayed an increase in the strength and the hydrogen bond lifetime within the solution. Moreover, the computations revealed that near the interface of amino acids (i.e., near the protein surface), water molecules are preferentially favored over the other co-solvents [50]. Thus, the stabilizing effect of TMAO occurs due to an increase of the hydration shell around the protein, especially around the hydrophobic groups of the biomolecules, and due to strengthening the hydrogen bond network of the solution. However, the destabilizing effect of urea manifests itself in a disruption of the protective interfacial water layers and decreasing the overall interaction between the amino acid residues and the solvent molecules.

Considering that DMSO and TMAO are both small dipolar compounds containing hydrophobic methyl groups and an oxygen atom capable of forming hydrogen bonds with water molecules (within the S=O and N–O groups, respectively), it is safe to assume that they could also share some similarities in their mechanisms of protein stabilization. A combined experimental and theoretical study of the interaction of TMAO and DMSO with a K-peptide, which is a fragment of hen egg white lysozyme [51], confirmed that both TMAO and DMSO prefer interactions with water molecules in diluted solutions. However, in presence of higher additive concentrations, the amount of water-TMAO interactions gradually increase and lead to a stronger TMAO hydration, while the hydration of DMSO weakens, which gives rise to the differences in the protein-additive interaction.

## 4. Conclusions

We used DSC to study the impact of two nonspecific organic compounds, urea, which is a conventional denaturant, and DMSO, which is a multilateral organic solvent, on the conformational

dynamics of OVA. Experimental results obtained in phosphate buffer solutions containing 0–30 % of additives revealed the destabilizing impact of both urea and DMSO in a mildly acidic media, as manifested in the gradual decrease of the thermal unfolding enthalpy and the transition temperature.

These results substantially differ from the calorimetric parameters observed in our study of OVA within mildly alkaline buffered solutions of DMSO, where the moderate stabilization of OVA was observed in presence 5–10% of DMSO. However, the overall pattern of the OVA interaction with urea and DMSO is consistent with our previous findings on the stability and conformational flexibility of another model globular protein, α-chymotrypsin, in similar medium conditions. Thus, our findings on the OVA stability could also be explained by preferential solvation patterns. The positive preferential solvation of a protein by urea in urea/water mixtures mainly weakens the hydrophobic interactions of the protein globule and eventually leads to the disruption of the tertiary structure within the whole range of urea concentrations. However, in DMSO/water mixtures, the positive preferential solvation by water molecules can be observed under certain experimental conditions. Low concentrations of DMSO remotely strengthen the bond water networks of the protein, thereby protecting it from unfolding, whereas a subsequent increase of DMSO concentration leads to a switch of preferential solvation and the gradual destabilization of the protein.

## Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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## Conflict of interest

The authors declare no conflict of interest.

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