Lysozyme in water-acetonitrile mixtures: Preferential solvation at the inner edge of excess hydration

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Preferential solvation/hydration is an effective way for regulating the mechanism of the protein destabilization/stabilization. Organic solvent/water sorption and residual enzyme activity measurements were performed to monitor the preferential solvation/hydration of hen egg-white lysozyme at high and low water content in acetonitrile at 25 °C. The obtained results show that the protein destabilization/stabilization depends essentially on the initial hydration level of lysozyme and the water content in acetonitrile. There are three composition regimes for the dried lysozyme. At high water content, the lysozyme has a higher affinity for water than for acetonitrile. The residual enzyme activity values are close to 100%. At the intermediate water content, the dehydrated lysozyme has a higher affinity for acetonitrile than for water. A minimum on the residual enzyme activity curve was observed in this concentration range. At the lowest water content, the organic solvent molecules are preferentially excluded from the dried lysozyme, resulting in the preferential hydration. The residual catalytic activity is ~80%, compared with that observed after incubation in pure water. Two distinct schemes are operative for the hydrated lysozyme. At high and intermediate water content, lysozyme is preferentially hydrated. However, in contrast to the dried protein, at the intermediate water content, the initially hydrated lysozyme has the increased preferential hydration parameters. At low water content, the preferential binding of the acetonitrile molecules to the initially hydrated lysozyme was detected. No residual enzyme activity was observed in the water-poor acetonitrile. Our data clearly show that the initial hydration level of the protein macromolecules is one of the key factors that govern the stability of the protein-water-organic solvent systems. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4984116>]

I. INTRODUCTION

Organic solvents are widely utilized in biochemical physics, biotechnology, and biomedicine to selectively modulate the protein properties. In particular, organic solvents may stabilize the partially folded conformations of proteins (amyloid fibrils and molten globules).^{1–5} These protein states may be responsible for the numerous debilitating diseases such as Parkinson's disease, type II diabetes, and Alzheimer's disease.

Protein-water interactions play a key role in determining the structure, functions, and stability of the enzyme molecules in the presence and absence of organic solvents.^{6–14} Enzyme activity is an intricate function of the water content in organic liquids. The typical functions of the enzyme activity on the water concentration in organic liquids can be delineated into three parts:^{15–17}

- (a) The mixtures with high water content constitute the first concentration region. One can observe hydrolytic activity in this range. However, numerous industrially important reactions including transesterification and peptide synthesis are suppressed in aqueous solutions as a result of the unfavorable shift of reaction equilibria.

- (b) A sharp decline in the enzymatic activity was observed after a certain threshold concentration of the organic solvent had been reached. The position of this minimum depends on the physicochemical properties of the solvent.¹⁶ Organic solvents may perturb the protein structure by altering the electrostatic interactions of the polar protein groups, by direct interaction with the biocatalysts, or through weakening of the hydrophobic interactions.
- (c) The third concentration range corresponds to the water-poor mixtures. The dried enzymes are in a glassy-like state at low water content.^{6,13,14,18,19} Due to the reduced conformational flexibility in organic solvents with low water content, the enzymes remain in the active conformation. There are numerous advantages in employing nonaqueous organic liquids, including the catalysis of the industrially important synthetic reactions (peptide synthesis and transesterification), the suppression of undesirable side reactions caused by water, the high solubility of hydrophobic reagents, and the enhanced thermostability.^{15–20}

Preferential solvation/hydration is an effective way for revealing the mechanism of the protein destabilization/stabilization in water-organic mixtures.^{21–30} Organic solvent and water molecules exist preferentially in the protein solvation shell. This difference in the solvent components

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between the solvation shell and bulk solvent has been described as preferential solvation.^{21–30} Preferential solvation is a thermodynamic quantity that describes the protein macromolecule occupancy by the organic solvent and water molecules. This is related to the actual numbers of organic solvent/water molecules in contact with the protein's surface.^{22,23,30} Preferential hydration is the excess of water at the protein surface relative to the water content in the bulk solvent. The preferential hydration does not always stabilize the native proteins.³⁰ The preferentially hydrating solvent systems can be divided into two groups. The first group always stabilizes the protein structure. The dominant interaction in the first group is the organic component exclusion. The protein remains essentially inert. The preferential interactions in the second group are determined by the chemical nature of the protein surface. This gives rise to a precise balance between the binding and exclusion of the organic solvent. The preferential binding depends strongly on the chemical nature of the organic solvent/water interface.³¹ The protein may adsorb and unfold at this interface. The exposure of the additional protein hydrophobic groups can be enhanced by the protein unfolding.³¹

The aim of our study is to simultaneously monitor the preferential solvation/hydration of the protein molecules at high and low water content in organic liquids at 25 °C. Our approach is based on the analysis of the organic solvent/water sorption and residual enzyme activity data. One of the most important advantages of our approach is the determination of the preferential interaction parameters in the entire range of water content in organic liquids.

Hen egg-white lysozyme was used as a model protein. This protein is one of the most applied and studied in biophysical and biotechnological investigations.^{32,33} Lysozyme is a small monomeric protein of 129 amino acid residues. The physiological role of lysozyme is to hydrolyze polysaccharide chains.^{32,33}

The choice of acetonitrile (AN) was determined by the following reasons:

- (a) Acetonitrile (AN) is a water-miscible organic solvent. Therefore, the effect of this low molecular weight substance on the hydration and functions of lysozyme can be studied in the entire range of water content.
- (b) Acetonitrile is able to form hydrogen bonds with various hydrogen donors. In contrast to water, however, it has no hydrogen bond donating ability.

II. EXPERIMENTAL

A. Materials

A hen egg-white lysozyme (EC 3.2.1.17; crystallized three times, dialyzed, and lyophilized) and dried *Micrococcus lysodeikticus* cells were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The molecular weight of the protein was taken as 14 300 Da. Acetonitrile (analytical grade, purity >99%) was purified and dried according to the recommendations.³⁴ Water used was doubly distilled. All water-organic mixtures were prepared gravimetrically using a Precisa balance (Swiss) with a precision of 0.000 01 g.

B. Initial protein states

1. Dried protein

The lysozyme powder was placed on the thermostated cell as shown in Fig. 1(a) and dried using a microthermoanalyzer “Setaram” MGD TD-17S ($\pm 0.000\,01$ g) at 25 °C and 0.1 Pa, until a constant sample weight was reached as shown in Fig. 1(a). The dried protein's water content was estimated as 0.002 ± 0.001 g water g⁻¹ protein using the Karl Fischer titration method, according to recommendations [Fig. 1(a)].³⁵ This value for lysozyme implies that at the **zero hydration level**, there are about two water molecules that strongly bound to each protein molecule.

2. Hydrated protein

The hydrated protein preparation was obtained by adding 50 mg of pure water to 10 mg of the dried lysozyme.

C. Organic solvent and water sorption measurements

The lysozyme samples were prepared as described previously.³⁶ The initially dehydrated as prepared in Fig. 1(a) or hydrated lysozyme samples were presented to water-organic vapor mixtures. The water-organic vapor mixture was flowed consecutively through a thermostated saturator filled with the water-organic mixture and a cell containing the lysozyme sample. Protein samples (7–10 mg) each were flushed by water-organic vapor mixtures until no further mass changes were detected as described previously.³⁶ Typically, the sorption equilibrium was reached after 6 h at 25 °C. Measurements of the protein-bound water (A_1) were conducted by the Karl Fischer titration with a Metrohm 831 KF coulometer. Organic solvent content of lysozyme (A_3) was calculated as the difference between the total sorption uptake ($A_1 + A_3$) and water

FIG. 1. Schematic presentation of the experimental setup of the sorption measurements. The components of the experimental setup are as follows: (a) 1, air pump; 2, thermostated glass tube with P_2O_5 ; 3, microthermoanalyzer “Setaram” MGD TD-17S; 4, thermostated cell; 5, Karl Fischer titrator. (b) 1, air pump; 2, thermostated glass tube with P_2O_5 ; 3, microthermoanalyzer “Setaram” MGD TD-17S; 4, thermostated cell; 5, Karl Fischer titrator; 6, thermostated saturator; 7, water-organic mixture.

content (A_1). The total sorption uptake ($A_1 + A_3$) was measured by microthermoanalyzer “Setaram” MGD TD-17S. Figure 1 presents the schematic representation of the experimental setup. The water activity (a_1) in the vapor phase was adjusted by altering the water content in the liquid water-acetonitrile mixture.

D. Residual enzyme activity

Residual enzyme activity was determined by measuring the enzyme activity after storage in water-organic mixtures as described previously.^{37,38} The lysozyme activity was determined as follows. The dried/hydrated lysozyme was immersed in an aqueous-organic mixture of required composition and was incubated at 25 °C for 3 h. This time period exceeded the time corresponding to the completion of the calorimetric heat effect accompanying the interaction of the dehydrated proteins with pure organic solvents and water-organic mixtures.^{35,39} The concentration of lysozyme in the water-organic mixtures was 1 mg/ml. Adding 100- μ l aliquots of the lysozyme solution in the water-rich acetonitrile (or the lysozyme suspension in the water-poor acetonitrile) to the aqueous solution of the substrate [*Micrococcus lysodeikticus* cells (2.9 ml, 0.3 mg/ml) in 0.1M potassium phosphate pH 7.0], we initiated the enzymatic reaction. Change in the absorbance at 450 nm was recorded using a Perkin-Elmer Lambda 35 double-beam scanning spectrophotometer. The reaction was followed for 300–1500 s. Each kinetic curve was reproduced not less than three times.

III. RESULTS AND DISCUSSION

A. Water and organic solvent sorption

Figure 2 presents the water (A_1) and organic solvent (A_3) vapor sorption isotherms for the dried and hydrated lysozyme at 25 °C. The acetonitrile and water sorption depends markedly on the initial hydration level of lysozyme. Three distinct effects were identified as follows:

- At high water content (water mass fraction in acetonitrile, $w_1 = 0.9$ – 1.0), the A_1 and A_3 values are similar for the dried and hydrated protein.
- At the intermediate water content, at a given w_1 , the A_1 values are higher for the initially hydrated lysozyme. The A_3 values are higher for the dried lysozyme.
- At low water content ($w_1 < 0.1$), the water sorption is lower for the initially hydrated lysozyme. The A_3 values are higher for the dried lysozyme.

B. Methodology. Excess functions

The protein solvation shell is composed of two parts: (i) nonideal (due to preferential solvation/hydration) and (ii) ideal. The nonideal effect of the solvation shell on the protein properties (residual enzyme activity and water and organic solvent sorption) can be expressed in terms of the excess functions, F^E ,^{40–42} i.e., the difference between the observed mixing function, F^M , and the function for an ideal binary mixture, F_{id}^M .

FIG. 2. (a) Water (A_1) sorption isotherms for lysozyme at 25 °C: 1, dried lysozyme; 2, hydrated lysozyme. (b) Organic solvent (A_3) sorption isotherms for lysozyme at 25 °C: 1, acetonitrile sorption by the dried lysozyme; 2, acetonitrile sorption by the hydrated lysozyme. The standard errors of estimation of the water/organic solvent sorption were 0.001–0.002 g/g. Each experiment was performed 3–4 times.

Deviations of the excess functions from zero indicate the extent to which the solvation shell differs from the pure binary water-organic system due to preferential interactions between water (component 1), protein (component 2), and organic solvent (component 3).

The F^E values were calculated using Eq. (1),

$$F^E = F^M - F_{id}^M. \quad (1)$$

The $F_{id,i}^M$ values can be calculated using Eq. (2),

$$F_{id,i}^M = F_i^M(w_i = 0) + w_i[F_i^M(w_i = 1.0) - F_i^M(w_i = 0)], \quad (2)$$

where $F_i^M(w_i = 1.0)$ is the observed mixing function of lysozyme at $w_i = 1.0$; $F_i^M(w_i = 0)$ is the observed mixing function of lysozyme at $w_i = 0$; w_1 is the water mass fraction in the binary water-organic mixtures; and w_3 is the organic solvent mass fraction in the binary water-organic mixtures ($w_1 + w_3 = 1.0$).

The $F_{id,i}^M$ values describe the situation when there are no preferential interactions between water, lysozyme, and organic solvent. In this case, the water mass fraction in the ideal part of the protein solvation shell is the same as in the pure water-organic mixture.

C. Excess sorption

Figure 3 presents the Z_1^M (water mass fraction in the lysozyme solvation shell) and Z_3^M (organic solvent mass fraction in the lysozyme solvation shell) values as a function of water mass fraction. The Z_1^M and Z_3^M values were calculated using Eqs. (3) and (4),

$$Z_1^M = \frac{A_1}{A_1 + A_3}, \quad (3)$$

$$Z_3^M = \frac{A_3}{A_1 + A_3}. \quad (4)$$

The simultaneous action of acetonitrile and water was characterized by the Z_1^E and Z_3^E values (Fig. 4). These excess sorption functions were calculated using Eqs. (5) and (6),

$$Z_1^E = Z_1^M - Z_{id,1}^M, \quad (5)$$

$$Z_3^E = Z_3^M - Z_{id,3}^M, \quad (6)$$

where Z_1^M is the mass fraction of water in the solvation layer for the real water-organic mixture. $Z_{id,1}^M$ is the mass fraction of water in the solvation layer for the ideal water-organic mixture. The $Z_{id,1}^M$ values were calculated using Eq. (7),

$$Z_{id,1}^M = Z_1^M(w_1 = 0) + w_1[Z_1^M(w_1 = 1.0) - Z_1^M(w_1 = 0)], \quad (7)$$

where $Z_1^M(w_1 = 1.0)$ is the water mass fraction in the solvation shell of lysozyme at $w_1 = 1.0$; $Z_1^M(w_1 = 0)$ is the water mass fraction in the solvation shell at $w_1 = 0$; and w_1 is the mass fraction of water in organic solvent.

Z_3^M is the acetonitrile mass fraction in the solvation shell for the real water-organic mixture; $Z_{id,3}^M$ is the organic solvent mass fraction for the ideal water-acetonitrile mixture. The $Z_{id,3}^M$ values can be calculated using Eq. (8),

$$Z_{id,3}^M = Z_3^M(w_3 = 0) + w_3[Z_3^M(w_3 = 1.0) - Z_3^M(w_3 = 0)], \quad (8)$$

where $Z_3^M(w_3 = 0)$ is the organic solvent mass fraction in the solvation shell of lysozyme at $w_3 = 1.0$; $Z_3^M(w_3 = 0)$ is the

FIG. 3. 1—Water mass fraction in the solvation layer of the dried lysozyme (Z_1^M); 2—organic solvent mass fraction in the solvation layer of the dried lysozyme (Z_3^M); 3—water mass fraction in the solvation layer of the hydrated lysozyme (Z_1^M); 4—organic solvent mass fraction in the solvation layer of the hydrated lysozyme (Z_3^M).

FIG. 4. 1—Excess water mass fraction in the solvation layer of the dried lysozyme (Z_1^E); 2—excess organic solvent mass fraction in the solvation layer of the dried lysozyme (Z_3^E); 3—excess water mass fraction in the solvation layer of the hydrated lysozyme (Z_1^E); 4—excess organic solvent mass fraction in the solvation layer of the hydrated lysozyme (Z_3^E).

organic solvent mass fraction in the solvation shell of lysozyme at $w_3 = 0$; and w_3 is the mass fraction of organic solvent in the binary water-acetonitrile mixture.

1. Dried lysozyme

As concluded from Fig. 4, the Z_1^E values are positive at high ($w_1 = 0.9-1.0$) and low ($w_1 = 0-0.2$) water content. A considerable decline in the water sorption was found in the intermediate range of water content. The Z_1^E values are negative in this concentration region. The most pronounced suppression was observed in the water mass fraction range from 0.5 to 0.8. On the other hand, the Z_3^E values are positive in the intermediate range.

2. Hydrated lysozyme

As shown in Fig. 4, the Z_1^E values are positive at high ($w_1 = 0.9-1.0$) and intermediate ($w_1 = 0.3-0.9$) water content. The most pronounced increase in the water content was found at $w_1 \sim 0.5$. However, the Z_1^E values are negative at low water content ($w_1 = 0-0.2$).

D. Residual enzyme activity

Figure 5(a) shows typical kinetic curves for the enzymatic reaction catalyzed by the dehydrated and hydrated lysozyme preliminary incubated in water-acetonitrile mixtures. The catalytic activity was characterized by the ratio of the extent of hydrolysis attained within 300 s with lysozyme incubated in a water-organic mixture to the same quantity measured using lysozyme incubated in pure water [Fig. 5(a), curve 1].

The residual activity values are presented in Fig. 5(b). As concluded from Fig. 5(b), AN affects the catalytic activity of the hydrated and dried enzyme in a complicated way.

1. Dried lysozyme

At high water content ($w_1 \sim 0.9-1.0$), the residual activity values are close to 100%. At $w_1 < 0.9$, there is a sharp

FIG. 5. (a) Typical kinetic curves for the enzymatic reaction catalyzed by the dried and hydrated lysozyme previously incubated in water-acetonitrile mixtures. Dried lysozyme. Water mass fraction in AN: (1) 1.0, (2) 0.76, (3) 0.49, (4) 0.4. Hydrated lysozyme. Water mass fraction in AN: (5) 0.39; (6) 0.23. (b) Residual activity of lysozyme in water-AN mixtures: 1, dried lysozyme; 2, hydrated lysozyme. All values are the averages of three measurements. Experimental errors were 1%-1.5%.

transition from the water-rich region to the intermediate one. The residual catalytic activity of lysozyme changes from 100% to 0% in the transition region. A minimum on the residual activity curve was observed at w_1 of ~ 0.5 in AN.

At $w_1 < 0.4$, the residual catalytic activity increases. At low water content in AN, the residual catalytic activity remains virtually constant, equal to $\sim 80\%$ - 85% compared with that observed after incubation in pure water.

2. Hydrated lysozyme

At high and intermediate water content ($w_1 \sim 0.4$ - 1.0), the residual activity values are close to 90%-100%. At $w_1 = 0.2$ - 0.4 , there is a sharp transition from the water-rich region to the water-poor one. The residual catalytic activity of the hydrated lysozyme changes from 100% to 0% in the transition region. At $w_1 < 0.1$, the residual catalytic activity is close to zero.

E. Excess residual enzyme activity

The effect of the excess hydration (Z_1^E) on the residual enzyme activity was characterized by the R_1^E values (excess residual enzyme activity). The R_1^E values were calculated using Eq. (9),

$$R_1^E = R^M - R_{id,1}^M, \quad (9)$$

where R^M is the observed residual enzyme activity; $R_{id,1}^M$ is the function for an ideal binary mixture.

The R_{id}^M values were calculated using Eq. (10),

$$R_{id,1}^M = R^M(w_1 = 0) + w_1[R^M(w_1 = 1.0) - R^M(w_1 = 0)], \quad (10)$$

where $R^M(w_1 = 1.0)$ is the observed residual activity of lysozyme at $w_1 = 1.0$; $R^M(w_1 = 0)$ is the observed residual activity of lysozyme at $w_1 = 0$; w_1 is the water mass fraction in acetonitrile; and w_3 is the acetonitrile mass fraction in the binary water-organic mixtures.

Figure 6 presents the dependencies of the R_1^E values on the water mass fraction in acetonitrile. In ideal binary mixtures [mixtures of two components, W (water) and S (organic solvent)], the average W-S interactions in the solvation shell are the same as the average W-W and S-S interactions in the bulk solvent. Nonideal mixtures are composed of particles for which the W-W, S-S, and W-S interactions are all different. As shown in Fig. 6, the R_1^E values differ significantly from zero, indicating that the effect of the water-organic solvation layer on the residual enzyme activity is nonideal in the entire range of water content. It is worth noting that the R_1^E values are consistent with the Z_1^E values (Fig. 6). Three different concentration regimes were observed for the dried lysozyme [Fig. 6(a)]:

- At $w_1 > 0.8$, the R_1^E and Z_1^E values are positive.
- At the intermediate water content ($w_1 = 0.3$ - 0.8), the R_1^E and Z_1^E values are negative. Acetonitrile augments

FIG. 6. (a) 1—Excess water mass fraction in the solvation layer of the dried lysozyme (Z_1^E); 2—excess residual activity of the dried lysozyme in water-AN mixtures; (b) 1—excess water mass fraction in the solvation layer of the hydrated lysozyme (Z_1^E); 2—excess residual activity of the hydrated lysozyme in water-AN mixtures.

the irreversible inactivation of lysozyme in this region for the dehydrated protein.

- (iii) At low water content, the R_1^E and Z_1^E values are positive.

Two distinct regimes are operative for the hydrated lysozyme [Fig. 6(b)]:

- (i) The R_1^E and Z_1^E values are positive at intermediate and high water content ($w_1 = 0.2-1.0$).
(ii) At $w_1 < 0.1$, the R_1^E and Z_1^E values are negative. The acetonitrile-induced irreversible inactivation was found at low water content for the hydrated lysozyme.

F. Preferential interaction parameters

The preferential interaction parameters^{21–23} [Eqs. (11) and (12)] describe the extent to which the protein solvation shell differs from the pure binary water-organic system due to preferential interactions between water (component 1), protein (component 2), and organic solvent (component 3). The preferential solvation of lysozyme was estimated using Eq. (11),

$$(\partial g_3 / \partial g_2)_{T, \mu_1, \mu_3} = A_3 - \frac{w_3}{w_1} A_1, \quad (11)$$

where A_1 is the lysozyme hydration, expressed as gram water per gram lysozyme; A_3 is the binding of acetonitrile, expressed as gram AN per gram lysozyme; w_1 is the water mass fraction in water-acetonitrile mixtures; and w_3 is the mass fraction of AN in water-organic mixtures ($w_1 + w_3 = 1.0$).

The preferential hydration was characterized using Eq. (12),

$$(\partial g_1 / \partial g_2)_{T, \mu_1, \mu_3} = -\left(\frac{w_1}{w_3}\right)(\partial g_3 / \partial g_2)_{T, \mu_1, \mu_3}. \quad (12)$$

The preferential interaction parameters calculated using Eqs. (11) and (12) are presented in Figs. 7 and 8. To show the reliability of our findings, the $(\partial g_1 / \partial g_2)_{T, \mu_1, \mu_3}$ and $(\partial g_3 / \partial g_2)_{T, \mu_1, \mu_3}$ values for the hydrated lysozyme [Figs. 7(a) and 8(a)] were compared with the published data for lysozyme dissolved in water-acetonitrile mixtures.²³ As concluded from Figs. 7(a) and 8(a), our results and the previously published findings exhibited strong agreement.

Gibbs energies of the transfer of water (ΔG_1^{pref}) and AN (ΔG_3^{pref}) from water-acetonitrile mixtures to the protein solvation shell were calculated using Eqs. (13) and (14),

$$\Delta G_1^{pref} = \mu_1^E(\text{solvation shell}) - \mu_1^E(\text{binary mixture}), \quad (13)$$

$$\Delta G_3^{pref} = \mu_3^E(\text{solvation shell}) - \mu_3^E(\text{binary mixture}). \quad (14)$$

The $\mu_1^E(\text{binary mixture})$ and $\mu_3^E(\text{binary mixture})$ values were estimated using Eqs. (15) and (16),

$$\mu_1^E(\text{binary mixture}) = RT \ln \gamma_1(\text{binary mixture}), \quad (15)$$

$$\mu_3^E(\text{binary mixture}) = RT \ln \gamma_3(\text{binary mixture}). \quad (16)$$

Water activity coefficients [$\gamma_1(\text{binary mixture})$, the mass fraction scale; the reference state is pure water] in water-acetonitrile mixtures were estimated using Eq. (17),

FIG. 7. (a) The preferential hydration parameters as a function of water mass fraction in acetonitrile ($(\partial g_1 / \partial g_2)_{T, \mu_1, \mu_3}$): 1, dried lysozyme; 2, hydrated lysozyme; 3, adapted data from Ref. 23. (b) Gibbs energy of the transfer of AN (ΔG_3^{pref}) from binary water-organic mixtures to the solvation shell: 1, dried lysozyme; 2, hydrated lysozyme.

$$\gamma_1(\text{binary mixture}) = \frac{a_1}{w_1}. \quad (17)$$

Organic solvent activity coefficients [$\gamma_3(\text{binary mixture})$, the mass fraction scale; the reference state is pure acetonitrile] in water-organic mixtures were calculated using Eq. (18),

$$\gamma_3(\text{binary mixture}) = \frac{a_3}{w_3}. \quad (18)$$

Water activity (a_1) and acetonitrile activity (a_3) were taken from the published data^{43,44} based on the vapor-liquid equilibrium. Additional details of the organic solvent and water activity are presented as the [supplementary material](#).

The $\mu_1^E(\text{solvation shell})$ and $\mu_3^E(\text{solvation shell})$ values were estimated using Eqs. (19) and (20),

$$\mu_1^E(\text{solvation shell}) = RT \ln \gamma_1(\text{solvation shell}), \quad (19)$$

$$\mu_3^E(\text{solvation shell}) = RT \ln \gamma_3(\text{solvation shell}). \quad (20)$$

Water activity coefficients [$\gamma_1(\text{solvation shell})$, the mass fraction scale] in the solvation shell were calculated using Eq. (21),

$$\gamma_1(\text{solvation shell}) = \frac{a_1}{Z_1^M}, \quad (21)$$

where Z_1^M is the mass fraction of water in the lysozyme solvation shell; $Z_1^M = \frac{A_1}{A_1 + A_3}$.

Organic solvent activity coefficients [$\gamma_3(\text{solvation shell})$, the mass fraction scale] in the solvation shell were calculated using Eq. (22),

FIG. 8. (a) The preferential solvation parameters as a function of water mass fraction in acetonitrile $((\partial g_3/\partial g_2)_{T,\mu_1,\mu_3})$: 1, dried lysozyme; 2, hydrated lysozyme; 3, adapted data from Ref. 23. (b) Gibbs energy of the transfer of water (ΔG_1^{pref}) from binary water-organic mixtures to the solvation shell: 1, dried lysozyme; 2, hydrated lysozyme; 3, excess Gibbs energy of water bound to lysozyme.¹⁴ Reference state is pure liquid water at 25 °C.

$$\gamma_3(\text{solvation shell}) = \frac{a_3}{Z_3^M}, \quad (22)$$

where Z_3^M is the mass fraction of AN in the lysozyme solvation shell; $Z_3^M = \frac{A_3}{A_1+A_3}$.

The ΔG_1^{pref} and ΔG_3^{pref} values are presented in Figs. 7 and 8. As concluded from Figs. 7 and 8, the ΔG_1^{pref} and ΔG_3^{pref} values correlate well with the preferential interaction parameters.

The ΔG_1^{pref} value at $w_1 = 0$ [Fig. 8(b)] was compared with the excess Gibbs energy of water bound to the dried lysozyme.¹⁴ This Gibbs energy was obtained from the water sorption experiments in the absence of AN. As shown in Fig. 8(b), a good agreement was found between our data and the previously published results. This result constitutes evidence that our calculations are reliable.

The lysozyme destabilization/stabilization due to the preferential solvation/hydration was characterized by the ΔG_2^{pref} values (Fig. 9). The ΔG_2^{pref} values were calculated using the Gibbs-Duhem equation for ternary systems [Eq. (23)],

$$\Delta G_2^{pref} = \frac{m_1 \Delta G_1^{pref} + m_3 \Delta G_3^{pref}}{m_2}, \quad (23)$$

$$\Delta G_2^{pref} = \mu_2^E(\text{protein in water} - \text{organic mixtures}) - \mu_2^E(\text{pure protein}), \quad (24)$$

FIG. 9. Gibbs energy (ΔG_2^{pref}) of the transfer of lysozyme from pure state to the water-organic mixtures: 1, dried lysozyme; 2, hydrated lysozyme.

where μ_1^E , μ_2^E , and μ_3^E are the excess chemical potentials of water, lysozyme, and acetonitrile; m_1 , m_2 , and m_3 are the masses of water, dried or hydrated lysozyme, and AN.

G. Effect of the preferential interactions on the lysozyme activity and hydration

The enzyme activity and sorption experiments can be summarized as follows. Three composition regimes were observed for the dried lysozyme:

- (i) At high water content ($w_1 = 0.9-1.0$), lysozyme is in the native state. The $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ and R_1^E values are positive. On the other hand, the ΔG_1^{pref} , ΔG_2^{pref} , and $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ values are negative. Lysozyme has a higher affinity for water than for acetonitrile. Our conclusion is in agreement with the previously published results for the water-rich acetonitrile mixtures. From the experiments on the equilibrium dialysis,²³ it was observed that lysozyme is preferentially hydrated at room temperature.
- (ii) At the intermediate water content ($w_1 = 0.4-0.8$), the preferential binding of AN to the dehydrated lysozyme was detected. The ΔG_1^{pref} , ΔG_2^{pref} , and $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ values are positive. On the other hand, the excess residual enzyme activity (R_1^E) values are negative.
- (iii) The dehydrated proteins are in a glassy-like (rigid) state.^{6,7,13,14} At the lowest water content, the acetonitrile molecules are not effective in solvating the dehydrated lysozyme alone. The organic solvent molecules are preferentially excluded from the dried lysozyme. This results in the preferential hydration. Therefore, the $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ values are positive (Fig. 7) at the lowest water content. At $w_1 < 0.2$, the dried lysozyme retains the catalytic activity after incubation in the water-poor acetonitrile (Figs. 5 and 6). The residual catalytic activity is $\sim 80\%$, compared with that observed after incubation in pure water.

Two distinct schemes are operative for the hydrated lysozyme:

- (i) At intermediate and high water content, the ΔG_2^{pref} values are negative. A deficiency of acetonitrile exists near

the lysozyme surface relative to its bulk phase concentration. The residual enzyme activity values are close to 90%-100%.

- (ii) At low water content ($w_1 = 0-0.1$), the preferential binding of the acetonitrile molecules to the initially hydrated lysozyme was found. No residual enzyme activity was observed in the water-poor acetonitrile. Therefore, the ΔG_1^{pref} , ΔG_2^{pref} , and $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ values are positive. On the other hand, the ΔG_3^{pref} and $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ values are negative.

IV. CONCLUSIONS

We investigated the preferential interactions of hen egg-white lysozyme with water-acetonitrile mixtures. Our approach is based on the analysis of the residual enzyme activity and the absolute values of the acetonitrile/water sorption. The degree of destabilization/stabilization due to the preferential interactions depends essentially on the initial hydration level of lysozyme and the water content in acetonitrile:

Three concentration regions were observed for the dehydrated protein:

- (i) At high water content, lysozyme is in the preferentially hydrated state.
- (ii) At the intermediate water content, the dehydrated lysozyme has a higher affinity for AN than for water. The residual enzyme activity is minimal in this concentration range.
- (iii) At low water content, the acetonitrile molecules are preferentially excluded from the protein surface, resulting in the preferential hydration. Dried lysozyme shows a high residual catalytic activity in the water-poor acetonitrile.

Two different regimes were found for the hydrated lysozyme. At high and intermediate water content, lysozyme shows the positive preferential hydration. However, in contrast to the dried protein, the initially hydrated lysozyme has the increased preferential hydration parameters at the intermediate water content. At the lowest water content, the preferential binding of the acetonitrile molecules to lysozyme was observed. Our data clearly show that the initial hydration level of the protein macromolecules is one of the key factors that control the stability of the protein-water-organic solvent systems.

SUPPLEMENTARY MATERIAL

See the [supplementary material](#) for additional details of the organic solvent and water activity.

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