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Electrodynamic Pressure Modulation of Protein Stability in Cosolvents

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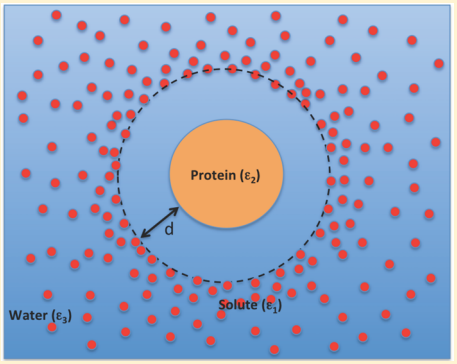
ABSTRACT: Cosolvents affect structural stability of proteins in aqueous solutions. A clear understanding of the mechanism by which cosolvents impact protein stability is critical to understanding protein folding in a biological milieu. In this study, we investigated the Lifshitz−van der Waals dispersion interaction of seven different solutes with nine globular proteins and report that in an aqueous medium the structure-stabilizing solutes exert a positive electrodynamic pressure, whereas the structure-destabilizing solutes exert a negative electrodynamic pressure on the proteins. The net increase in the thermal denaturation temperature (ΔT_d) of a protein in 1 M solution of various solutes was linearly related to the electrodynamic pressure (P_{vdW}) between the solutes and the protein. The slope of the P_{vdW} versus ΔT_d plots was protein-dependent. However, we find a positive linear relationship ($r^2 = 0.79$) between the slope (i.e., $d(\Delta T_d)/dP_{vdW}$) and the adiabatic compressibility (β_s) of the proteins. Together, these results clearly indicate that the Lifshitz's dispersion forces are inextricably involved in solute-induced stabilization/destabilization of globular proteins. The positive and/or negative electrodynamic pressure generated by the solute−protein interaction across the water medium seems to be the fundamental mechanism by which solutes affect protein stability. This is at variance with the existing preferential hydration concept. The implication of these results is significant in the sense that, in addition to the hydrophobic effect that drives protein folding, the electrodynamic forces between the proteins and solutes in the biological milieu also might play a role in the folding process as well as in the stability of the folded state.



Several small molecular weight solutes, such as sugars, polyhydric alcohols, urea, poly(ethyleneglycol), and certain amino acids, are known to affect the structural stability of globular proteins.[1−6] The fundamental mechanism involved in this process has not been satisfactorily resolved. At the molecular level, two opposing forces maintain the structural stability of globular proteins in aqueous solutions: the solvophobic force ($-T\Delta S_s$) that drives protein folding and the chain entropy ($-T\Delta S_c$) that tends to unfold the structure. The solvophobic force arises from the interaction of bulk water with hydrophobic groups in proteins. As a result of these opposing forces, the net stability of globular proteins is typically in the range of about 2−10 kcal/mol. A consequence of this delicate force balance is that any change in the magnitude of the solvophobic force will cause a corresponding change in the thermodynamic stability of the protein structure. On the basis of this premise, it has been proposed that solutes influence protein stability via causing alterations in bulk water structure, which in turn affects the strength of the solvophobic force.[5,7−9] Timasheff and co-workers have proposed that solute-induced alteration in water structure affects hydration of the protein surface.[4,5,10,11] According to this view, solutes that enhance hydrogen-bonded structure of water promote preferential hydration of proteins and thereby stabilize protein structure,[5,12] which by extension implies that solutes that breakdown water structure might destabilize protein structure by causing dehydration of the protein surface.

Although there is copious evidence showing that "structure making" solutes cause preferential hydration of proteins and "structure breaking" solutes tend to bind to the protein surface,[12,13] no correlation has been established between the extent of preferential hydration and protein stability. For instance, a critical examination of the data on preferential hydration values of lysozyme in aqueous solutions of various solutes and its thermal transition temperature[10] showed no correlation. Moreover, a pressure perturbation calorimetric study on water structure clearly demonstrated that there was no fundamental correlation between a solute's impact on water structure and its effect on protein stability.[14] Thus, the preferential hydration hypothesis cannot unambiguously explain a solute's effect on protein stability.

In a recent report, we have reasoned that in order for several chemically disparate classes of solutes to have an effect on protein stability the fundamental driving force involved in the phenomenon ought to be universal and it must be rooted in the three-body quantum electrodynamic interactions between the protein, water, and the solute in the system.[15] The three-body quantum electrodynamic interaction is best described by the Lifshitz−van der Waals theory.[16] In the previous report,[15] we showed that the structural stability of bovine serum albumin in aqueous solutions of various structure stabilizing and

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destabilizing solutes, as measured by changes in its thermal denaturation temperature (T_d), was linearly correlated with the Lifshitz–van der Waals electrodynamic (dispersion) interaction energy between the protein and the solutes across the water medium. The structure stabilizing solutes exhibited repulsive electrodynamic interaction, whereas the structure destabilizing solutes exhibited attractive electrodynamic interaction with the protein. In this three-body interaction, the solute's hydration shell apparently played an essential role as a modulator of these interactions.

In the present study, we investigated the Lifshitz–van der Waals electrodynamic interaction between seven structure stabilizing and destabilizing solutes and nine globular proteins. We report that a solute's impact on protein stability is directly related to the electrodynamic pressure exerted on the protein by dispersion interactions between the protein phase and the solute across the water medium. Additionally, the electrodynamic pressure-induced net change in thermal stability of globular proteins is dependent on their adiabatic compressibility.

BACKGROUND

According to the classical van der Waals theory, two molecules interact with each other in a medium when the dipole (electromagnetic) field created by quantum fluctuations in molecule 1 is reflected back by nearby molecule 2 that has been polarized by this field. It does not take into consideration the polarizability of the intervening medium. However, according to the Lifshitz's theory, when such interactions occur across a polarizable medium 3, such as water, the dipole field of molecule 1 also polarizes molecules in medium 3, and the dipole field of molecule 3 also acts on molecule 2, as shown schematically in Figure 1. These multiple electromagnetic

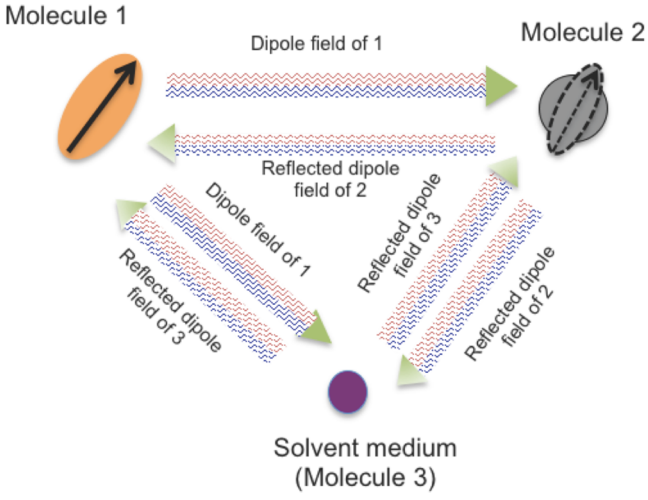

Figure 1. Schematic representation of Lifshitz–van der Waals electrodynamic interaction in a ternary system. The solid arrow denotes dipole. The dotted arrow indicates induced dipole.

reflections in a ternary system generate additional repulsive and attractive electrodynamic (dispersion) forces, depending on the frequency-dependent polarizability of the three components. On the basis of this Lifshitz theory of van der Waals interactions, a generalized formulation for interaction between components 1 and 2 in a polarizable medium 3 can be expressed as[16]

$$E_{vdw} = -\frac{C}{d^6} \quad (1)$$

where

$$C = \frac{6kT}{(4\pi\varepsilon_0)^2}\sum_{n=0}^{\infty}{}^{*}\frac{\alpha_1(i\xi_n)\alpha_2(i\xi_n)}{\varepsilon_3^2(i\xi_n)} \quad (2)$$

where k is the Boltzmann constant, T is the absolute temperature, ε_0 is the permittivity of free space, d is the distance between molecules 1 and 2, $\alpha_1(i\xi_n)$ and $\alpha_2(i\xi_n)$ are excess polarizabilities of molecules 1 and 2 at imaginary frequencies $i\xi_n$ (n = 0, 1, 2,...), respectively, and $\varepsilon_3(i\xi_n)$ is the dielectric permittivity of medium 3 at imaginary frequencies. The star on the summation (Σ^*) denotes that the zero frequency term is divided by 2.

McLachlan's[16,17] formulation of Lifshitz–van der Waals electrodynamic interaction has been successfully applied to explain the difference between the adsorption behavior of proteins at the air–water and oil–water interfaces.[18] A similar approach can be applied to understand the interaction between a solute (component 1) and protein (component 2) in water (medium 3), as depicted in Figure 2. In this model, a globular

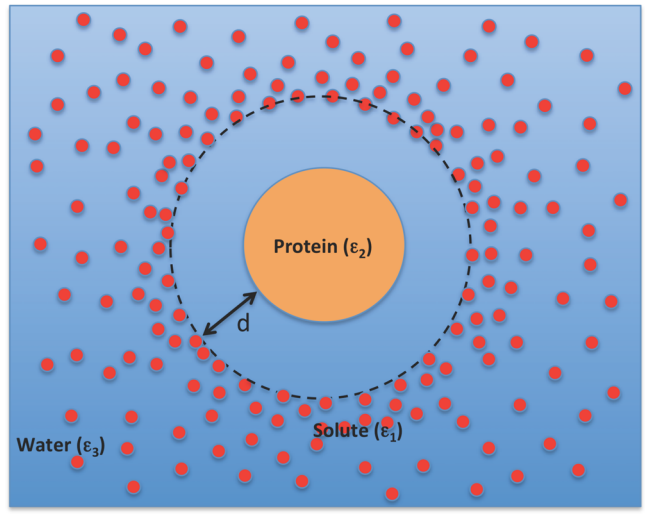

Figure 2. Schematic representation of three-body electrodynamic interactions between a protein (large orange circle), water (the blue region), and a solute (filled red circles). ε_1, ε_2, and ε_3 are dielectric permittivity of the solute, protein, and water, respectively; d is the surface-to-surface distance between the solute and the protein across the water medium.

protein with an enormous number of atoms buried in the interior can be treated as a condensed phase. If R_1 is the radius of the solute, its excess polarizability $\alpha_1(i\xi_n)$ at imaginary frequencies $i\xi_n$ in water is[15]

$$\alpha_1(i\xi_n) = \frac{3\varepsilon_0\varepsilon_3(i\xi_n)}{\rho_1}\Delta_{13} \quad (3)$$

where

$$\Delta_{13} = \left(\frac{\varepsilon_1(i\xi_n) - \varepsilon_3(i\xi_n)}{\varepsilon_1(i\xi_n) + 2\varepsilon_3(i\xi_n)}\right)$$

and $\varepsilon_1(i\xi_n)$ and $\varepsilon_3(i\xi_n)$ are the dielectric permittivity at imaginary frequencies $i\xi_n$ of solute and water, respectively, and $1/\rho_1$ is the volume of the solute molecule (= $4\pi R_1{}^3/3$). If

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R_2 is the radius of the protein, and if $R_2 \gg R_1$, then the interaction between the solute and the protein can be regarded as interaction of a spherical particle with a flat surface of medium 2 across medium 3. For this condition, the excess polarizability of protein in water is[19]

$$\alpha_2\varepsilon_3(i\xi_n) = \frac{2\varepsilon_0\varepsilon_3(i\xi_n)}{\rho_2}\Delta_{23} \tag{4}$$

where

$$\Delta_{23} = \left(\frac{\varepsilon_2(i\xi_n) - \varepsilon_3(i\xi_n)}{\varepsilon_2(i\xi_n) + \varepsilon_3(i\xi_n)}\right)$$

and $1/\rho_2$ is the volume of the protein molecule.

The generalized van der Waals interaction energy for a solute molecule interacting with a flat surface of medium 2 (protein) at a distance d across medium 3 (water) is[20]

$$E_{vdw}(d) = -\frac{\pi C\rho_2}{6d^3} \tag{5}$$

Substituting eqs 2, 3, and 4 in eq 5, it can be shown that the Lifshitz–van der Waals electrodynamic (dispersion) interaction energy is

$$E_{vdw}(d) = \frac{kTR_1^3}{2d^3}\sum_{n=0}^{\infty}{}^{*}\Delta_{13}\Delta_{23} \tag{6}$$

The relationship between frequency ξ_n and the Planck's constant (h) is given by

$$\xi_n = 2\pi nkT/h \tag{7}$$

where n is zero or positive integer. According to eq 7, the first nonzero frequency ($n = 1$) at which polarization of molecules can commence is 3.9×10^{13} Hz, which is in the infrared region. Frequencies from 3.9×10^{13} Hz through 1.6×10^{16} Hz ($n = 1$ through 420) were used in this study to calculate the dielectric permittivity of the solute, protein, and water at imaginary frequencies.

Equation 6 can be split into zero (Debye and Keesom interactions) and nonzero frequency terms (dispersion interaction) as

$$E_{vdw}(d) = -\frac{kTR_1^3}{4d^3}\left(\frac{\varepsilon_1 - \varepsilon_3}{\varepsilon_1 + 2\varepsilon_3}\right)\left(\frac{\varepsilon_2 - \varepsilon_3}{\varepsilon_2 + \varepsilon_3}\right) - \frac{kTR_1^3}{2d^3}\sum_{n=1}^{\infty}\Delta_{13}\Delta_{23} \tag{8}$$

where ε_1, ε_2, and ε_3 are static dielectric permittivity of the solute, protein, and water, respectively. Since the static dielectric permittivity of water (ε_3) is usually greater than ε_1 and ε_2, the first term in eq 8 is always negative, and therefore the contribution of Debye–Keesom interaction to van der Waals force is always attractive. On the other hand, in the case of the dispersion forces, the dielectric permittivity of the solute ($\varepsilon_1(i\xi_n)$), protein ($\varepsilon_2(i\xi_n)$), and water ($\varepsilon_3(i\xi_n)$) may cross each other over imaginary frequencies $i\xi_n$ of the electromagnetic spectrum. This will affect the sign and magnitude of the summation term (Σ) in eq 8. If $-\Sigma$ is positive, the dispersion contribution to van der Waals force will be repulsive. Thus, the electrodynamic (dispersion) forces would determine whether the interaction between a solute and a protein is attractive or repulsive.

When several solute molecules surround a protein molecule as depicted in Figure 2, each solute molecule will interact with the protein phase. In such a situation, the electrodynamic pressure generated by the solute–protein interaction at a distance d from the protein surface is

$$P_{vdw}(d) = -\frac{kTR_1^3}{4d^6}\left(\frac{\varepsilon_1 - \varepsilon_3}{\varepsilon_1 + 2\varepsilon_3}\right)\left(\frac{\varepsilon_2 - \varepsilon_3}{\varepsilon_2 + \varepsilon_3}\right) - \frac{kTR_1^3}{2d^6}\sum_{n=0}^{\infty}\Delta_{13}\Delta_{23} \tag{9}$$

Since all the solutes considered in this study are electrically neutral at pH 7, the contribution of electrostatic force to the interaction energy was not considered in the present analysis.

MATERIALS AND METHODS

Materials. Ribonuclease A (RNase A), lysozyme, ovotransferrin, β-lactoglobulin, α-lactalbumin, ovomucoid, chymotrypsinogen, myoglobin, ovalbumin, and all the solutes (sucrose, glycerol, PEG200, alanine, proline, urea, serine, and betaine) used in this study were obtained from Sigma Aldrich Co. (St. Louis, MO. USA).

Differential Scanning Calorimetry. The thermal denaturation temperature of proteins was determined using a Micro DSC VII (Setaram, Caluire, France). Protein solutions (10% w/w) were made in 10 mM phosphate buffer (pH 6.8) containing 1 M solute. Samples were thermally scanned from 20 to 120 °C at a constant heating rate of 1 °C/min. The temperature at the peak of the endothermic profile (T_d) was determined using the Setsoft software (version 1.40) supplied by the DSC manufacturer. The DSC was calibrated using cyclohexane, phenyl ether, and *o*-terphenyl standards recommended by the DSC manufacturer. Thermal analysis was carried out at least in duplicate for all samples.

Dielectric Permittivity Calculations. The method for calculating the dielectric permittivity of proteins, solutes, and water at imaginary frequencies has been described in detail elsewhere.[15] Briefly, a two oscillators model representing contributions from ultraviolet (UV) and infrared (IR) frequencies (eq 10) was used for the solutes and proteins.[21,22]

$$\varepsilon_i(i\xi_n) = 1 + \frac{C_{UV}}{1 + (\xi_n/\omega_{UV})^2 + (g_{UV}\xi_n/\omega_{UV})^2} + \frac{C_{IR}}{1 + (\xi_n/\omega_{IR})^2 + (g_{IR}\xi_n/\omega_{IR})^2} \tag{10}$$

where C_{UV} and C_{IR} are the UV and IR oscillator strength, respectively, g_{UV} and g_{IR} are the damping coefficient of the UV and IR oscillators, respectively, and ω_{UV} and ω_{IR} are the dominant UV and IR absorption frequencies, respectively, ξ_n is the real frequency. The contribution from microwave frequencies was omitted.

Proteins have strong absorption bands at 193.6 nm (6.4 eV) in the UV region and at 1652 cm^{-1} wavenumber (0.205 eV) in the IR region.[23] Thus, a two-oscillator model was used to calculate the dielectric permittivity at imaginary frequencies of all proteins, except myoglobin. Myoglobin is a heme protein with strong absorption bands at 205 and 577 nm in the UV/vis range, and at 1652 cm^{-1} wavenumber in the IR region. Therefore, a three-oscillator model was used to determine the dielectric permittivity of myoglobin. The oscillator strengths C_{205} and C_{577} were calculated using the formulation,[24]

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Table 1. Molecular Parameters of Proteins, Water, and Solutes Used in the Dielectric Permittivity Representations and the Calculation of Electrodynamic Interaction between the Solutes and Proteins in Water Medium[a]

additive	MW	P (cm^3)	R (nm)	δ (l/g)	ε_∞	ε_s	n^2	$\omega_{UV} \times 10^{15}$ (Hz)	$\omega_{IR} \times 10^{14}$ (Hz)	C_{UV}	C_{IR}
water	18		0.14			78	1.777	3.035	1.028	0.78	0.075
									0.488		0.014
									0.222		0.152
									0.166		0.737
									0.05		1.464
ovalbumin	42,866	29.58		0.17	2.67	3.085		1.548	0.495	1.67	0.415
RNase A	13,692	28.78		0.17	2.75	3.19		1.548	0.495	1.745	0.445
lysozyme	14,315	29.22		0.17	2.64	3.06		1.548	0.495	1.64	0.42
conalbumin	75,718	29.07		0.17	2.62	3.037		1.548	0.495	1.64	0.417
β-Lg	18,283	29.81		0.17	2.66	3.07		1.548	0.495	1.66	0.41
α-La	14,002	29.84		0.17	2.64	3.06		1.548	0.495	1.64	0.42
ovomucoid	22,261	27.99		0.17	2.64	3.07		1.548	0.495	1.64	0.43
chymotryps-inogen	25,700	27.60		0.17	2.61	3.025		1.548	0.495	1.61	0.415
myoglobin	16,954	29.62		0.17	2.65	3.06		1.462	5.195, 0.495	1.53, 0.119	0.41
sucrose	342		0.44			3.3	2.434		1.015	1.433	0.87
glycerol	92		0.31			42.5	2.174		0.882	1.174	1.99
urea	60		0.26			3.5	2.222	1.665		1.222	
PEG200	200		0.34			21.1	2.129	1.578		1.129	
betaine	117.14	31.57	0.338	0.186	2.425	2.77			1.012	1.435	0.335
proline	115.1	27	0.324	0.2	2.37	2.80			1.068	1.37	0.428
alanine	89.1	18.63	0.296	0.258	2.2	2.71			1.047	1.2	0.51

[a]MW, molecular weight; P, average molar polarizability per amino acid residue; R, molecular radius; δ, dielectric increment;[47,48] ε_∞ relative permittivity at high frequencies; ε_s, static dielectric constant; n, refractive index; ω_{UV}, ultraviolet relaxation frequency; ω_{IR}, Infrared relaxation frequency; C_{UV}, ultraviolet oscillator strength; C_{IR}, infrared oscillator strength.

Table 2. Thermal Denaturation Temperatures (T_d) of Various Proteins in 1 M Solutions Various Solutes

	T_d (°C)								
solute (1 M)	α-lactalbumin	β-lactoglobulin	chymotrypsinogen	ovotransferrin	ovalbumin	lysozyme	myoglobin	RNase A	ovomucoid
buffer	64.2	72.3	56.6	61.5	77.7	72.3	77.0	64.1	75.2
sucrose	67.0	78.5	60.3	65.9	82.8	78.3	80.9	68.1	79.2
glycerol	64.6	73.8	57.4	62.0	77.8	73.8	77.0	64.7	75.4
alanine	68.3	80.4	58.2	64.2	81.4	77.1	77.9	67.0	78.2
serine	67.6	83.0	58.7	64.8	81.9	76.8	78.8	ND[a]	ND
betaine	66.5	75.3	58.6	63.3	79.0	75.5	75.8	66.0	75.9
proline	67.0	74.7	59.4	62.1	78.3	ND	75.7	64.9	76.2
urea	63.2	67.4	54.6	57.7	74.7	70.0	73.4	60.9	72.5
PEG200	61.2	65.6	ND	57.4	71.6	71.2	71.2	62.6	73.6

[a]ND = not determined.

$$\frac{C_{205}}{C_{577}} = \frac{f_{205}}{f_{577}}\frac{\omega_{577}}{\omega_{205}} \tag{11}$$

and

$$C_{205} + C_{577} = n^2 - 1 = \varepsilon_\infty - 1$$

where f_{205} and f_{577} are absorption strengths (extinction coefficients) at 205 and 577 nm, respectively, ω_{205} and ω_{577} are frequencies at 205 and 577 nm wavelength, respectively, n is the refractive index and ε_∞ is the dielectric permittivity at high frequencies. The f_{205} of myoglobin was determined as described elsewhere[25] using a molar extinction coefficient value of 13 980 M^{-1} cm^{-1} at 280 nm. The f_{577} of myoglobin is 14 600 M^{-1} cm^{-1}. The C_{205} and C_{577} values of myoglobin calculated in this manner are given in Table 1.

Sucrose, glycerol, proline, alanine, betaine, and serine do not have absorption bands in the visible and UV regions other than the first ionization potential but have strong absorption bands in the IR region.[26,27] The use of the first ionization potential in lieu of a specific UV absorption band has been shown to overestimate the contribution of UV-relaxation to dielectric permittivity (eq 10).[24] Furthermore, it has been suggested that a hydration shell around a solute quenches UV frequencies and dampens the contribution of UV relaxation to its dielectric permittivity (eq 10).[15] For these reasons, the dielectric permittivity of sucrose, glycerol, proline, alanine, betaine, and serine at imaginary frequencies, $\varepsilon_i(i\xi_n)$, was calculated using eq 10 by setting the UV oscillator strength $C_{UV} = 0$. Poly-(ethyleneglycol)-200 (PEG200) has a strong UV absorption at 190−200 nm and a very weak IR absorption (85% transmission) at 3314 cm^{-1}. Similarly, urea has a strong absorption at about 180 nm[28] and a very weak absorption (>50% transmission) at 3450 cm^{-1}. Since the IR absorption for PEG200 and urea is very weak, the IR contribution was omitted and only the UV relaxation was considered in the dielectric permittivity representation. For water, five infrared frequencies and one ultraviolet frequency reported in the literature[22] were used in this study. The ω_r values (eq 10) of various solutes,

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proteins, and water used in this study are listed in Table 1. The details of the calculation of static dielectric constants (ε_s), relative permittivity at high frequencies ($\varepsilon_\infty = n^2$), and molar polarization values (P) of proteins and solutes are described in our previous work.[15]

RESULTS

With the exception of ovalbumin and ovotransferrin, the proteins chosen for this study are single domain globular proteins. The solutes used in this study (Table 1) represent chemically different classes of compounds that have been previously shown to stabilize (sucrose, glycerol, betaine, alanine, serine, and proline) and destabilize (urea and PEG200) proteins.[10] While betaine, proline, serine, and alanine are amino acids, sucrose and glycerol are polyhyderic alcohol-type solutes. With regard to their ability to affect the surface energy of water, sucrose, alanine, serine, proline, and urea increase the surface tension, whereas glycerol and betaine decrease the surface tension of water.[29] Thus, if Lifshitz's electrodynamic interaction is the universal mechanism by which these solutes impact protein stability, then their influence should be independent of their chemical nature, their effect on surface energy/tension of water, and structural differences among the proteins, but dependent only on the nature and magnitude of the electrodynamic force generated between the solute and the protein in the aqueous medium.

Table 2 shows the thermal denaturation temperature (T_d) of the proteins in 1 M solutions of various solutes at pH 6.8. The T_d of all the proteins studied was higher than the control in sucrose, glycerol, betaine, proline, serine, and alanine solutions, indicating that these solutes stabilized the protein structure. On the other hand, the T_D was lower in urea and PEG200 solutions than the control, indicating that these solutes destabilized the protein structure. If we assume a two-state model of protein structure, that is, only folded (N) and unfolded (D) states, then the T_d (or T_m) represents the melting temperature at which [N] = [D] and the free energy change $\Delta G_{N\rightarrow D} = 0$. Since $\Delta G_{N\rightarrow D} = \Delta H - T\Delta S$, it follows that at $T = T_d$, $T_d = \Delta H/\Delta S$, and at $T \neq T_d$, $\Delta G_{N\rightarrow D} = (T_d - T)\Delta S$. If ΔS is a constant for the process N ↔ D, then the solute-induced change in the T_d of a protein is a direct measure of the solute's effect on the thermodynamic stability (i.e., $\Delta G_{N\rightarrow D}$) of the protein at any given temperature T.

Figure 3 shows a plot of the frequency-dependent dielectric permittivity ($\varepsilon(i\xi)$) of proteins and solutes against log ξ. The dielectric permittivity curves of the proteins were above that of the water curve (Figure 3A), whereas the curves of most of the solutes were below that of the water curve, except for urea and PEG200, which were above that of water (Figure 3B). As discussed earlier, according to eq 9, the dispersion interaction between a solute and a protein will be repulsive when $\varepsilon_1(i\xi_n) < \varepsilon_3(i\xi_n)$, and it will be attractive when $\varepsilon_1(i\xi_n) > \varepsilon_3(i\xi_n)$. Since the Debye–Keesom interaction term in eq 9 is always attractive in an aqueous medium, the sign and magnitude of the dispersion term will determine whether or not the electrodynamic pressure P_{vdW} for a solute-protein pair is repulsive (positive) or attractive (negative).

The electrodynamic pressure as a function of surface-to-surface distance (d) between a solute and a protein in water medium was calculated for various protein–solute pairs, and the results are shown in Figure 4 for lysozyme and ovotransferrin as representative examples. As predicted, interaction of sucrose, glycerol, proline, alanine, serine, and

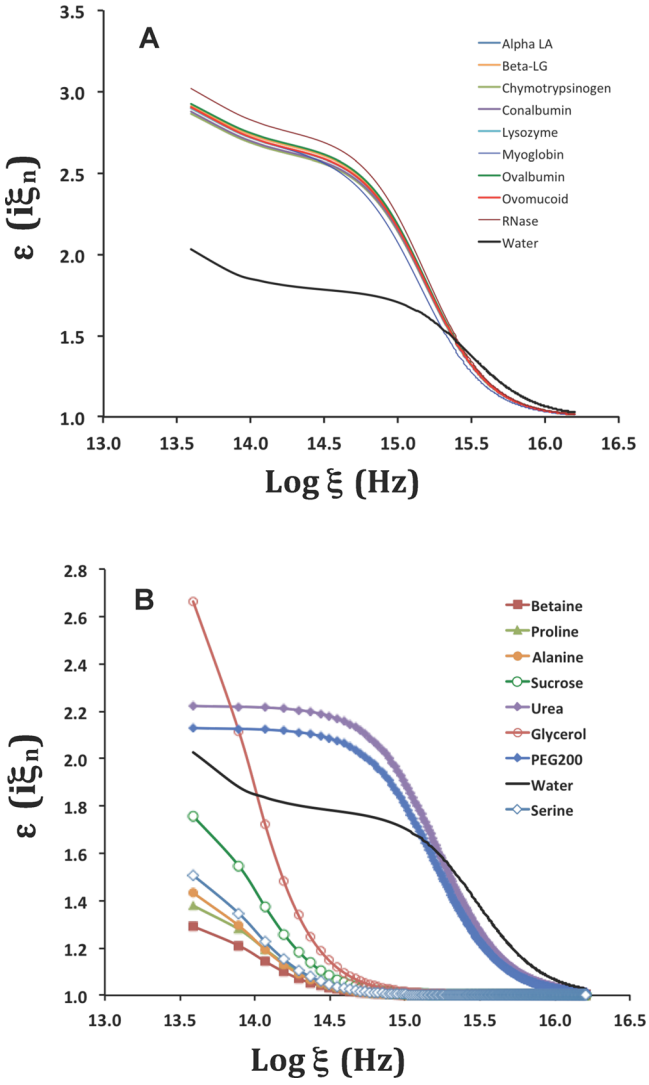

Figure 3. (A) Dielectric permittivity ($\varepsilon(i\xi)$) as a function of frequency (ξ) of water and the nine proteins used in this study. Both ultraviolet and infrared relaxations (eq 10) were included in the construction of the dielectric permittivity curves. (B) Dielectric permittivity ($\varepsilon(i\xi)$) as a function of frequency (ξ) of water and the seven solutes used in this study. The values of the parameters used in the calculation of dielectric permittivity at imaginary frequencies of the solutes are given in Table 1.

betaine with the proteins was repulsive, whereas interaction of PEG200 and urea with the proteins was attractive. The positive and/or negative pressure arising from repulsive/attractive electrodynamic interaction, respectively, was >1 atm even at distances greater than ∼1 nm. This implicitly means that sucrose, glycerol, proline, alanine, serine, and betaine would exclude themselves from the protein–water interfacial region, whereas PEG200 and urea would accumulate at the protein–water interface and bind to the protein surface. It should be noted that the positive pressure arises from repulsive electrodynamic interactions between the protein and the solute at molecular scale distances, and it is not related to the osmotic pressure arising from the concentration gradient generated by self-exclusion of the solute from the protein–solvent interfacial region. Likewise, the negative pressure arises from attraction of the solute (PEG200 and urea) to the protein surface, and not from the reverse concentration gradient generated by

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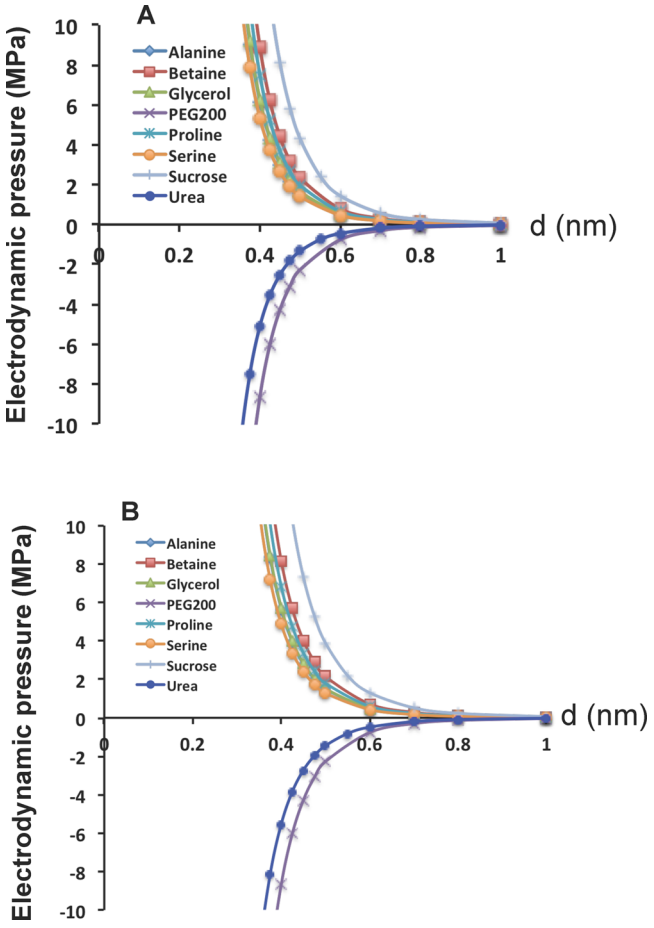

Figure 4. Electrodynamic pressure as a function of surface-to-surface separation distance between the solute molecule and lysozyme (A) and ovotransferrin (B) across the water medium. These calculations were based on eq 9 using the molecular parameters listed in Table 1.

accumulation at or binding of these solutes to the protein surface. This negative pressure gradient in the protein−solvent interfacial region might act in concert with the protein chain entropy ($T\Delta S_c$) to unfold the protein structure.

To examine what impact a solute has on protein−water interaction in the ternary system, the electrodynamic interaction between water (ε_1) and protein (ε_2) across the solute medium (ε_3) was calculated for all protein−solute pairs, and the results are shown in Figure 5 for lysozyme as a representative example. The water−lysozyme interaction was attractive in sucrose, glycerol, betaine, proline, serine, and alanine media but was repulsive in urea and PEG200 media. Similar results were obtained for all other proteins (data not shown). To determine if the repulsive interaction of sucrose, glycerol, betaine, proline, serine, and alanine with the protein phase actually caused a net increase in the affinity of water to the protein phase, the electrodynamic interaction between water (ε_1) and protein (ε_2) across air (ε_3) medium (i.e., water(ε_1)−air(ε_3)−protein(ε_2) system) was calculated as a reference and compared it with the water(ε_1)−solute(ε_3)−protein(ε_2) systems. The rationale is that since the dielectric susceptibility of air is invariant at all $i\xi_n$, any difference between the electrodynamic pressure profiles of water(ε_1)−air(ε_3)−protein(ε_2) and the water(ε_1)−solute(ε_3)−protein(ε_2) systems should be indicative of the influence of the dielectric permittivity of the solute on protein−water interaction. The

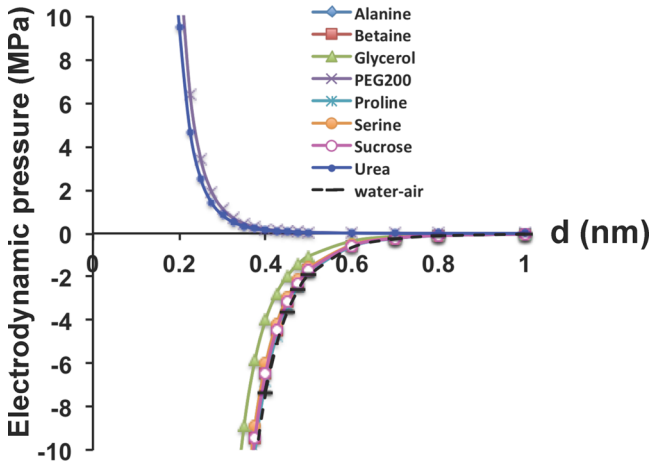

Figure 5. Electrodynamic pressure as a function of surface-to-surface separation distance between water and lysozyme in various water(ε_1)−solute(ε_3)−lysozyme(ε_2) systems. The interaction between water and lysozyme in air medium, i.e., water(ε_1)−air(ε_3)−lysozyme(ε_2) system also is shown (- - -).

data in Figure 5 show that the attractive electrodynamic interaction of water with the protein phase in the water(ε_1)−air(ε_3)−protein(ε_2) system (dotted line in Figure 5) was almost identical to the water(ε_1)−solute(ε_3)−protein(ε_2) systems of sucrose, glycerol, betaine, proline, serine, and alanine, implying that the structure stabilizing solutes (sucrose, glycerol, betaine, proline, serine, and alanine) do not impose enhanced (i.e., preferential) interaction of water with the protein. That is, the net increase in protein stability occurs primarily as a result of repulsive interaction between the protein phase and the stabilizing solutes, and not because of any preferential interaction of water with the protein as a result of solute-induced changes in bulk water structure.

If Lifshitz's electrodynamic pressure is the dominant mechanism by which solutes influence protein stability, then one should expect a correlation between the net increase in the thermal denaturation temperature (ΔT_d) of proteins in solutions of various solutes and the electrodynamic pressure generated by the repulsive/attractive protein-solute interaction. Figure 6 shows a linear relationship between ΔT_d of various proteins in 1 M solutions of various solutes and the calculated electrodynamic pressure (P_{vdW}) at an arbitrary distance of 0.5 nm from the protein surface. This was the case at any other chosen distance d, albeit with a different slope: the slope decreased as the distance increased. The scatter in the plot, especially in the data points of amino acids, might be due to the spherical approximation used in the calculation of P_{vdW} (eq 9). The linear relationship with a high correlation coefficient for most of the proteins studied, regardless of the chemical nature and their structure stabilizing or destabilizing nature, clearly indicates that the Lifshitz's electrodynamic forces are inextricably involved in solute-induced stabilization and/or destabilization of globular proteins.

It is notable that the slopes ($d\Delta T_d/dP_{vdW}$) of the P_{vdW} versus ΔT_d plots (Figure 6) (at d = 0.5 nm) varied among the proteins studied, suggesting that the impact of the electrodynamic pressure on thermal stability of proteins was to some extent protein-dependent. Conceptually, the slope (°C/MPa) of these plots represents the net increase in the thermal energy needed to unfold the protein against the compression caused by 1 MPa electrodynamic pressure (at an arbitrarily selected d = 0.5 nm).

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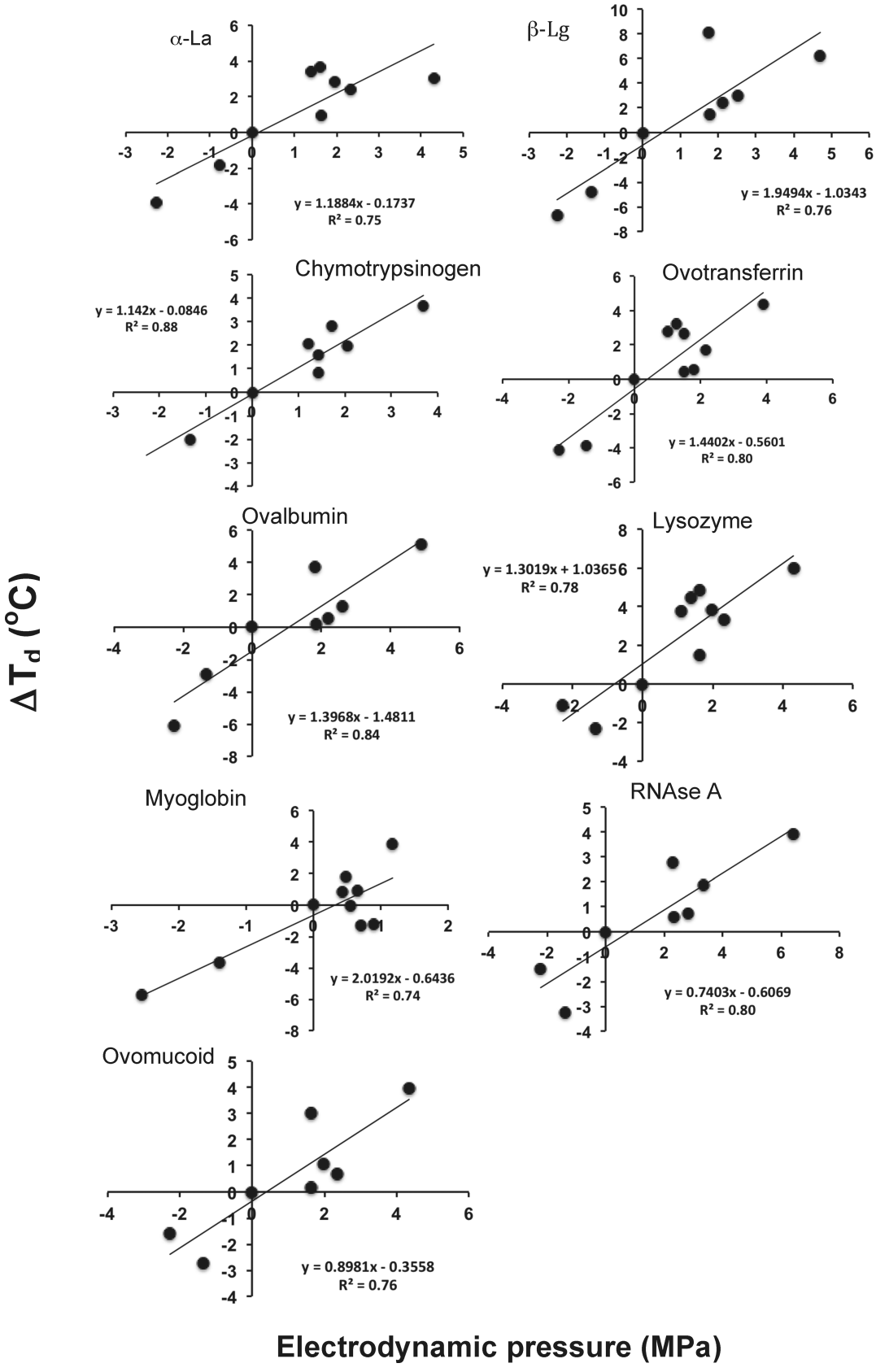

Figure 6. Relationship between the net change in thermal denaturation temperature (ΔT_d) of various proteins in 1 M solutions of various solutes and the electrodynamic pressure (P_{vdW}) in solute(ε_1)−water(ε_3)−protein(ε_2) systems at an arbitrary separation distance of d = 0.5 nm between the solute and the protein. The T_d of the proteins in 1 M solutions of various solutes is presented in Table 2.

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The variation in $d(\Delta T_d)/dP_{vdW}$ among proteins, which ranged from 0.74 to 2.02 °C/MPa at d = 0.5 nm (Figure 6), might be related to certain molecular properties, e.g., adiabatic compressibility, of the proteins. It has been reported that glycerol decreased the specific volume and compressibility of several globular proteins.[30] In the light of the present study the above observation might be attributed to the electrodynamic pressure-induced compression of the proteins in glycerol solution. As a corollary, we should expect that the negative electrodynamic pressure caused by attractive interaction of urea and PEG200 with proteins might elicit an increase in the specific volume of proteins.

If the volume change in globular proteins by solutes is principally due to the electrodynamic pressure gradient generated by solute−protein interactions, then the variation in $d\Delta T_d/dP_{vdW}$ (Figure 6) among proteins must be a function of the compressibility of these globular proteins: The greater the compressibility, the greater would be the volume change and the slope. Figure 7 shows a plot of $d\Delta T_d/dP_{vdW}$ of various

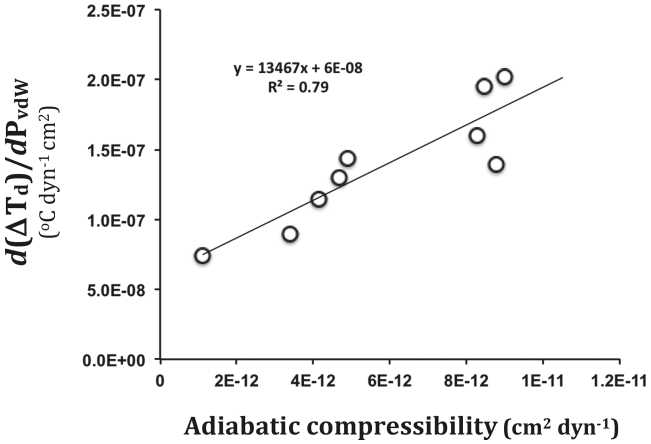

Figure 7. The relationship between the slope $d(\Delta T_d)/dP_{vdw}$ (from Figure 6) and the adiabatic compressibility of the proteins. The adiabatic compressibility values were from ref 31.

proteins versus their adiabatic compressibility (β_s) reported in the literature.[31] The remarkable linear correlation coefficient (r^2 = 0.79) clearly indicates that the variations in thermal stability response of globular proteins to electrodynamic pressure might indeed be related to differences in their adiabatic compressibility. Shown in Figure 8 is the ΔT_d versus P_{vdW} plot of all the nine proteins in 1 M solutions of seven different solutes investigated in this study. The $d\Delta T_d/dP_{vdW}$ of this plot indicates that on an average the thermal denaturation temperature of a typical globular protein is increased by about 1.3 °C per MPa electrodynamic pressure at a solute−protein distance of 0.5 nm.

DISCUSSION

According to the prevailing view, the solutes that stabilize protein structure do so via promoting preferential hydration of the protein surface, whereas the solutes that destabilize protein structure do so via binding to the protein surface. The central thesis of this preferential hydration hypothesis is that solutes alter the bulk water structure, and this in turn alters the thermodynamics of protein−water interaction and thereby protein stability.[2,8,10,13] One of the recurring arguments in favor of this preferential hydration hypothesis is the correlation

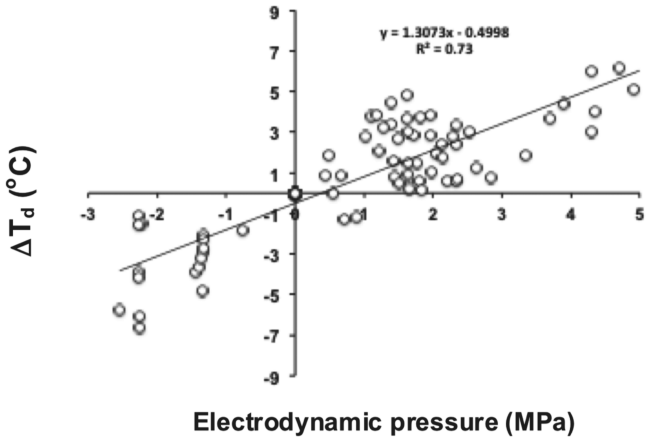

Figure 8. An aggregate plot of P_{vdW} at d = 0.5 nm versus ΔT_d of all the nine proteins in 1 M solutions of all seven solutes.

between molal surface tension increment of solutes and protein stability.[4,10,29,32] According to this suggestion, elevation of the surface free energy of water by solutes, such as sugars, increases the free energy (heat) of formation of a cavity to house a protein molecule, which leads to a greater tendency of water to interact with the protein surface. The opposite effect is believed to occur in the case of structure destabilizers. A fundamental flaw in this argument is that it assumes that solute-induced changes in the protein−water interfacial tension are similar in sign and magnitude to changes in the air−water interfacial tension. There is no physical basis to support this argument. Furthermore, this narrative does not explain why urea and salts such as NaI, $NaClO_4$, and NaSCN, which increase the surface tension of water, invariably destabilize protein structure. Similarly, even though glycerol decreases the surface tension of water, it is known to stabilize the native structure of several proteins.[5,29] Justification of such anomalies as due to non-specific weak binding of these solutes to protein surface negates the universality of the preferential hydration mechanism. Furthermore, if preferential hydration is the mechanism by which "structure making" solutes stabilize proteins, then one should expect a correlation between the extent of preferential hydration, that is, positive values of $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$ (where g_1 is grams of water bound to the protein, g_2 is the grams of protein, T is the temperature, μ_1 and μ_3 are chemical potentials of water and solute, respectively) and the thermal stability of a protein in various solute solutions. However, no such correlation exists. For instance, the $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$ values for lysozyme and other proteins in 1 M solution of various solutes were in the range of 0.3−0.6 g/g,[4,10,32] which were similar to the usual monolayer hydration values of most proteins,[33] and there was no correlation between the $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$ values and the thermal transition temperature (T_m) of lysozyme in 1 M solutions of various solutes.[10] Similarly, in a study on the stabilization of proteins by sucrose,[34] while the transition temperature (T_m) of chymotrypsinogen, α-chymotrypsin, and ribonuclease increased linearly with increasing sucrose concentration, there was absolutely no correlation between T_m and the $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$ values. On the other hand, one does observe a correlation between $(\partial g_3)/\partial g_2)_{T,\mu_1,\mu_3}$ (where g_3 is grams of solute bound to the protein) and the stability of the protein.[34] In essence, a critical evaluation of the data in the literature[4,5,32,34] actually indicates the following facts: (1) In the case of structure

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stabilizers, the stability of a protein increases as the solute concentration is increased. This is accompanied by an increase in the exclusion of the solute from the protein−solvent interfacial region. This suggests that the increased stability of the protein is associated with the exclusion of the solute from the protein−solvent interfacial region. (2) An increase in the solute concentration does not result in a proportional increase in preferential hydration; that is, the $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$ remains constant within the experimental error at all solute concentrations. Therefore, the observed increase in protein stability with increase of solute concentration is not due to $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$. Taken together, the stabilization of proteins by solutes is related only to the degree of exclusion of the solute, that is, $(\partial g_3)/\partial g_2)_{T,\mu_1,\mu_3}$, from the protein−water interfacial region and not to preferential hydration, that is, $(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$. Thus, a re-examination of the existing data in the literature in fact suggests that the forces involved in the exclusion of a solute from the protein−solvent interfacial region are also fundamentally responsible the stabilization of the protein as well. None of the studies in the literature has explained the forces responsible for the exclusion of structure stabilizing solutes from the protein-water interfacial region. Given the fact that the size of a typical structure stabilizing solute is much smaller than a protein and is only slightly larger than a water molecule, Schellman's[35] proposal that the exclusion of a solute from the protein−water interfacial region is due to the excluded volume effect is not very convincing.

A clear molecular level understanding of the mechanism by which a solute impacts protein stability is critical to understanding protein folding in a biological milieu. Fundamentally, and conceptually, an uncharged solute's influence on protein stability in an aqueous solution must be due to the force field generated by the quantum electrodynamic interaction between the solute and the protein phase across the aqueous medium. The sign and magnitude of this force depend on the relative dielectric susceptibility of the protein, solute, and water at imaginary electromagnetic frequencies. The results of the present study clearly reveal that the repulsive electrodynamic interaction between a structure-stabilizing solute and a protein is the root cause of the exclusion of the solute from the protein−solvent interfacial region, and the attractive electrodynamic interaction between a structure-destabilizing solute and a protein is the reason for the accumulation of the solute at the protein surface. At high solute concentrations, that is, at small distances between the solute and the protein, the repulsive electrodynamic interaction exerts a significant positive pressure (not related to the osmotic pressure) on the protein molecule, which compresses the protein and increases the thermal energy needed to unfold the protein structure. It should be pointed out that the exclusion of the solute from the protein−solvent interfacial region is not because of the excluded volume or the molecular crowding effect, as suggested by others,[35,36] but is due to the repulsive electrodynamic interaction between the solute and the protein. This self-exclusion of the solute allows water to accumulate near the protein surface. However, the thermodynamic affinity of water under this situation is about the same as that in the absence of the solute (Figure 5). This explains why the preferential hydration ($(\partial g_1)/\partial g_2)_{T,\mu_1,\mu_3}$) values did not change significantly in the cases of chymotrypsinogen, α-chymotrypsin, and ribonuclease in 0.05−1.0 M sucrose and other polyol solutions.[4,5,34] Thus, the stabilizing effect of the solute arises solely from the electrodynamic pressure emanating from the protein-solute interaction and not from preferential hydration of the protein.

It is also apparent that the destabilizing effects of urea and PEG200 on protein structure arise from the negative electrodynamic pressure created by the attractive force between these solutes and the *protein phase*, and it is not necessarily driven by nonspecific binding of these solutes to functional groups on the protein surface as suggested previously,[37,38] though the electrodynamic attraction might lead to casual binding of these solutes to certain groups on the protein surface and partial dehydration of the protein surface. The negative pressure gradient might act in concert with the protein chain entropy ($T\Delta S_c$) to shift the N ↔ D equilibrium in favor of the D state. The global linear relationship between ΔT_d and P_{vdW} (Figures 6 and 8), regardless of the stabilizing or destabilizing nature of the solutes, supports this interpretation.

There is incontrovertible spectroscopic and molecular dynamics simulation evidence in the literature for the notion that solutes alter bulk water structure. For instance, Raman spectroscopic studies have shown that polyols, such as sucrose and glycerol, enhance the tetrahedrally hydrogen bonded water units in bulk water as well as in hydration shells around them, whereas urea destroys the tetrahedrally hydrogen bonded water structure.[39,40] Molecular dynamics simulation studies also have shown that urea destabilizes the tetrahedrally hydrogen bonded structures in bulk water.[41] Neutron diffraction studies have shown that although urea mixes well and substitutes for water in the hydrogen bonded water network, its large molecular volume (compared to water) disrupts water−water hydrogen bonding as evidenced from the complete disappearance of the second nearest neighbor peak at 4.5 Å in the radial distribution function.[42] Thus, solutes induce changes in bulk water structure and in the hydration shell water, and therefore any theory on the mechanism of solute-induced stabilization/destabilization of protein structure should address the role of water structure in the process.

In the previous study,[15] we proposed that water structure, especially the hydration shell of a solute, plays an important role as a modulator of the electrodynamic interaction between the solute and the protein. In its original formulation, the Lifshitz theory assumes that the intervening medium between two interacting particles is a continuum and does not form any ordered structure around the interacting particles.[43] These assumptions are not valid for solvent water. The "flickering cluster" structure of bulk water and water's propensity to form hydration shell around dissolved solutes defies the above assumptions. A direct consequence of this nonideal property of water as a medium is that it might alter the relative contributions of UV and IR relaxations to the dielectric permittivity of the solute (eq 10). We hypothesize that in the case of 'structure making' solutes (e.g., sucrose, glycerol, and amino acids) the hydration shell around the solute quenches the contribution of UV frequencies to its dielectric susceptibility (eq 10) and this results in a repulsive electrodynamic interaction with the protein. In contrast, in the case of "structure breaking" solutes (e.g., urea and PEG200), the lack of a well-defined tetrahedrally hydrogen bonded hydration shell permits the UV frequencies to contribute to their dielectric susceptibility and this results in attractive electrodynamic interaction with the protein phase. This has been demonstrated in our previous report.[15] For instance, if the far UV frequency

corresponding to the first ionization potential (for the lack of an UV absorption frequency) is used for the dielectric permittivity representation of sucrose, glycerol, and amino acids (eq 10), the electrodynamic interaction of these solutes with proteins becomes attractive, which means that all these solutes should bind to the protein phase.[15] However, this goes against the irrefutable experimental evidence in the literature that sucrose, glycerol, betaine, alanine, proline, and serine do not bind to proteins and are excluded from the protein-water interfacial region.[2,3,5,10,32,34] The implication is that the water structure, especially the quality of the hydration shell (i.e., its thickness and whether or not it is tetrahedrally hydrogen bonded) around 'structure making' solutes, modulates the electrodynamic interaction between the protein and the solute by selectively affecting the UV relaxation contribution to its dielectric permittivity[15] as depicted schematically in Figure 9. This modulation might occur through alterations in the UV oscillator strength (i.e., $C_{UV} \ll n^2 - 1$) or the damping factor g_{UV} ($>10^{18}$) or both in eq 10.

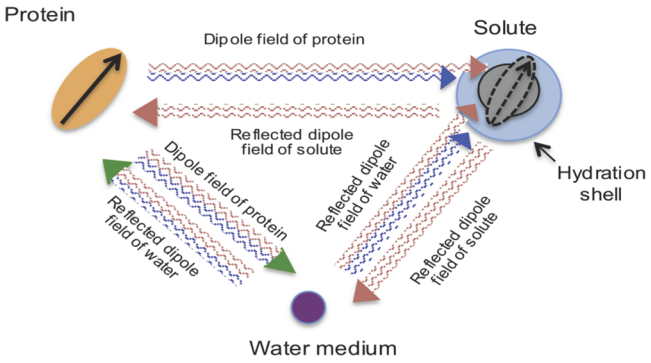

Figure 9. Schematic representation of quenching of ultraviolet frequencies by the hydration shell of the solute in the protein–water–solute ternary system. The solid arrow denotes dipole. The dotted arrow indicates induced dipole. The blue lines represent ultraviolet frequencies and the red lines represent infrared frequencies. The ultraviolet frequencies are depicted as blocked (quenched) by the solute's hydration shell.

Quenching or dampening of high frequency electromagnetic radiation from the excited state of a molecule by the hydration shell has been experimentally shown in the case of fluorescent probes. For example, 1-anilino-8-napthalene sulfonate (ANS) is not fluorescent in water, but it becomes highly fluorescent in water–ethanol mixtures and in organic solvents.[44] The zero quantum yield of fluorescence of ANS in pure water is attributed to the interaction of its hydration shell with the excited state of the chromophore, resulting in localized deactivation of the excited state without emission of radiation.[44]

A corollary of the proposed hypothesis is that it may provide a quantum mechanical definition of the "hydration forces". In the classical treatment of colloidal interactions using the DLVO theory, any discrepancy between the experimental and predicted interaction energies is usually ascribed to "hydration forces".[45,46] According to this concept, when two hydrated solutes approach each other, the energy needed to disrupt the hydration layers around the solutes manifests itself in the form of repulsion.[45] However, to our knowledge, there is no theoretical formulation in the literature on a potential energy function describing the magnitude of this hydration force. In the context of the Lifshitz theory, we suggest that the hydration force may not be a distinct physical force arising from repulsion between two hard sphere hydration shells, but it may be regarded as the hydration shell-modulated repulsive electrodynamic force between hydrated solutes. If this interpretation is correct, it will have enormous implications for our understanding of solute–solute interactions in a biological milieu. That is, the attractive and repulsive interactions between solutes in a highly concentrated biological milieu might be mediated simply via modulation of the electrodynamic interactions between the solutes by hydration shells. This supposition however needs to be experimentally verified.

SUMMARY

The results of this study provide a radically different view of the mechanism of stabilization/destabilization of protein structure by solutes. The currently accepted preferential hydration mechanism asserts that solutes affect protein stability indirectly via altering the bulk water structure and by enhancing the hydration of the protein surface. In contrast, the results of the present study clearly demonstrate that solutes affect protein stability directly via quantum electrodynamic interaction with the protein. Structure stabilizers exert a positive electrodynamic pressure, whereas structure destabilizers exert a negative electrodynamic pressure on the protein. In this three-body interaction, the bulk water structure and more importantly the solute's hydration shell might play an essential role as a modulator of the electrodynamic interactions. The implication of these results is significant in the sense that, in addition to the hydrophobic effect that drives protein folding, the electrodynamic forces between protein and solutes present in the biological milieu also might play a role in the folding process and in the stability of the folded state. In a broader context, by selectively manipulating the UV frequencies emanating from dipole fields of solutes, the hydration shells of solutes may control solute–solute and solute–protein interactions in a biological milieu.

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