Viscous cosolvent effect on the ultrasonic absorption of bovine serum albumin

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ABSTRACT Protein–ligand binding and enzyme activity have been shown to be regulated by solvent viscosity, induced by the addition of viscous cosolvents. This was indirectly interpreted as an effect on protein dynamics. However, viscous cosolvents might affect dynamic, e.g., viscosity, as well as thermodynamic properties of the solution, e.g., activity of solution components.

This work was undertaken to examine the effect of viscous cosolvent on the structural dynamics of proteins and its correlation with dynamic and thermodynamic solution properties. For this purpose we studied the effect of viscous cosolvent on the specific ultrasonic absorption, $\Delta\mu$, of bovine serum albumin, at pH = 7.0 and at 21°C, and frequency range of 3–4 MHz. Ultrasonic absorption (UA) directly probes protein dynamics related to energy dissipation processes.

It was found that the addition of sucrose, glycerol, or ethylene glycol increased the BSA $\Delta\mu$. This increase correlates well with the solvent viscosity, but not with the cosolvent mass concentration, activity of the solvent components, dielectric constant, or the hydration of charged groups.

On the grounds of these results and previously reported findings, as well as theoretical considerations, we propose the following mechanism for the solvent viscosity effect on the protein structural fluctuations, reflected in the UA: increased solvent viscosity alters the frequency spectrum of the polypeptide chain movements; attenuating the fast (small amplitude) movements, and enhancing the slow (large amplitude) ones. This modulates the interaction strength between the polypeptide and water species that "lubricates" the chain's movements, leading to larger protein–volume fluctuation and higher ultrasonic absorption.

This study demonstrates that solvent viscosity is a regulator of protein structural fluctuations.

INTRODUCTION

The energetics of protein structural fluctuations involves continuous exchange of thermal energy between the protein structure and the surrounding molecules. According to the principles of the Brownian theory of motion (1), the thermal motion of a flexible chain in a liquid can be described as a random motion in a viscous medium (2). The energy balance consists of influx of energy due to collisions, or structural rearrangement of the solvent molecules, as the "external" forces, and of an energy dissipation due to viscosity and frictional forces. The relaxation time of the process depends on both the medium viscosity and the chain elasticity (2). The energy balance of the system is described by the so-called "fluctuation-dissipation theorem" (3), which attributes to the medium viscosity a central role in determining the system energetics. The same principles led Kramers (4) to derive an expression for the absolute rate k for a process of overcoming a potential energy barrier U by a diffusion process taking place in a medium with viscosity η. The expression is:

$$k = (A/\eta)\exp(-U/RT), \quad (1)$$

in which A is a constant and R is the gas constant, and T is the absolute temperature. Kramers' theory models the kinetics of chemical reactions and relates the reaction rate k to viscosity of the reactants' environment. The validity of Kramers' law has been confirmed experimentally for polymer dynamics (5), and by computer simulations (6). The viscosity-dependent exchange of energy between the protein and the solvent has been proposed to be a source of energy for the protein structural fluctuations determining its function (7, 8).

In practice, the solvent viscosity is elevated by adding viscous cosolvents. These substances, mainly polyols, have been shown to slow down kinetic coefficients of ligand–protein interactions and subsequent biochemical processes, such as peptide hydrolysis by carboxypeptidase A (8), binding of O_2 or CO to respiratory proteins (9–12), some steps in the bacteriorhodopsin cycle (13), kinetics of lactate dehydrogenase (14), ester hydrolysis by substilisin BPN (15), and the activity of cell surface phospholipase A_2 (16, 17), as well as membrane lipid metabolism (16, 18) and cellular secretion (19). A similar trend has been observed in the viscous cosolvents effect on hydrogen isotope exchange in lysozyme (20, 21). Deviations from the viscosity dependence predicted by

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Eq. 1 toward weaker (9, 13–15, 20–22) or stronger (21) dependence on viscosity were observed and studied theoretically (23, 24).

The majority of the above mentioned studies of the viscosity effect on protein reactions generally did not consider thermodynamic parameters to play an appreciable role in this effect. However, Gregory et al. (21), who studied kinetics of hydrogen exchange, concluded that the cosolvent effect on the exchange of slow protons could not be explained in terms of solvent viscosity. They suggested that the viscosity might play a role in the kinetics of only some protons, and proposed a thermodynamic source for the cosolvent effect on hydrogen exchange. Moreover, polyols, such as glycerol, which are used as viscous agents, have a profound effect on the thermodynamic properties of proteins and increase their chemical potential, leading to the preferential hydration of the protein by exclusion of the polyol from the immediate vicinity of the protein (25–27). In order to distinguish between the "dynamic" and the "thermodynamic" effect of the cosolvent on a measurable property X of a tested protein, one should measure the effect of a variety of cosolvents on X and examine its correlation with both solution viscosity as a dynamic parameter, and thermodynamic solution parameters, such as the activity coefficients of solution components.

Absorption of ultrasonic energy by proteins probes those aspects of protein or polymer dynamics that are associated with volume-dependent relaxation processes. Thus, ultrasonic spectroscopy is an appropriate tool for studying relaxation processes in a protein, expressed by its specific ultrasonic absorption, $\Delta\mu$. This is derived by measuring the excess UA of protein solution over that of the pure solvent (28).

The objective of this study was to investigate the effect(s) of viscous cosolvents on protein dynamics, and to analyze the contribution of the solvent viscosity as a dynamic property, and of a number of solution thermodynamic parameters, i.e., the activity coefficient of the solution components and the solution dielectric constant. As noted above, ultrasonic absorption (UA) spectroscopy is a unique tool, suitable for studying dynamic processes of protein related to energy dissipation. For this purpose we have studied the effect of various viscous cosolvents on the UA of bovine serum albumin, a globular protein that has been well characterized by UA (29–32).

MATERIALS AND METHODS

Solutions. Viscous solutions were prepared by the addition of sucrose (J. T. Baker Chemical Co., Phillipsburg, NJ), glycerol (Frutarom, Israel), and ethylene glycol (Sigma Chemical Co., St. Louis, MO) dissolved in PBS buffer and degassed for at least 24 h. The same procedure was applied for NaCl 1 M solution. Solutions of 1, 2, and 4% (wt/vol) of fatty acid free BSA (Sigma Chemical Co.) were prepared by adding 20% BSA dissolved in PBS to the various solutions. The solution was further degassed overnight. Solutions of 4% glycin were prepared by adding 20% glycine dissolved in PBS to either PBS, glycerol, or sucrose solutions. All experiments were performed at pH 7.

Viscosity measurements. The solvent viscosity was measured using an Ostwald capillary viscometer, at 21° ± 0.1°C, and compared with values reported in literature.

Ultrasonic measurements. The apparent value of the ultrasonic absorption of the BSA at 21 ± 0.1°C was determined using the "resonator method" described by Eggers and Funck (33). The solution was placed in a plexiglass cell (5 ml) bounded by two parallel quartz transducers (5 MHz, 1 inch diameter purchased from Valpey Fisher Co., Hopkinton, MA). One of the crystals, the transmitter, generates ultrasound while being driven by a constant amplitude oscillator (model 3325A Frequency Synthesizer; Hewlett Packard Co., Palo Alto, CA). The other crystal, the receiver, converts the ultrasound back into an electric signal. By varying the frequency linearly with time, in a periodic manner, the so-called "frequency sweep" is generated. By monitoring the periodic pattern of amplitude versus time, with an oscilloscope, one can obtain a series of resonance peaks at frequencies f_n ($n = 1, 2 \ldots$) having half-power band width Δf_n. The relative energy dissipation per cycle μ is given by the following equation (33):

$$\mu = \alpha\lambda = \pi\Delta f_n/f_n, \tag{2}$$

where α is the amplitude attenuation, i.e., the inverse value of a typical decay distance of an ultrasonic wave having wavelength λ. μ is called the "'ultrasonic absorption per wavelength." In the case of protein concentration at volume fraction x, the observed Δf_n can be expressed as:

$$\Delta f_n(x) = g(f_n) + x\Delta f_n^p + (1 - x)\Delta f_n^s, \tag{3}$$

where $g(f_n)$ is the contribution of the cell itself (34). Δf_n^p and Δf_n^s are, respectively, the apparent values of $\Delta f_n(x)$ for pure protein and solvent. By measuring $\Delta f_n(x)$ for a number of protein concentrations we can eliminate $g(f_n)$ and verify the ideality of the mixture. The excess ultrasonic absorption per wavelength $\Delta\mu$ of the "pure protein" over "pure solvent" can be obtained from Eqs. 2 and 3 as follows:

$$\Delta\mu = \pi[\Delta f_n(x)/f_n - \Delta f_n(0)/f_n]/x = \pi(\Delta f_n^p - \Delta f_n^s)/f_n, \tag{4}$$

where $\Delta f_n(0)$ is the value of $\Delta f_n(x)$ for protein-free solution, whether buffer or mixed solvent. It should be mentioned that the method is less reliable near f_R, $3f_R$, $5f_R \ldots$, where f_R is the resonance frequency of the crystals (32), and the signal is difficult to detect near the frequencies 0, $2f_R$, $4f_R$ (f_R = 5 MHz in our system).

The measurements took place, for convenience, at the frequency range of 3 to 4 MHz. In practice, protein concentrations were expressed by w = mg protein per ml. However, $x = w/\rho$ where ρ is the apparent density of the protein, taken to be 1.36 g/ml for BSA (35). Thus,

$$\Delta\mu = \pi\rho[\Delta f_n(w)/f_n - \Delta f_n(0)/f_n]/w. \tag{5}$$

Analysis. Because $f_n(w)/f_n$ undergoes very little variation over the measured frequency range, we have replaced it by its average value $\langle\Delta f_n(w)/f_n\rangle$. $\Delta\mu$ is obtained from the slope of the regression line in the plot of $\langle\Delta f_n(w)/f_n\rangle - \langle\Delta f_n(0)/f_n\rangle$ versus w.

RESULTS

Effect of viscous cosolvents on ultrasonic absorption of BSA. The ultrasonic absorption of BSA solutions was measured in buffer and in viscous solvents, the viscosity of which was modified by the addition of glycerol, sucrose, or ethylene glycol.

The excess ultrasonic absorption, $\Delta\mu$, for BSA in buffer at pH 7, 21°C, and in the frequency range of 3 to 4 MHz, was found to be 0.025 ± 0.007. This value is compatible with that reported by Barnes et al. (32) for BSA.

As shown in Fig. 1 $\Delta\mu$ increased by increasing the cosolvent concentration. (This Figure includes $\Delta\mu$ obtained for BSA in the presence of 1 M NaCl, which will be discussed hereafter). Because a separate curve was obtained for each cosolvent, it is clear that $\Delta\mu$ cannot be correlated with the cosolvent weight fraction, as a parameter independent of the cosolvent nature. We have therefore examined the possible correlation of $\Delta\mu$ with the solvent parameters, which might be influenced by the cosolvent and affect the protein, as follows.

Solvent viscosity. Fig. 2 depicts the relation between $\Delta\mu$ and the solvent viscosity η on log–log scale. It clearly demonstrates that for all tested cosolvents, $\Delta\mu$ exhibits a single power-low dependence on the solvent viscosity, independent of the cosolvent nature, given by

$$\Delta\mu \alpha \eta^{\epsilon}, \quad (6)$$

where $\epsilon = 0.46 \pm 0.07$.

Activity coefficient of the solution components. The activity of the solution components plays an important role in determining the thermodynamic properties of proteins in mixed solvent, as already discussed in the Introduction.

In Fig. 3 $\Delta\mu$ was plotted versus the log of activity coefficient of glycerol, sucrose, and 1 M NaCl. RT log γ is, by definition, the chemical-potential change arising from the solvent–cosolvent interaction. It is evident that $\Delta\mu$ does not correlate with log γ as a parameter independent of the cosolvent nature.

As for the water, it has been shown that the dependence of log γ on sucrose concentration is negligible; 40% sucrose changes log γ of water by −0.003 (36). It is therefore clear that the observed cosolvent effect on $\Delta\mu$ cannot be attributed to changes in water activity.

Protein hydration. Change of state of water molecules from the "free" state in the bulk to a "bound" state associated with the protein hydration, is accompanied by a decrease in volume, Δv, of the interacting water species (37). Because cosolvents like sucrose, glycerol, and ethylene glycol might modify equilibrium properties

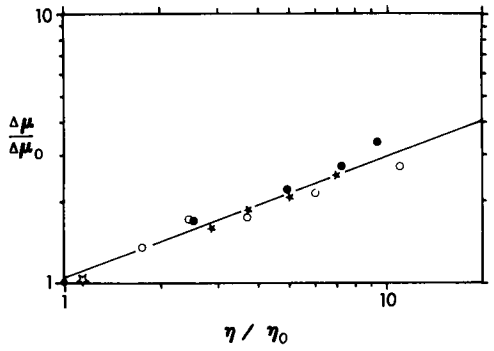

FIGURE 2 Log–log presentation of $\Delta\mu$ of BSA versus solvent viscosity (η). $\Delta\mu$ values of Fig. 1 were plotted against η, corresponding to the above cosolvent concentrations, the values of which, at 20°C were taken from literature (57). Both variables are expressed in relative units, where $\Delta\mu_0 = 0.025$ and $\eta_0 = 1$ cp correspond to the buffer. Linear regression gave the equation $\Delta\mu/\Delta\mu_0 = (\eta/\eta_0)^{\epsilon}$ (Eq. 6), with $\epsilon = 0.46 \pm 0.07$, and a correlation coefficient of 0.97. Symbols are the same as in Fig. 1.

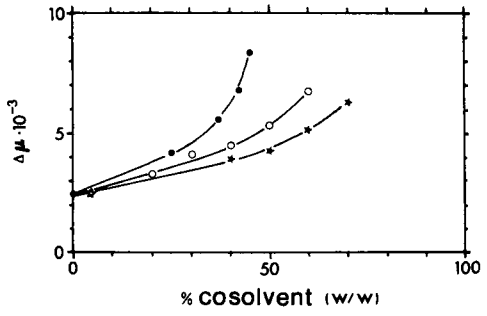

FIGURE 1 Excess ultrasonic absorption, $\Delta\mu$, of BSA versus cosolvent mass concentration. $\Delta\mu$ at 21°C and pH 7 was plotted versus the mass concentration of sucrose (●), glycerol (○), ethylene glycol (*), and 1 M NaCl (☆). Each datum is an average of two series of measurements at three protein concentrations (see Methods).

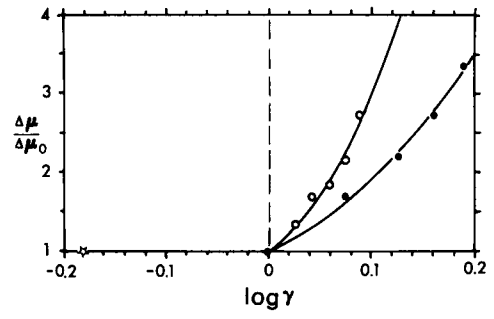

FIGURE 3 $\Delta\mu$ versus the activity coefficient γ of the cosolvents. $\Delta\mu$ values of Fig. 2 were plotted as a function of logγ for glycerol, sucrose, and NaCl. Values of logγ were derived from literature date at 20°C (58). The curves are best-fitted second-order polynomes. Symbols are the same as in Fig. 2.

of protein-solvent interaction (25–27), we expect that a direct cosolvent effect on Δv will affect $\Delta\mu$, because UA is specifically sensitive to changes in the state of water (38). To examine this possibility the following experiment was performed.

Effect of cosolvents on glycine absorption. Ultrasonic absorption is known to peak at the pK values of titratable groups (28, 30, 39) for the following reason: the binding of water to charged groups is tight and accompanied by a relatively large volume decrease (electrostriction) (40). At the pK values these groups fluctuate between "free" and bound states and display the highest rate of the coupled volume-fluctuation. Because volume fluctuations dissipate acoustic energy, the UA reaches a maximum at the pK value, where the water state is not well defined (neither "free" nor "bound"). Therefore, a possible effect on electrostricted water should be examined at pH values distant from the solute pKs, where the water is in a well defined, bound state. At the neutral pH, applied here, glycine withstands these conditions, because its pK values are 2.4 and 9.8. At pH 7, BSA has a potential for a maximum of 182 charged groups (41), while glycine has ~14-fold more charged groups at the same mass concentration. Thus, if the cosolvent effect on the BSA $\Delta\mu$ is related to electrostricted water it should be much more pronounced with glycine.

We have compared the UA of 4% glycine in cosolvent-free solvent with that of 4% glycine in 55% glycerol and 43% sucrose isoviscous solutions, at pH 7.4 and 21°C. It was found that the addition of the cosolvents did not affect the glycine $\Delta\mu$. These results are consistent with those of Lang et al., who demonstrated that the addition of denaturants, e.g., 8 M urea, did not affect the UA of diglycine ~pH 7 (42). This clearly suggests that the increase of the BSA $\Delta\mu$ induced by the addition of the viscous cosolvents is not due to an effect on electrostricted water.

In general, volume changes (which affect UA) associated with electrostatic interactions depend on the solution dielectric constant D, which is decreased upon the addition of cosolvents. Dielectric constant is well known to affect kinetics of chemical reactions that involve charged intermediates (43). Yet its contribution to kinetics of protein reactions has been shown to be considerably smaller than that of the solvent viscosity (8). Therefore, we examined the correlation of $\Delta\mu$ with D in the presence of the cosolvents. As shown in Fig. 4, for each cosolvent $\Delta\mu$ displayed a linear dependence on $1/D$, but with a clearly different slope for each cosolvent. This suggests that the increase in $\Delta\mu$ induced by the cosolvents is not due to an effect on the dielectric constant.

In light of these findings and considerations, it is plausible to conclude that the cosolvent effect on the UA of BSA, can be attributed to the modification of the solvent viscosity.

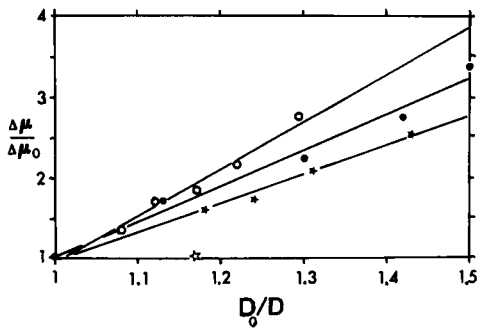

FIGURE 4 $\Delta\mu$ versus the solvent dielectric constant D. $\Delta\mu$ value of Fig. 2 were plotted as a function of D_0/D, where D and D_0 are the dielectric constant of the mixed solvent and the buffer, respectively. The corresponding correlation coefficients are 0.993, 0.990, and 0.995, for glycerol, sucrose, and ethylene glycol, respectively. D values at 20°C were taken from literature for glycerol (59), sucrose (60), ethylene glycol (61), and NaCl (62). Symbols are the same as in Fig. 2.

DISCUSSION

The results of this study demonstrate that the excess ultrasonic absorption, $\Delta\mu$, of BSA is increased in the presence of various cosolvents.

The cosolvent effect on $\Delta\mu$ did not correlate with the cosolvent mass concentration, water activity, cosolvent activity coefficient, or the solution dielectric constant (Figs. 1, 3, 4), as parameters independent of the cosolvent nature. The finding that the cosolvents did not affect the glycine $\Delta\mu$ suggests that the increase in BSA $\Delta\mu$ did not stem from an effect of the cosolvents on electrostricted water. This conclusion is compatible with that of other researchers, stating that the origin of the UA in proteins is not electrostricted water (44).

The increase in BSA $\Delta\mu$ was found to be a single function of the solvent viscosity, regardless of the nature of the applied cosolvent (Fig. 2). This finding supports the theoretical prediction that solvent viscosity may modify the energy dissipative processes in the protein (23).

It therefore seems that the observed cosolvent effect on the UA of BSA has a dynamic, rather than thermodynamic origin. Obviously, this does not rule out cosolvent effects of thermodynamic origin as demonstrated by previous studies, using other methods (21, 25–27). However, the data presented here clearly show that the observed effect on the UA of BSA can be explained in terms of solvent viscosity. A similar conclusion was

drawn by Ng and Rosenberg for cosolvent effect on enzymatic catalysis (15).

What processes can account for the UA of proteins in our tested range of pH (7.0) and frequency (3–4 MHz)?

To answer this question we will first discuss previously suggested mechanisms.

Proton transfer. Proton transfer processes are pH-dependent and induce pH-dependent UA, predominantly around the protein pKs. Thus, for BSA and some other proteins, the UA is insensitive to pH changes around neutral pH (distant from their pKs) (28, 29, 38, 42). Furthermore, quantitative analysis of the pH-dependence of $\Delta\mu$ suggests that near neutral pH internal proton exchange does not contribute significantly to $\Delta\mu$. This includes charged groups having near-neutral pK values, e.g., histidine (45). Thus, proton transfer processes do not contribute appreciably to UA of BSA near neutral pH.

Helix-coil transition. Relaxation processes derived from helix-coil transition have been demonstrated in polyglutamic acid (46) and proposed to contribute to BSA UA, but as a relaxation process observed at 70 kHz and pH = 4.2, which are out of our range of frequency and pH (31).

Water exchange coupled to polymer dynamics. The UA of aqueous solutions of dextran and polyethylene glycol at pH 7 (47–50) show volume relaxation processes in the MHz region. The origin of these processes is suggested to be a coupling between thermal motion of the polymer segments, and changes in the binding strength of water, interacting with the polymer (47, 50) (see also Fig. 5). Thus, the volume fluctuations probed by the UA correspond to water–polymer interaction modulated by the polymer motion. The same range of relaxation frequency of ~2 to 15 MHz was observed in BSA (30) and other proteins (29), which supports the relevance of this mechanism to this study.

Further evidence for the possible role of water exchange in relaxation phenomenon in proteins at the 1–10 MHz range is found in the following studies.

Careri et al. (51) have demonstrated that upon addition of water to dry lysozyme the frequency of dielectric relaxation increases and enters the MHz region upon reaching ~35% water content. At this degree of hydration the protein restores its enzymatic activity (52). Dielectric relaxation originates from molecular dynamic events that contribute to UA as well (53). This further assesses the role of hydration in the dissipative processes probed by UA.

In accord with that, Kato et al. have shown that the UA of dried dextran increases significantly with increasing its water content (50).

It therefore seems that at the frequency and pH range used here, the protein hydration plays an important role

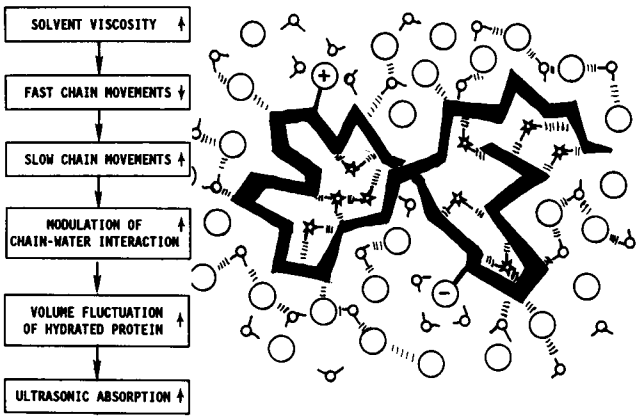

FIGURE 5 A model for the cosolvent effect on $\Delta\mu$. The Figure shows a schematic illustration of the polypeptide chain (*thick wavy line*), forming a protein embedded in a mixture of water (*V-like elements*) and cosolvent (*empty circles*). Stacks of dashes indicate hydrogen-bonding interactions. Water molecules, that can be described as "lubricating" the chain movements, are marked with stars. Charged groups with electrostricted water are shown as well. A conceptual flowchart for the proposed mechanism is presented on the left. By increasing the solvent viscosity, the frequency spectrum of the chain movements is altered; fast small-amplitude movements are attenuated, while the slow, large-amplitude movements are enhanced. As a result, the interaction strength between water, that "lubricates" the chain movements, and the chain itself, is modulated more intensively by the high amplitude movements, leading to larger protein–volume fluctuations and higher ultrasonic absorption (see Discussion).

in the effect of viscous cosolvents on the protein ultrasonic absorption.

What type of water could be relevant to the observed cosolvent effect of the ultrasonic absorption of BSA? Zaks and Klibanov studied the cosolvent effect on enzyme catalysis (54, 55) and suggested that some of the hydration water acts as "lubricant" or "plasticizer" that provides the enzyme with sufficient conformational flexibility needed for catalysis. This lubrication is attributed to the ability of water molecules to form hydrogen bonding with the functional groups on the polypeptide chain, and thereby enables the conformational mobility, i.e., the sliding of adjacent polypeptide segments (56). This water species is distinct from that tightly-bound to charged groups which are immobilized by electrostriction (40).

In light of the considerations summarized above we suggest the following (Fig. 5).

(*a*) The "lubricating" water is a determinant of the internal friction of the protein molecule, i.e., the energy dissipation associated with internal motions of the polypeptide chain. Thus, it could be identified with the type of water responsible for the UA, due to the coupling between water and the polypeptide chain, as previously proposed (47, 50). This conclusion is sup-

ported by the findings that the UA of denatured proteins is markedly low, suggesting that protein UA is associated with the tertiary structure (28).

(*b*) The elevation of the solvent viscosity increases the viscous damping of parts of polypeptide chain exposed to the solvent. Following the principles of the Brownian theory (1), this should result in attenuation of the small-amplitude high-frequency components of the chain motions. In order to comply with the equipartition of thermal energy, this will induce increasing the large-amplitude, low-frequency components of the chain motion (2).

(*c*) Because local modes of polymer motion at the MHz frequencies can be considered as "slow components," these will be intensified by the damping of the high-frequency motions, induced by the elevated solvent viscosity. Subsequently, energy dissipation due to the segmental motion and the water-coupled volume changes expressed in the UA is increased, as demonstrated in this study.

In conclusion, this study demonstrates that solvent viscosity affects BSA ultrasonic absorption. We propose that this phenomenon reflects an indirect effect of solvent viscosity on water-coupled protein dynamics.

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