Dielectric Behavior of Lysozyme and Ferricytochrome-c in Water/Ethylene-Glycol Solutions

A. Bonincontro,* S. Cinelli,† G. Onori,† and A. Stravato*
*INFM-Dipartimento di Fisica, Università "La Sapienza," I-00185 Rome, Italy; and †INFM-Dipartimento di Fisica, Università di Perugia, I-06123 Perugia, Italy

ABSTRACT This work deals with a dielectric study at radio frequencies of the influence at room temperature of two organic molecules, known as cryo-protectants, ethylene-glycol and glycerol, on conformational and dynamic properties of two model proteins, lysozyme (lys) from chicken egg-white and ferricytochrome-c (cyt-c) from horse heart. Cyt-c is a compact globular protein whereas lys is composed of two structural domains, separated by the active site cleft. Measurements were carried out at the fixed temperature of 20°C varying the concentration of the cosolvent up to 90% w/w. From the analysis of the dielectric relaxation of the protein solution, the effective hydrodynamic radius and the electric dipole moment of the protein were calculated as a function of the cosolvent concentration. The data show that glycerol does not modify significantly the conformation of both proteins and cyt-c is also stable in the presence of ethylene-glycol. On the contrary ethylene-glycol strongly affects the dielectric response of lysozyme denoting a specific effect on its conformation and dynamics. The data are coherently interpreted hypothesizing that glycol molecule wedges between and separates the two domains of lys making them rotationally independent.

INTRODUCTION

This work deals with a dielectric study at radio frequencies of ethylene-glycol and glycerol influence on conformational and dynamic properties of two well-known proteins, representing very useful models for folding studies: lysozyme (lys) from chicken egg-white and ferricytochrome-c (cyt-c) from horse heart.

Ethylene-glycol, like sucrose or glycerol, is commonly used as cryo-protectant for subzero temperature fractionation and long-term low-temperature conservation of various biomaterials (Carpenter and Crowe, 1988; Hancock and Hsu, 1996). The mechanisms whereby cryo-protectants preserve protein structure under low temperature remain obscure (Huang et al., 1995). The interest for the effects of these cosolvents on conformational and dynamic properties of proteins is more general. For example, these cosolvents are used in studying protein folding and also used as additive to stabilize protein structure during crystallization (Sawano et al., 1992; Rariy and Klibanov, 1998; Sousa, 1995; Farnum and Zukoski, 1999; Rubinson et al., 2000). However their role in these processes is poorly understood. Despite extensive studies on the interactions among proteins and cosolvents, few investigators have given their attention to conformation alterations in the presence of ethylene-glycol or glycerol at room temperature. The majority of studies has been focused mainly on the aspects of thermal unfolding of protein molecules (Arakawa and Timasheff, 1985; Arakawa et al., 1990; Gekko and Timasheff, 1981; Fink, 1986). Knowledge of the effects of these cosolvents on the protein structure is fundamental to better understand their role in these processes.

Dielectric spectroscopy (DS) is a noninvasive, very sensitive technique to investigate complex systems and it is particularly suitable in studying biological systems. Protein solutions exhibit at radio frequencies typical dielectric relaxations due to orientation polarization. From the dispersion curve it is possible to determine two significant parameters characterizing conformation and structure of a protein: the effective hydrodynamic radius and the electric dipole moment. In our laboratories a research line, devoted to study small conformational changes of proteins in solution near conditions of biological activity, is in progress from some years (Bonincontro et al., 1998; 2000; 2001). Our experiments have shown that dielectric spectroscopy is really a valid tool in studying structural and conformational modifications of proteins promoted by different agents, such as pH, temperature, and solvent composition. The technique is highly sensitive and able to evidence small effects on the overall conformation of the macromolecule.

In this article, following our previous works, we consider the effect of ethylene-glycol and glycerol at room temperature on two small globular proteins with very different structural and functional properties. Cyt-c is a compact globular protein whose structure as a single particle is well established both in crystalline and solution state (Scott and Mauk, 1996). On the contrary, the three-dimensional structure of lys consists of two domains: an α-domain with helical structure and a β-domain with predominantly β-sheets, separated by the active site cleft (Mc Cammon et al., 1976). It has been suggested that bifunctional molecules like alcohol can penetrate into the hydrophobic core of lys modifying its structural and dynamic properties (Segawa and Sugihara, 1984).

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Address reprint requests to A. Bonincontro, E-mail: adalberto.bonincontro@uniroma1.it.

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A peculiar behavior of lysozyme in water/ethylene-glycol mixtures is the main result of this work.

MATERIALS AND METHODS

Chicken egg-white lysozyme and horse heart cytochrome-c were obtained from Sigma (St. Louis, MS) and used without ulterior purification. The solutions for dielectric measurements were prepared by weighing and dissolving the proteins in water/ethylene-glycol and water/glycerol mixtures at different concentrations of the cosolvent (up to ~90% w/w). The concentrations of protein (lys 15 mg/ml; cyt-c 10 mg/ml) were chosen with reference to their solubility in ethylene-glycol (Knubovets et al., 1999). These values of concentration were low, but sufficient to ensure a good signal-noise ratio. The pH was controlled by a Crison micro-pH 2000. The lys solutions assumed a value of pH $\approx$ 6. At this pH value, the protein has a positive net charge of ~10 electron units and it is in the native state. The pH value of cyt-c solutions was adjusted to 6 adding microvolumes of concentrated HCl. The ionic strength of the samples was equivalent to a 2–4-mM NaCl solution, as deduced from conductivity measurements. The values of viscosity of the mixtures were obtained by literature (Tsierkezos and Molinou, 1998; Bonincontro et al., 2001).

In dielectric experiments permittivity ε' and dielectric loss ε'' were measured by means of a computer controlled Hewlett-Packard impedance analyzer, model 4194A, in the frequency range 10^5–10^8 Hz. The measuring cell, previously described, is a section of a cylindrical wave guide, which can be partially filled with the sample solution (Bonincontro et al., 1996). The system behaves as a wave guide excited far beyond its cut-off frequency mode and therefore only the stray field of the coaxial line–wave guide transition is used in the measurement. Cell constants were determined by measurements with electrolyte solutions of known conductivity, following well-defined procedures of literature (Athey et al., 1982). The errors on $\varepsilon'$and ε'' are within 1%. The relaxation contribution ε_d'' to the dielectric loss was calculated subtracting the conductivity term: $\sigma/\varepsilon_0\omega$, where σ is the ionic conductivity, $\omega = 2\pi f$ (f is the frequency of the applied electric field) and ε_0 is the vacuum dielectric constant.

A JASCO V-750 spectrophotometer with cells of 1-mm light path was used for the circular dichroism measurements. Both dielectric and optical experiments were performed at 20.0 $\pm$ 0.1°C.

RESULTS AND DISCUSSION

We measured permittivity ε' and dielectric loss ε'' of lysozyme dissolved in mixtures water/ethylene-glycol in the frequency range 10^5–10^8 Hz, varying the content of cosolvent from 0 to 90% in weight fraction. The concentration of the protein was 15 mg/ml.

At the frequencies used in the experiment the lysozyme solutions showed a well-defined dielectric dispersion (one curve is reported in Fig. 1 as an example). This behavior was just observed in water solution and interpreted with success as due to the orientation polarization of the dipole moment of the protein (Bonincontro et al., 1999, 2001). The experimental data were fitted with the real and imaginary part of the Cole-Cole equation (Hasted, 1973):

$$\varepsilon = \varepsilon_\infty + \frac{\Delta\varepsilon}{1 + i(f/f^*)^{(1-\alpha)}}, \qquad (1)$$

where ε is the complex dielectric constant, f is the measuring frequency, $f^* = 1/2\pi\tau$ is the relaxation frequency, i is the imaginary unit, $\Delta\varepsilon$ is the dielectric increment, ε_∞ is the permittivity extrapolated at high frequency, and α is an empirical parameter taking into account a spread of relaxation times.

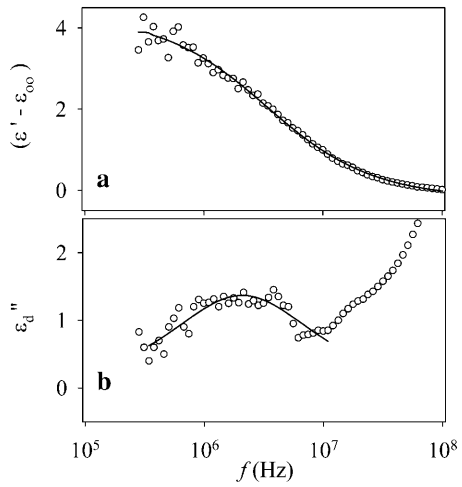

FIGURE 1 Increment of permittivity ($\varepsilon' - \varepsilon_{00}$) (*a*) and relaxation dielectric loss ε_d'' (*b*) of a solution of lysozyme in water/ethylene-glycol mixture. Fractional concentration of cosolvent 0.91 w/w. In the plot of ε_d'', the beginning of relaxation of the cosolvent is evident. Solid line is a Cole-Cole fit.

It has to be considered that the reliability of relaxation dielectric loss ε_d'' is very low in comparison to ε'. As a matter of fact the ionic conductivity term (see Materials and Methods section) is prevailing with respect to the dielectric contribution in the relaxation range of frequencies for samples with ionic conductivity of the order of 10^{-2} S/m as our protein solutions. This explains the large scattering of data in Fig. 1 *b*. The results of best fit on ε_d'' are in agreement, within the errors, with the ones of ε'. However, because of their large errors, we decided to use the values obtained by fitting the data of the real part ε' of the complex dielectric constant. These values are reported in Table 1.

Recently, we measured in the same range of frequency the permittivity of lysozyme dissolved in water-glycerol mix-

TABLE 1 Dielectric parameters of lys-water/ethylene-glycol solutions at different concentrations *X* (w/w) of cosolvent

X (w/w)	$\Delta\varepsilon$	τ (10^{-8} s)	α
0	4.2 ± 0.4	2.0 ± 0.1	0.19 ± 0.04
0.204 ± 0.004	3.4 ± 0.3	2.4 ± 0.1	0.30 ± 0.05
0.243 ± 0.005	3.3 ± 0.3	2.3 ± 0.1	0.30 ± 0.05
0.398 ± 0.008	3.8 ± 0.4	1.6 ± 0.1	0.33 ± 0.06
0.52 ± 0.01	3.9 ± 0.4	2.0 ± 0.1	0.38 ± 0.06
0.61 ± 0.01	3.5 ± 0.4	2.0 ± 0.1	0.37 ± 0.05
0.71 ± 0.01	3.2 ± 0.3	2.6 ± 0.2	0.38 ± 0.04
0.82 ± 0.02	3.8 ± 0.4	4.1 ± 0.2	0.39 ± 0.05
0.87 ± 0.02	4.1 ± 0.4	3.5 ± 0.2	0.40 ± 0.05
0.91 ± 0.02	3.9 ± 0.4	4.7 ± 0.3	0.41 ± 0.04

The uncertainties are statistical errors derived by the best fit procedure employed to process the experimental data of ε'. Level of confidence 95%.

tures, varying the content of the cosolvent up to 70% w/w (Bonincontro et al., 2001). It has to be noted that glycerol and ethylene-glycol are generally considered equivalent as cryo-protectants with no regard for their specific interactions with proteins. However the dielectric behavior of lysozyme in the two cosolvents is very different. In Fig. 2 we report the ratio η/τ between viscosity of the solvent and relaxation time of the protein as a function of the weight fraction of cosolvent for glycerol (Fig. 2 *a*) and ethylene glycol (Fig. 2 *b*). In water-glycerol mixtures τ is proportional to η. This means that the size and the geometry of the protein remain substantially unchanged. On the contrary, in ethylene-glycol, the ratio η/τ is not constant, denoting that a conformational modification occurs. As a matter of fact we may calculate the effective hydrodynamic radius r of the globular protein from the relaxation time τ, using the equation:

$$\tau = \frac{4\pi\eta r^3}{KT}, \tag{2}$$

where η is the viscosity of the solvent, K the Boltzman's constant, and T the absolute temperature (Grant et al., 1978). The result is shown in Fig. 3 *a*. The use of Eq. 2 in the case of mixtures may be critical, involving macro versus micro viscosity considerations. However we verified in a recent work that the diffusive behavior of lysozyme in water-glycerol mixtures is coherent with the Debye-Stokes-Einstein model, at least for glycerol concentrations up to 60–70% w/w (Bonincontro et al., 2001). The estimated value of hydrodynamic radius is in agreement with literature and it is not influenced by the glycerol concentration.

In Fig. 3 *b* we report the electric dipole moment μ, estimated from the dielectric increment $\Delta\varepsilon$ by the Oncley formula:

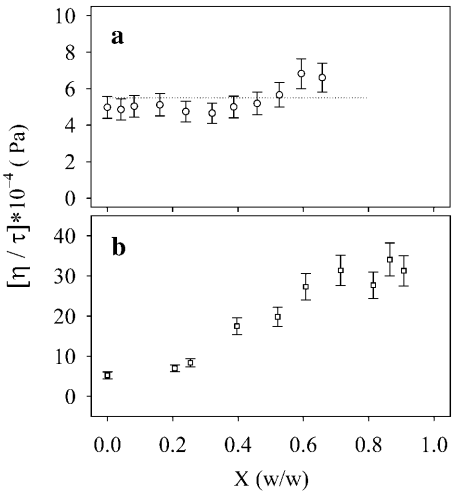

FIGURE 2 Ratio between solvent viscosity and relaxation time of solute for lysozyme in water/glycerol (*a*) and water/ethylene-glycol (*b*) mixtures as function of weight fraction of cosolvent. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data. Level of confidence 95%. Dotted line for visual aim.

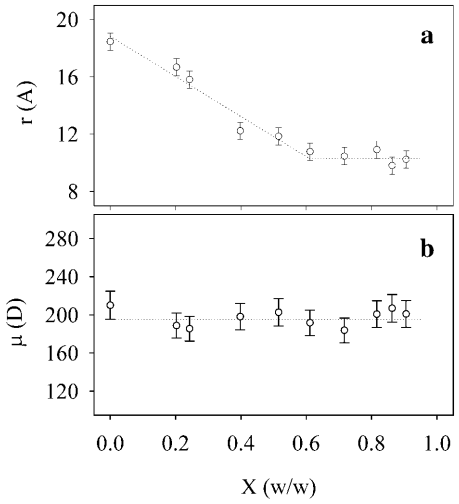

FIGURE 3 Hydrodynamic radius (*a*) and electric dipole moment (*b*) of lysozyme in water/ethylene-glycol mixtures as function of weight fraction of cosolvent. Calculations on the basis of the dielectric parameters of Table 1, obtained fitting the experimental data with one Cole-Cole dispersion. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data. Level of confidence 95%. Dotted lines for visual aim.

$$\mu^2 = \frac{2\varepsilon_0 MKT\Delta\varepsilon}{Ncg}, \tag{3}$$

where M is the protein molecular mass expressed in kilodalton, T is the temperature expressed in Kelvin, K is the Boltzmann constant, ε_0 is the vacuum dielectric constant, N is the Avogadro number, c is the protein concentration expressed in kg/m^3, and g is the molecular correlation parameter generally assumed as 1 in dilute protein solutions (Pethig, 1979). Our panorama appears really inconsistent. A so very large decrease of dimension (the hydrodynamic radius assumes almost a half-value) is impossible for a very compact protein such as the lysozyme. In any case the trend almost constant of the dipole moment is inconsistent with the variation of r.

It has to be observed, moreover, that measurements performed at 60% w/w in the far-ultraviolet (UV) region indicate that the secondary structure of the protein is well preserved under these experimental conditions (Fig. 4 *a*). The small changes in the molar ellipticity detected in the near-UV region (Fig. 4 *b*) between water and water/ethylene-glycol solutions might be ascribed to minor tertiary structure variations.

This behavior seems peculiar of the system lysozyme/ethylene-glycol and it could be related to specific structural characteristics of this protein. As a matter of fact, an experiment carried out on cytochrome-c dissolved in water/ethylene-glycol mixture shows a very different panorama. In Fig. 5 we report the hydrodynamic radius (*a*) and the electric dipole moment (*b*) of cytochrome as a function of content of ethylene-glycol in the solvent, obtained by dielectric

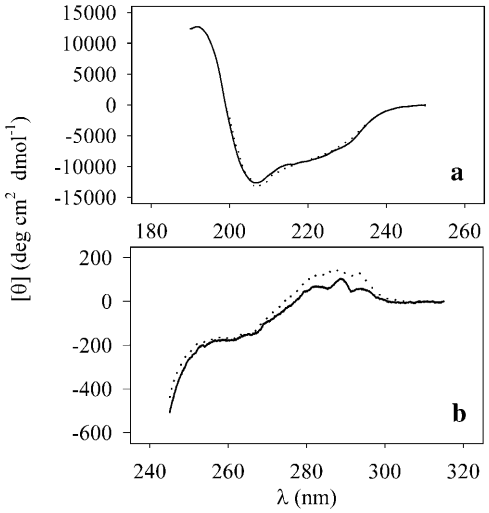

FIGURE 4 Circular dichroism spectra in the far-UV (*a*) and near-UV (*b*) in water (*solid line*) and in water/ethylene-glycol solutions (*dotted line*). Fractional concentration of cosolvent 0.61 w/w.

relaxation measurements as done with lysozyme. The measurements were carried out at $pH \approx 6$ and with a protein concentration of 10 mg/ml, varying the fraction of ethylene-glycol in the range 0–60% w/w where the lysozyme has shown the maximum hydrodynamic radius variation. In particular the pH fixed to 6 is well far from the value of 9, assumed by the cytochrome in water and very near to the conditions of the alkaline transition (Bonincontro et al., 2001). As we can see, both hydrodynamic radius and dipole moment of this protein with a monodomain structure remain constant within the errors, denoting the absence of any

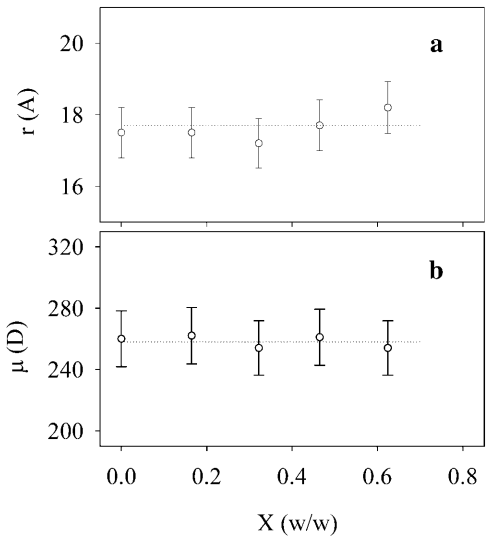

FIGURE 5 Hydrodynamic radius (*a*) and electric dipole moment (*b*) of cytochrome-c in water/ethylene-glycol mixtures as function of weight fraction of cosolvent. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data. Level of confidence 95%. Dotted lines for visual aim.

structural and conformational effect. The same result was obtained in an analogous experiment performed on cyt-c dissolved in water/glycerol mixtures (data not shown).

As it is known, the three-dimensional structure of lysozyme is formed by two domains (subunits) with prevailing α-helix and β-sheet organizations, respectively. The two subunits are connected by two segments constituted by peptide residues and realize a pocket containing the active site of the enzyme (Mc Cammon et al., 1976). The structure allows to the domains a hinge-bending mode that appears in the native state as vibrations at very high frequencies. The model has been used in simulations of molecular dynamics, but few experimental evidences exist (Hayward et al., 1997; Haliloglu and Bahar, 1999).

The cleft between the two domains includes the binding site for the substrate, which is a polysaccharide and is largely hydrophilic. The ethylene-glycol is rather hydrophilic itself and might actually mimic a substrate. We then hypothesized, as a possible interpretation, that ethylene-glycol enters in the pocket reducing the strength of bonds between the two subunits in such a way that they can respond to the action of an applied electric field as free particles. This means that we measure a sort of average radius between compact particles in the native state and single subunits. Therefore a pseudo-transition occurs. As a matter of fact, a partial opening of the protein structure could explain the observed small changes of CD spectra in the near-UV region (Fig. 4 *b*).

We decided to apply the classical model used in the interpretation of denaturant processes considering the co-existence of two states, native and denatured, respectively, which maintain their conformation until only one species remains present. This means that in the interval where the radius changes from 18.7 Å (protein in native state) to 10.5 Å (free subunits) the measured dielectric constant has to be considered as a linear combination between the contributions of the two populations existing during the transition. In particular:

$$\varepsilon = \varepsilon_\infty + X_s \frac{\Delta\varepsilon_s}{1 + i(f/f_s^*)^{(1-\alpha_s)}} + (1 - X_s)\frac{\Delta\varepsilon_n}{1 + i(f/f_n^*)^{(1-\alpha_n)}}, \tag{4}$$

where the subscript s refers to the open protein population of molar fraction X_s and the subscript n refers to native particles. The transition appears completed at a weight concentration of ethylene-glycol in the solvent of ~60%. Over this concentration r remains constant to the value of 10.5 Å up to 90% of ethylene glycol, as a consequence of the constant value of the quantity (η/τ) in this range of concentration of the cosolvent.

We inserted the values of dielectric parameters of the two coexisting species in the real part of Eq. 4. The dielectric parameters $\Delta\varepsilon_n$, α_n and $\Delta\varepsilon_s$, α_s, referred to native proteins and subunits, respectively, were considered constant coherently with the adopted model of transition, whereas the

frequencies of the two species were corrected for the viscosity of the solvent. The choice of the real part of the equation was due to the better definition of ε' as respect to ε_d''. As a matter of fact, ε' is directly measured whereas ε_d'' is obtained by subtracting the ionic contribution (see Materials and Methods section). For any content of cosolvent Eq. 4 describes very well the experimental data with a defined appropriate value of the X_s parameter. Therefore we calculated the molar fraction X_s of the open proteins with separated subunits as a function of the cosolvent concentration. The results are shown in Fig. 6 *a*. The typical trend of a cooperative process, characterized by a two-state transition is evident. The actual concentration of oscillating dipoles is then

$$\frac{c'}{M} = 2X_s\frac{c}{M} + (1 - X_s)\frac{c}{M} = (1 + X_s)\frac{c}{M}. \qquad (5)$$

We calculated again the dipole moments μ by Eq. 4 using the concentrations derived by Eq. 5. The results are in Fig. 6 *b*. As it can be seen, the trend of μ is now consistent with *r*. The value decreases up to 60% of ethylene-glycol and after it remains constant.

CONCLUSIONS

In this work we compared the effects at room temperature of two solvents known as cryo-protectans, ethylene-glycol and glycerol, on conformational and dynamical properties of two well-known proteins, lys and cyt-c, with distinctive structural properties. Our data show that glycerol does not modify significantly the conformation of lysozyme. On the contrary ethylene-glycol strongly affects the dielectric response at radio frequencies of lysozyme, denoting a relevant structural effect. Moreover cytochrome-c is stable in the presence both of ethylene-glycol and glycerol. Therefore, a specific effect of ethylene-glycol on conformation and dynamic of lys exists. The data are coherently interpreted hypothesizing that glycol molecule wedges between and separates the two domains of lys making them rotationally independent. This mechanism, of course, has to be further verified by measurements on other systems.

It has to be noted that such effect is evidenced by variations of the dielectric response whereas only small changes in the tertiary structure are observed in the near-UV circular dichroism spectra.

Finally dielectric spectroscopy again appears as a unique tool for studying subtle conformational changes resulting from interactions of proteins with the solvent.

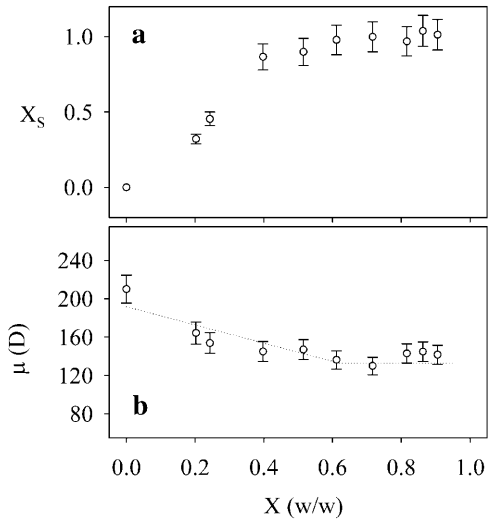

FIGURE 6 Molar fraction of open lysozyme molecules calculated by Eq. 4 in the text (*a*) and average electric dipole moment of lysozyme in water/ethylene-glycol mixtures, calculated considering the transition of the protein from native to open state (see text) (*b*) as function of weight fraction of cosolvent. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data. Level of confidence 95%. Dotted line for visual aim.

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