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Water and Protein Dynamics in Protein – Water Mixtures over Wide Ranges of Composition

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ABSTRACT

Water and protein dynamics in two globular protein-water systems, water-lysozyme and water-BSA (bovine serum albumine), were studied by differential scanning calorimetry (DSC), dielectric relaxation spectroscopy (DRS) and thermally stimulated depolarization currents (TSDC) techniques. Water equilibrium sorption isotherms (ESI) measurements were also recorded at room temperature. The samples covered a wide range of composition, from practically dry solid pellets (2wt% of water) to dilute solutions (82wt% of water). Crystallization and melting events of water were studied by DSC and the amount of uncrystallized water was calculated. The evolution of dynamics with hydration level was followed for various dielectric relaxation processes, the emphasis being given to relaxation processes of polar groups on the surface of the proteins and of uncrystallized water molecules. A relationship between the formation of a conductive percolating water cluster and the saturation of the water ν process was found.

Index Terms — **hydrated protein, uncrystallized water, glass transition, dielectric relaxation**

1 INTRODUCTION

The biological function of globular proteins is inextricably interdependent with water [1]. The hydration properties of proteins and water and protein dynamics in protein-water mixtures have been studied by a variety of experimental techniques. The results indicated the significant influence of protein-water interactions on the structure, the dynamics and the biological function of proteins [2-7]. A minimum amount of water is necessary for enzymatic activity of a protein and dielectric results indicated a correlation between the onset of enzymatic activity and of a percolation type displacement process of protons on single macromolecules [4]. Thermal and dynamic studies of hydrated proteins revealed the presence of a thermal glass transition in the temperature range from about -110 to -70 ^{o}C, depending on the protein, the hydration level and the experimental technique employed [5-7]. A dynamical transition was also observed and was associated with an abrupt onset of atomic displacements on the microscopic length and time scale, usually probed by quasielastic neutron scattering in the range from about -75 to -35 ^{o}C [8]. The mechanism of the dynamical transition, which should not be confused with the glass transition, remains a subject of debate [7, 8].

Dielectric spectroscopy is particularly suited to the investigation of partially hydrated proteins [2, 9]. The wide frequency range of the dielectric spectrum means that it is possible to characterize the material over broad ranges of both time and length scale. Short-range charge hopping along a single macromolecule, protein backbone flexibility and the reorientation polarization of surface hydration water can be used to understand the properties of the system at the molecular scale. All of these properties are dependent to some degree on the residual concentration of water in the protein and the specific interaction between the water of hydration and the protein.

In this work, glass transition and water and protein dynamics were studied in mixtures of water and two globular proteins, BSA and lysozyme, in wide ranges of water content, both solutions and hydrated solid samples. Preliminary results on aqueous mixtures of lysozyme and BSA proteins have already been published [10, 11]. Thermal glass transition and crystallization and melting events of water were studied by

differential scanning calorimetry (DSC). Water and protein dynamics were followed over wide ranges of frequency and temperature by two dielectric techniques, broadband dielectric relaxation spectroscopy (DRS) [12] and thermally stimulated depolarization currents (TSDC). TSDC is a special dielectric technique in the temperature domain, which corresponds to measuring dielectric loss at a fixed low frequency in the range of 10^{-2}-10^{-4} Hz and is characterized by high sensitivity and high peak resolving power [13]. In addition, equilibrium water sorption isotherms (ESI) measurements were performed at room temperature. The results obtained by the various techniques are discussed in analogy to similar results obtained for polymers and other glass-forming systems. Our purpose here is mainly to identify underlying molecular processes which are common in the two hydrated proteins and to follow their evolution with increasing water content.

2 EXPERIMENTAL

2.1 MATERIALS

Lysozyme from chicken egg white (62970 Fluka, Mr~14.600) in the form of dialyzed and lyophilized powder and albumin from bovine serum (BSA) in the form of lyophilized powder (Sigma 3294, Mr~66.000) were bought from Sigma-Aldrich and used as received. Water with low conductivity (in the order of 10μS/cm) was used for preparation of solutions for DSC and for dielectric measurements. In this article we refer to the water fraction h_w (grams of water per grams of hydrated protein) or to the water content h_d (grams of water per grams of dry protein) of the samples, depending on the method of analysis. For dielectric measurements, mixtures of protein and water were prepared either in the form of solution ($h_w > 0.4$) or in the form of compressed solid pellets ($h_w \leq 0.4$). For solutions, protein was dissolved in water and, for better dissolution, the mixtures were kept at 4°C for two days before the dielectric measurements. In the case of the solid samples (hydrated pellets) an amount of protein powder ~100 mg was compressed to a cylindrical pellet of thickness 0.6-0.8mm and diameter of about 13mm. Each solid sample was hydrated to the required degree by equilibration for more than 3 days above saturated salt solutions in sealed jars.

2.2 EXPERIMENTAL TECHNIQUES

DSC measurements were performed in a Mettler Toledo 823e calorimeter on samples between 5 and 15 mg. For DSC measurements BSA powder was dried in vacuum for 72h to remove traces of humidity. 30% wt aqueous solution of BSA was prepared by dissolving a specified quantity into water. The solution was placed into open aluminium pans (40 μl) at room ambient for different times to obtain several water contents. After that, the pans with hydrated protein were sealed. The hydrated protein samples were cooled down from 25°C to -150°C followed by a heating scan up to 40°C, both at 10°C/min.

For dielectric measurements the solid samples were placed between two electrodes forming a cylindrical capacitor 12mm in diameter. The solutions were placed between electrodes 20 mm in diameter kept apart by silica spacers 50 μm in thickness. TSDC measurements were carried out in the temperature range 123-273K using a Keithley 617 electrometer in combination with a Novocontrol sample cell. For DRS measurement an Alpha Analyzer in combination with the Novocontrol Quatro Cryosystem were used. Measurements of the complex dielectric function were taken isothermally as a function of frequency in the range of 10^{-1} to 10^{6} Hz for different temperatures in the range 123-273K in steps of 5K.

3 RESULTS AND DISCUSSION

3.1 ESI MEASUREMENTS

Results of water sorption measurements are shown in Figure 1: water content h_d against water activity α_w at room temperature (25°C) for a lysozyme and a BSA sample. As seen in Figure 1, an initial linear region for water activity up to 0.8 is observed, followed by a departure from linear behaviour for α_w >0.8, which is typical for hydrogels and is explained in terms of clustering of water molecules [14].

The Guggenheim-Anderson-de Boer (GAB) equation [15] :

$$\frac{h}{h_m} = \frac{cfa_w}{(1 - fa_w)[1 + (c-1)fa_w]} \quad (1)$$

was fitted to the experimental data. In equation (1) h_m is the water content corresponding to water molecules directly attached to sorption sites (primary hydration sites, first sorption layer), whereas c and f are parameters related to the energy difference between water molecules in the first sorption layer and in the second and higher sorption layers, and between water molecules in the second and higher sorption layers and bulk water, respectively. The GAB fit is satisfactory. For h_m, which is the most significant parameter in the equation, values of 0.088 and 0.073 were obtained for lysozyme and BSA, respectively. Taking into account that the numbers of amino acid residues corresponding to each lysozyme and BSA macromolecule are 129 and 607, respectively, we estimate that the primary hydration sites (accessible to molecularly distributed water molecules) are 71 amino residues in lysozyme and 270 amino residues in BSA macromolecules.

Interestingly, the observed water uptake is similar for both systems at low water activities where clustering of water is expected to be negligible [14]. At higher water activities BSA absorbs higher amounts of water, in terms of h_d, compared to lysozyme, and this occurs for water content values higher than 0.15. This fact supports the idea that despite the difference in molecular weights the water uptake is different only if there is a concentrated amount of water molecules out of the hydration shell (here for α_w >0.8), reflecting the increasing swelling degree with increasing molecular weight, as shown previously for various synthetic polymers [16].

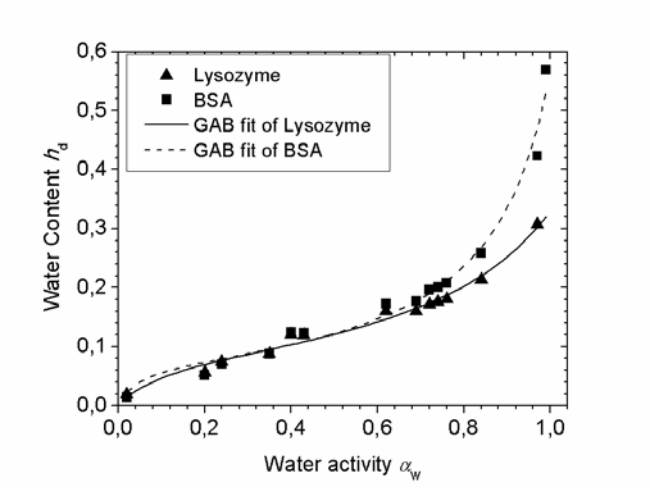

Figure 1. Water content h_d against water activity α_w at 25°C for a lysozyme and a BSA sample. Points are experimental data and the solid and dashed lines are the fits of GAB equation (1) to the BSA and lysozyme data, respectively.

3.2 DSC MEASUREMENTS

DSC cooling and heating scans, both at a rate of 10°C/min, were recorded for protein-water mixtures at various hydration levels. Cooling and heating thermograms recorded on several BSA-water mixtures, characterized by water fraction h_w, are shown in Figures 2a and 2b, respectively. The scale of Figure 2 allows us to observe clearly the crystallization and melting peaks of water. The first water fraction h_w in which crystallization is observed on cooling is 0.295 whereas a clear melting peak on heating is first observed at h_w=0.230. In the heating thermograms in Figure 2b we also observe a broad glass transition step for most of the samples, as well as a cold crystallization exothermic peak above the glass transition, for water fractions that exhibit no crystallization of water during cooling. A magnification of the thermograms during heating for five characteristic water fractions, h_w=0.070, 0.102, 0.184, 0.23 and 0.26, is shown in Figure 2c. For h_w=0.102 and 0.184, only the glass transition can be seen, as a heat capacity step. No glass transition had been found for the lower water fractions studied, that is for h_w= 0.070 and the dry sample. For h_w=0.23 and 0.26, the glass transition is followed by a cold crystallization peak of water and a subsequent melting peak, the former being more clear in case of h_w=0.26.

DSC results on lysozyme-water mixtures showed a cold crystallization region in the range of 0.179<h_w<0.21, which is comparable to but at lower h_w values than the one corresponding to BSA-water mixtures (0.23<h_w<0.30, see above), whereas no glass transition step was observable in the heating thermograms [10]. Another difference between BSA- and lysozyme-water mixtures was the shape of the crystallization and melting peaks, which were more complex in the case of lysozyme-water mixtures at high hydration levels where bulk water dynamics is dominant, i.e. where the melting temperature was close to 0 °C, resembling the one of bulk water. By comparative studies with DSC and FTIR measurements it is shown that multiple fusion peaks correspond to distinct water populations with respect to their connectivity [17, 18] whereas the maximum temperature of a single fusion peak is independent of the host material surface and gives information on the size distribution of the crystal phase, in case of water within the pores of organosilane-modified silica [19]. By these observations we may assume that, for lysozyme-water mixtures crystallization of different water populations with respect to their connectivity or their size distribution occurs, leading to the coexistence of distinct melting peaks and this holds for almost all the water fraction range studied here. On the contrary, BSA-water mixtures show a single melting peak, which suggests that the population of water that crystallizes upon cooling consists mainly of one kind of water clusters, with respect to their connectivity and/or their size at each hydration level. This may be explained in terms of the higher degree of swelling for BSA, as shown by ESI measurements (see above), which causes a more homogeneous distribution of water at high water contents.

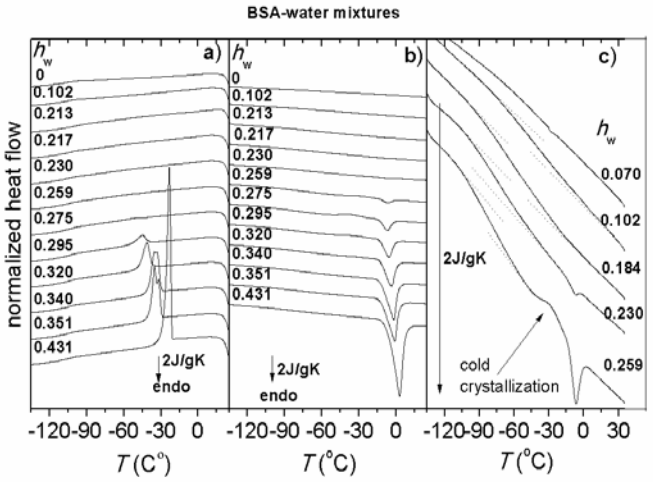

Figure 2. Normalized heat flow during cooling (a) and during heating (b), both at 10°C/min, in BSA-water mixtures at different water fractions h_w indicated on the plot. c) A magnification in the area of the glass transition during heating scan, for characteristic BSA-water mixtures of water fraction h_w indicated on the plot.

The fraction of uncrystallized water in the BSA and lysozyme-water mixtures, $h_{w,ucw}$, has been calculated by the enthalpy of melting and the melting enthalpy of bulk water (333.55 J/g [20]) as described in [10]. The composition diagram of Figure 3 shows that the fraction of uncrystallized water in the hydrated BSA samples remains stable at about 25% in the cold crystallization region and then is equal to about 24% for hydration levels where crystallization occurs during cooling, whereas for lysozyme-water mixtures the fraction of uncrystallized water remains almost constant at about 20% in the whole h_w range studied.

Our data allow us to estimate the number of crystallized and uncrystallized water molecules per amino acid residue of both globular proteins as a function of the total water fraction and the corresponding plots are shown in Figure 4.

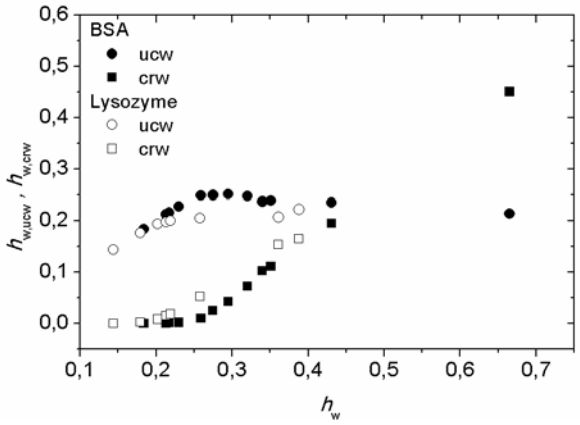

Figure 3. Fractions of uncrystallized water ($h_{w,ucw}$, circles) and crystallized water ($h_{w,crw}$, squares) in the lysozyme-water (open symbols) and BSA-water (closed symbols) mixtures, against total water fraction h_w.

We observe that at h_w~0.38 for both proteins the ratio $n_{UCW}/Res_{protein}$ is equal to 2. Recalling that this water fraction corresponds to the formation of the primary hydration shell of the globular proteins [3, 4] we conclude that the formation of the primary hydration shell is completed when 2 water molecules correspond to each amino acid residue of the swelled protein macromolecule. For further increase of total water fraction the fraction of uncrystallized water remains constant implying the continuous reorganization of adsorbed water.

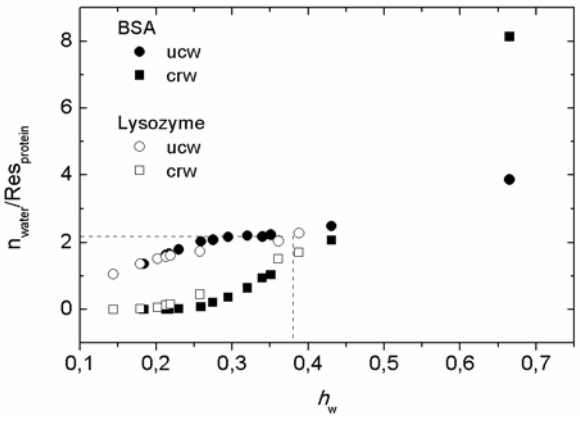

Figure 4. The ratio of adsorbed water molecules per proteinic residue in the case of uncrystallized water (ucw, circles) and crystallized water (crw, squares) in the lysozyme-water (open symbols) and BSA-water (closed symbols) mixtures, against water fraction h_w.

3.3 TSDC MEASUREMENTS

Figure 5 shows normalized TSDC thermograms for several lysozyme- and BSA-water samples at comparable hydration levels. Starting at low water fractions and low temperatures, a broad peak is observed in the solid pellets, already for samples of h_w=0.020 (at about -100°C for lysozyme and -110°C for BSA), which shifts to lower temperatures with increasing water fraction (at about -125°C for hydrated lysozyme and BSA proteins with h_w=0.148 and 0.152, respectively) and increases in magnitude. In agreement with previous work on proteins [21, 22] this peak is attributed to a local, secondary relaxation of small polar groups of the biopolymer, plasticized by water. For $h_w > 0.20$, it is likely that additional contributions, probably due to either uncrystalized or crystallized water, are interfering in the low temperature side of the TSDC diagram, overlapping with the plasticized peak observed at low h_w, as is suggested by the superlinear increase of peak magnitude for that h_w region. In the two solutions with the highest water fractions, h_w=0.82 and 0.80, for lysozyme and BSA, respectively, a strong peak is observed at about -125°C. Based on previous work with TSDC measurements on various forms of polycrystalline ice (pure ice, ice microcrystals dispersed in oil and frozen aqueous saccharide solutions) [21], this peak is attributed to relaxation in bulk ice.

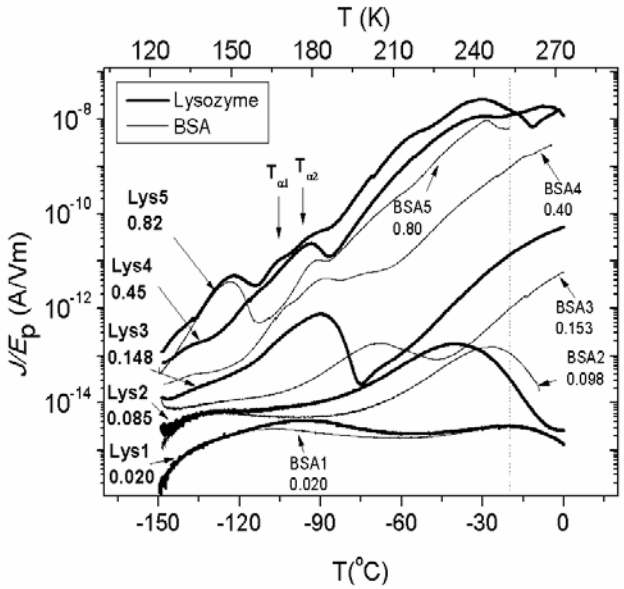

Figure 5. Normalized TSDC thermograms (density of depolarization current divided by polarizing electric field, J/E_p) against temperature T for the lysozyme-water and BSA-water samples of water fractions h_w indicated on the plot. The vertical line at -20°C indicates the polarization temperature T_p.

For the lysozyme sample with h_w=0.085 the peak which is attributed to the α relaxation associated with glass transition of the protein [10] is seen at about -40°C. It is known that the peak temperature, T_α, in TSDC thermogram recorded at the glass transition temperature region is a good measure of the calorimetric T_g due to similar time scales of DSC and TSDC methods [13, 21]. An analogous peak for the BSA sample of h_w=0.098 is located at about -30°C. It is obvious by this point that the glass transition temperature (T_g) seems to be higher for BSA. This trend holds also for lysozyme and BSA samples of h_w=0.148 and 0.153, respectively. For hydration levels where a part of water crystallizes during cooling, a splitting of the α peak was observed for both proteins (indicated by the two arrows in Figure 5), the evolution with increasing water fraction being, however, different in the two systems. In [10] and for lysozyme-water mixtures the additional contribution

(the low temperature peak) was attributed to a population of ice crystals with larger amount of defects than in bulk ice. For the BSA-water mixtures the results suggest rather the occurrence of microphase separation [11]. For the clarification of that point more experimental data are needed. Nevertheless, the strong plasticization of the α peak and the stabilization of the contributions for hydration levels where crystallization events of water set in, are evident for both systems. This fact probes the discussion of the data in terms of the phase diagram of the system as it is described in reference [23].

3.4 DRS MEASUREMENTS

Figure 6 shows isochronal plots of dielectric loss, which were recorded by DRS isothermally, at a low frequency, f=0.2 Hz. These preliminary DRS data indicate that the processes that were identified employing TSDC method are also present in DRS spectra and in good agreement with TSDC. A relaxation peak of polar groups plasticized by water for samples with h_w=0.020 and 0.070 is seen for both systems at about -80 ^oC and -100 ^oC, respectively. The α relaxation peek is located at about 0 ^oC for BSA and -10 ^oC for lysozyme of h_w=0.07and shifts to lower temperatures with increasing h_w. Finally, for higher hydration levels, the peek attributed to bulk ice is located at about -110 ^oC and contributions due to the α relaxation process are observed at higher temperatures for both systems.

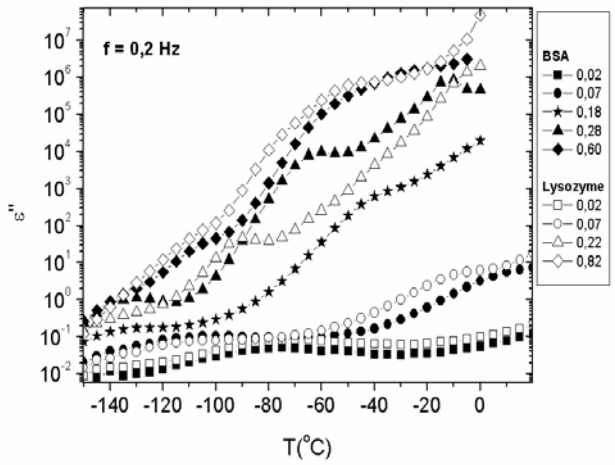

Figure 6. Isochronal plots of dielectric loss at the frequency of f=0.2Hz for the lysozyme-water and BSA-water samples of water fractions h_w indicated on the plot

Focusing now on low temperatures and low hydrations we study the dynamics of the secondary relaxation process of the polar groups on the protein surface and its relationship with the adsorbed water molecules. In Figure 7 we show the real part, ε′ (Figure 7a) and the imaginary part, ε′′ (Figure 7b) of the dielectric permittivity measured on lysozyme-water samples at various water fractions indicated on the plot. The corresponding plots measured on BSA-water samples are shown in Figures 8a and 8b.

The spectra of dielectric losses show that the secondary relaxation peak is strongly plasticized by the water for both proteins. The peak is shifted to higher frequencies with increasing h_w and becomes water fraction independent located at f ~ 10kHz for 0.13> h_w >0.18 and 0.07> h_w >0.20 in the case of BSA and lysozyme, respectively. In addition, we observe that the saturation of the secondary relaxation peak at elevated water fractions is accompanied with the increase of dielectric losses at the low frequency side of the spectra shown in Figures 7b and 8b. Indeed, for BSA with water fraction of 0.18 we observe that the secondary peak is shifted to the frequency of ~ 10 kHz while the dielectric loss is increasing at low frequencies implying that conductivity effects contribute to the spectra. Similar characteristics of the frequency dependence of the dielectric loss with increasing water fraction are also observed for the lysozyme-water samples (the water levels in this case being of limited number, however).

The increase of the dielectric losses is accompanied with increasing polarization effects as is indicated by the increase of the measured dielectric permittivity at low frequencies and high water fractions (Figures 7a and 8a).

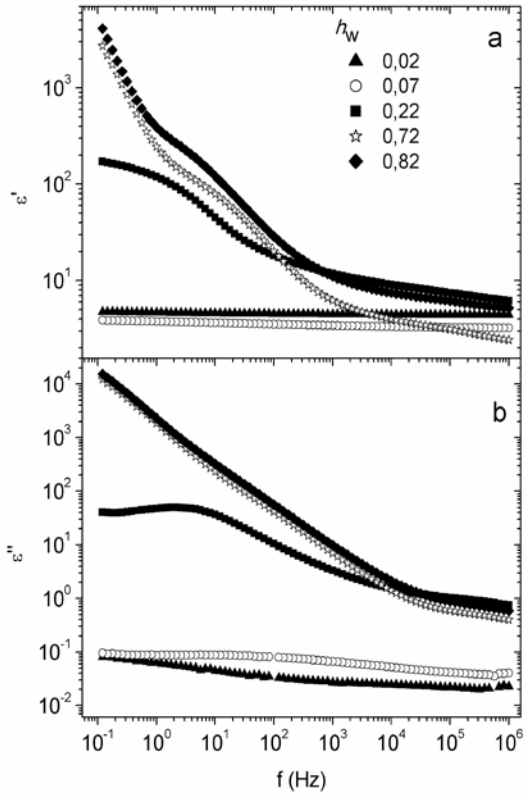

Figure 7. Frequency dependence of real (a) and imaginary part (b) of dielectric function measured at -80 ^oC on Lysozyme-water samples with the water fractions indicated on the plot.

The abrupt increase of electrical conductivity for water fractions in the range of 0.07 – 0.22 for lysozyme and 0.13 – 0.18 for BSA (which will be discussed in the next paragraph and supported also by TSDC measurements [10, 11]) is clearly shown in Figure 9, where the frequency dependence of ac conductivity measured on lysozyme- and BSA-water systems at several water fractions for both systems is shown.

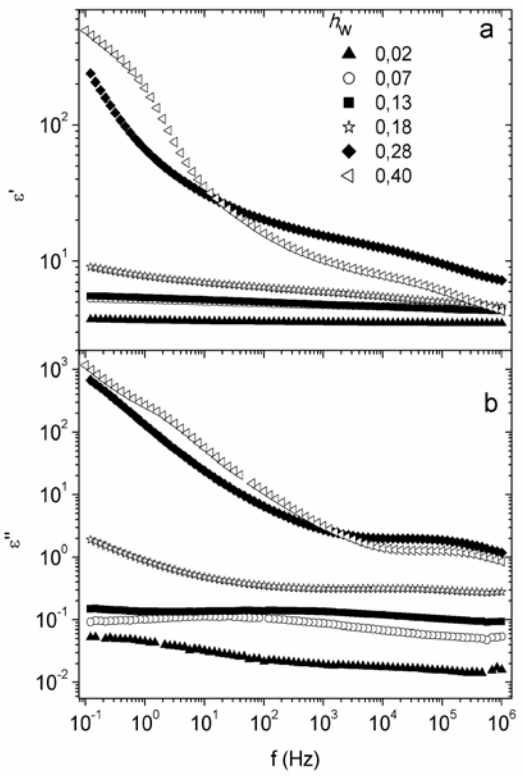

Figure 8. Frequency dependence of real (a) and imaginary part (b) of dielectric function measured at -80 °C on BSA-water samples with the water fractions indicated on the plot.

ac conductivity (actually the real part of the complex conductivity) was calculated from the measured dielectric loss ε′′(f) by

$$\sigma_{ac}(f) = \varepsilon_o (2\pi f)\, \varepsilon''(f) \quad (2)$$

where ε_o is the permittivity of free space. In Figure 9 we can observe that the frequency dependence of conductivity changes significantly for the BSA/water system when h_w increases from 0.13 to 0.18. Similarly, the $\sigma_{ac}(f)$ curves are significantly different for the lysozyme/water system with h_w =0.07 and h_w =0.22. The change of the $\sigma_{ac}(f)$ curves pointing to a dc plateau for water fractions higher than a critical value implies the change of conduction mechanism at high hydration levels with the formation of a percolating conductive path. The activation of such long length scale conduction process leads also to polarization effects (charge accumulation) as is revealed by the increase of the ε′ values at low frequencies and high hydrations (Figures 7a, 8a). This polarization process might be of the form of double layer polarization process, often observed in dielectric studies of hydrogels, biomacromolecules and other water containing systems [2, 12, 24, 25]. The interesting point here is the observation that the formation of the conductive percolating cluster and the low frequency polarization processes are related to each other, with the secondary dipolar process shown in Figures 7b and 8b to saturate at frequencies close to 10 kHz at -80°C, i.e. at the time scale of the so called ν process of water molecules [26]. Our results suggest that the ν process of water is no more plasticized by further addition of water beyond the percolation threshold. This result is consistent with the proposal that the ν process of water molecules includes rotation of molecules combined with translation after the breakage of two hydrogen bonds [26].

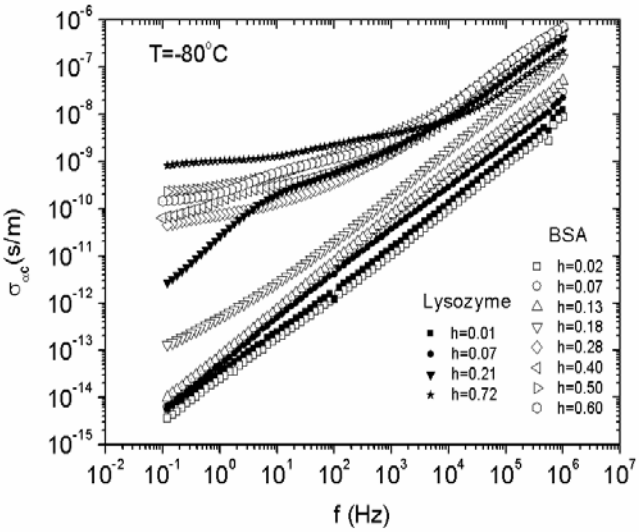

Figure 9. Frequency dependence of σ_{ac} conductivity measured at -80°C for hydrated BSA and lysozyme samples with the water fractions indicated on the plot.

To further investigate the interrelation between conduction and polarization effects in hydrated proteins with h_w higher than a critical value, we plot in Figure 10 the values of ε′ and σ_{ac} measured at the frequency of 1Hz, at -80°C for BSA and lysozyme samples as a function of the water fraction. We observe that ε′ and σ_{ac} values show clearly similar h_w dependence indicating that conductivity and low frequency polarization processes are interrelated. The plot of BSA data shows clearly that the h_w range where both polarization and

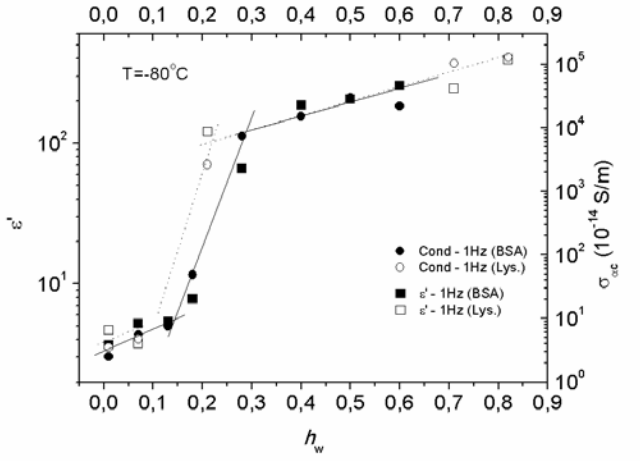

Figure 10. ε′ and σ_{ac} values measured at f = 1Hz at the temperature of -80°C on hydrated BSA and lysozyme samples as a function of water fraction h_w. The lines are guides for the eye.

conductivity effects increase drastically, is between 0.13 and 0.25. The data of lysozyme, although more rare, agree well with those of BSA, especially at high water fractions, implying the common nature of conduction process in globular proteins. Closer inspection of the data for low water fractions may suggest that in the case of lysozyme the formation of the water conductive percolating cluster occurs at lower h_w values than in BSA, i.e. within the range 0.10 – 0.20.

4 CONCLUSIONS

Glass transition and water and protein dynamics were studied in two systems of hydrated globular proteins, lysozyme and BSA, by differential scanning calorimetry (DSC), dielectric relaxation spectroscopy (DRS), thermally stimulated depolarization currents technique (TSDC) and water equilibrium sorption isotherms (ESI) measurements at room temperature. BSA exhibited a larger swelling degree as compared to lysozyme at high hydration levels. Dielectric measurements revealed the α relaxation process associated with the glass transition of the hydrated protein for both systems. For water fractions where no crystallization of water occurs during cooling our results showed a strong plasticization of T_g.

Our results indicate the existence of interrelations between the formation of a conductive percolating water cluster and the saturation of the reorientation process of uncrystallized water molecules at the time scale of the water ν process. The corresponding critical water fraction ranges have been determined. For higher water fractions polarization processes strongly related to charge conduction processes have been recorded.

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