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Lysozyme Hydration in Concentrated Aqueous Solutions. Effect of an Equilibrium Cluster Phase

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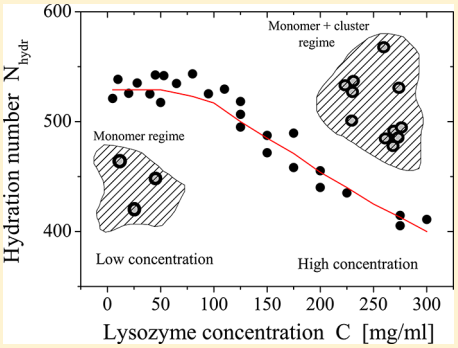
ABSTRACT: Water close to proteins plays a key role in determining their structural and functional properties. Despite being a subject of considerable interest, the characterization of hydration water, as far as its total amount is concerned, is still controversial and its influence on protein structure and folding is not yet fully understood. In this work, we have investigated the dielectric properties of lysozyme aqueous solutions over the frequency range where the orientational polarization relaxation of the aqueous phase occurs (from 500 MHz to 50 GHz). Measurements extend over a wide concentration range, up to 300 mg/mL, corresponding to a volume fraction of the order of 0.4. The analysis of the dielectric spectra, based on the decrease of the dielectric increment of the γ-dispersion as a function of protein concentration, allows us to estimate the *total* amount of hydration water (both *bound* water and *loosely bound* water) present in the system investigated. We observe a decrease of the hydration number as a function of the protein concentration. This behavior is well accounted for by considering the formation of small equilibrium clusters with aggregation number of some units, as recently reported by Stradner et al.[1] on the basis of small-angle X-ray and neutron scattering measurements.



1. INTRODUCTION

Because water bound to proteins plays a key role in determining various biochemical properties, the hydration of proteins is a subject of considerable interest and has been a field of intense research over several decades,[2−6] and hydration dynamics of macromolecules, particularly proteins and DNA, has been widely investigated.[7−11]

The hydration shell surrounding a protein molecule encompasses different types of water, the main of which can be referred to as *bound water*, consisting of water molecules rigidly bound to the protein surface, and as *loosely bound water*, whose molecules experience a faster rotational and translational diffusion. Between the two species, a dynamical exchange may occur. These two water constituents are called by Nandi and Bagchi[12] *biological water*. A quantitative estimation of the hydration water is of considerable biochemical and physicochemical interest.

Properties of hydration water have been extensively studied experimentally through a number of techniques which include X-ray crystallography,[13] NMR,[14] neutron scattering,[15] dielectric relaxation,[16] and time-resolved fluorescence[17] and computationally through molecular simulation.[18] While the first hydration shell, representing the monolayer coverage of the protein surface, is formed by rather strongly bound water molecules, water outside this monolayer is perturbed to a significantly smaller extent and its quantitative evaluation cannot be easily performed by measurements of thermodynamic properties such as heat capacity or specific volume.

Fortunately, among the different experimental techniques, dielectric relaxation spectroscopy may provide a potential tool to characterize, as a whole, hydration water against the background of bulk water because, depending on the frequency range of interest, the technique allows both a direct observation, through the orientational polarization of the water molecules in the hydration layer, and an indirect observation, through the lack of bulk water dielectric response compared to the effective water present in the system.

However, because of the complexity of the relaxation spectra observed in aqueous protein solutions, where different partially overlapping relaxation regions are simultaneously present,[19] the extraction of the contribution associated to the hydration water is not an easy task. Moreover, sometimes, the quantity of hydration water may depend on the particular region of the dielectric spectrum analyzed, either because hydration water can be characterized by a distribution of relaxation times or because the overlapping of the dielectric dispersions makes the separation of the different contributions difficult.

In principle, hydration water, or, more correctly, biological water, affects all the regions of the dielectric relaxation falling in the frequency interval from 1 kHz to 50 GHz. In the low frequency tail, hydration water at the protein surface makes the effective hydrodynamic radius larger than that of the bare

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protein, resulting in a shift toward smaller relaxation frequencies of the β-dispersion. In the intermediate frequency range, where the δ-dispersion dominates, the orientational polarization of the bound water molecules directly contributes to the relaxation process. Moreover, the bimodal nature of the δ-dispersion allows us to separate, at least in principle, the contribution of the tightly bound water (roughly at 100 MHz, δ_1-component) from the loosely bound water (roughly at 4−5 GHz, δ_2-component). Finally, in the higher frequency range, the γ-dispersion reflects the total number of water molecules (bulk water) unaffected by the protein. In this case, the strength of the γ-dispersion decreases with the increase of the protein concentration much more than expected for the presence of the bare protein, indicating that further water molecules do not contribute to the orientational polarization of the aqueous phase.

In our recent paper,[20] we have investigated in detail the three main relaxation processes occurring in a lysozyme aqueous solution and we have estimated the hydration number both from the deconvolution of the δ-dispersion and from the decrease of the strength of the γ-dispersion. However, this analysis was confined to a protein concentration (C = 120 mg/mL) that was not particularly high. In this concentration range, we have observed a total hydration number N_{hydr} roughly independent of the protein concentration, the amount of the hydration water being directly proportional to the protein concentration.

In the present paper, we have extended the protein concentration to higher values, up to 300 mg/mL; meanwhile, the dielectric measurements have been limited to the high-frequency tail of the spectrum, say from 500 MHz to 50 GHz, so that only the γ-dispersion was investigated. This choice was in part forced by the fact that the protein solution, at concentrations so high as those investigated here, displays a very marked ionic conductivity which prevents measurements at lower frequencies to be known with the necessary accuracy. In fact, in highly conducting systems, when the d.c. electrical conductivity exceeds a value of 0.8−1 mho/m as in the present case, the ion accumulation at the electrode interface introduces a further dielectric dispersion, known as the electrode polarization effect,[21,22] characterized by a giant dielectric strength and by a relaxation frequency that shifts toward higher frequencies with the increase of the d.c. electrical conductivity. This effect masks completely the β-dispersion and partially the δ-dispersion, preventing the possibility of extracting their relevant parameters.[23] On the other hand, at frequencies investigated here, i.e., frequencies higher than 500 MHz, the electrode polarization effect is negligible and the γ-dispersion is practically free from this effect.

In this work, we have measured the γ-dispersion of concentrated lysozyme aqueous solutions (up to 300 mg/mL) and, from the analysis of the dielectric strength $\Delta\epsilon_\gamma$ as a function of the protein concentration C, we have estimated the *total* amount of the hydration water, i.e., the water in the system whose orientational relaxation frequency ν_γ differs from the one of the pure aqueous phase. This definition encompasses both types of hydration water (*bound* water and *loosely bound* water) interacting with the protein and in exchange with bulk water.

We observe that the amount of hydration water maintains approximately constant in the low concentration range, showing a marked decrease as the protein concentration is higher than 120−150 mg/mL. At protein concentrations higher than these values, the hydration number N_{hydr} decreases linearly with the concentration, suggesting that the approaching of the hydration shells favors the partial destroy of the more external water layer, maintaining essentially the first hydration layer (*bound* water). This effect is greatly promoted by the formation of small protein clusters, as recently experimentally observed by Stradner et al,[1] with the aggregation number of the order of some units which favors the formation of a *bound* water hydration shell at the expense of the secondary hydration shell (*loosely bound* water).

2. EXPERIMENTAL SECTION

2.1. Sample Preparation. Hen egg white lysozyme was obtained from Sigma Chem. Co. (dialyzed and lyophilized, 95% protein by weight with the remainder as sodium acetate and sodium chloride) and used without further purification. The protein solutions have been prepared by dissolution of lysozyme powder at the desired concentration in deionized water (electrical conductivity less than 1×10^{-6} mho/cm). The pH values of the solutions were around 5.0. Under these conditions, the protein is in its native state and bears a positive net charge of about 10 elementary charge.

2.2. Dielectric Measurements. In the frequency range from 500 MHz to 50 GHz, measurements were carried out by means of an Agilent N5230 Vector Network Analyzer (VNA), together with a dielectric kit probe Agilent 85070E. The probe is immersed into a protein solution under investigation, contained in a glass vessel. The solution is the electrical termination of the probe, that is a section of a transmission line.

The analyzer measures the complex reflection coefficient $\Gamma^*(\omega)$, from which the complex dielectric constant $\epsilon^*(\omega)$ is obtained following the procedure reported by Bao et al.,[24] through the relationship[24]

$$\epsilon^*(\omega) = \frac{A_1^*(\omega)\Gamma^*(\omega) - A_2^*(\omega)}{A_3^*(\omega) - \Gamma^*(\omega)} \quad (1)$$

where $A_j(\omega)$ (j = 1, 2, 3) are frequency-dependent complex constants which can be obtained from a calibration procedure performed with air, a short connection, and Millipore water (electrical conductivity less than 10^{-6} mho/cm). All the measurements have been carried out at a temperature of 25 °C, controlled within 0.1 °C. Details of the experimental procedure together with the overall uncertainty are reported elsewhere.[20,24,25]

2.3. Analysis of the Dielectric Spectra. Over the frequency range from 1 kHz to 50 GHz, the dielectric and conductometric spectra of aqueous protein solutions present a rather complex behavior, showing dielectric relaxation regions that clearly derive from different and, partially overlapping, polarization mechanisms.[20,26] At least four or even more dielectric relaxations have been observed, and more or less sophisticated algorithms have been developed in order to make the appropriate deconvolution of the spectra.[27] However, in the high frequency tail of this interval, say from 500 MHz to 50 GHz, the spectra are dominated by the so-called γ-relaxation, undoubtedly attributed to the orientational polarization of the water molecules. This assignment is based on the fact that the relaxation frequency ν_γ does not depend on the protein concentration[6] and, above all, its value is very close to the one of the aqueous phase. Moreover, the parameter α which governs the spread of the relaxation times is very close to the one of the aqueous phase and, in any case, very small (α of the order of 0.01−0.04).

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Consequently, we have modeled the whole relaxation function according to Cole−Cole-type relaxation, which reads

$$\epsilon^*(\omega) = \epsilon_\infty + \frac{\Delta\epsilon_\gamma}{1 + (i\omega\tau_\gamma)^{1-\alpha}} + \frac{\sigma}{i\omega\epsilon_0} \quad (2)$$

Here, $\Delta\epsilon_\gamma$ and $\tau_\gamma = 1/(2\pi\nu_\gamma)$ are the dielectric increment (the dielectric strength) and the relaxation time of the relaxation precess (ν_γ, the relaxation frequency), ϵ_∞ the high-frequency limit of the permittivity and σ the d.c. electrical conductivity, and α is the parameter which takes into account the distribution of the relaxation times. In the limit $\alpha = 0$, eq 2 reduces to the Debye relaxation function with a single exponential polarization decay. Finally, ϵ_0 is the permittivity of free space and ω the angular frequency of the applied electric field.

Before going on, we want to justify why, in the presence of concentrated solutions, we were forced to restrict our investigation to the high frequency range (from 500 MHz to 50 GHz). In the absence of the electrode polarization effect, the total dielectric loss ϵ''_{tot} contains two contributions, ϵ''_d due to the dielectric response of the sample and a conductive response ϵ''_{cond} due to the losses associated to the bulk ionic conductivity, i.e.,

$$\epsilon''_{tot} = \epsilon''_d + \epsilon''_{cond} = \epsilon''_d + \frac{\sigma}{\epsilon_0\omega} \quad (3)$$

Figure 1 shows the frequency dependence of these two contributions for a lysozyme solution at a concentration of C = 200 mg/mL. In this sample, the d.c. electrical conductivity is σ = 0.9 mho/m. As can be seen, the experimental values are very close to the ones expected from a single dielectric contribution to which a conductivity contribution has been added. In other words, the presence of the δ-dispersion, which should fall around 10^8−10^9 Hz, is largely masked by the conductivity losses; i.e., its contribution (with dielectric strength of the order of unity) is heavily masked by the conductivity losses.

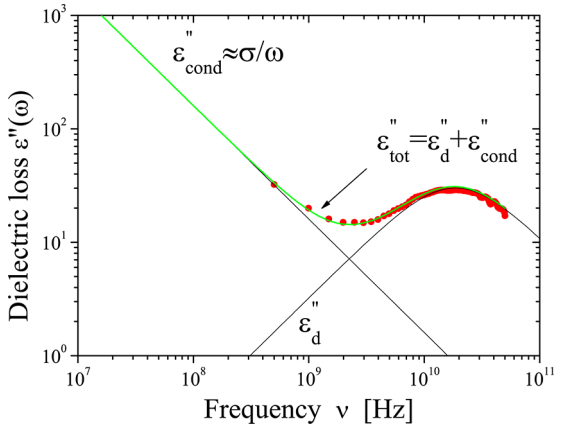

Figure 1. The total dielectric loss $\epsilon''_{tot}(\omega)$ of lysozyme aqueous solution (protein concentration C = 200 mg/mL) as a function of frequency. The contribution due to the d.c. electrical conductivity, ϵ''_{cond}, and the contribution due to the polarization of the aqueous phase, ϵ''_d, are shown as continuous lines. The total dielectric loss $\epsilon''_{tot} = \epsilon''_{cond} + \epsilon''_d$ approaches very closely the experimental values (red full circles). This agreement means that the d.c. losses prevent the possibility of extraction contributions of the β- and δ-dispersion from the measured total loss $\epsilon''_{tot}(\omega)$ at frequencies lower than about 500 MHz.

Consequently, the analysis of the dielectric spectra, taking solely into account the contribution of the γ-dispersion, has been carried out on the basis of eq 2 by means of a nonlinear least-squares minimization for complex functions on the basis of the Levenberg−Marquardt algorithm.[28]

A typical result is shown in Figure 2, where we report the permittivity $\epsilon'(\omega)$ and the dielectric loss $\epsilon''(\omega)$ as a function of frequency for a lysozyme solution of concentration C = 200 mg/mL at a temperature of 25 °C.

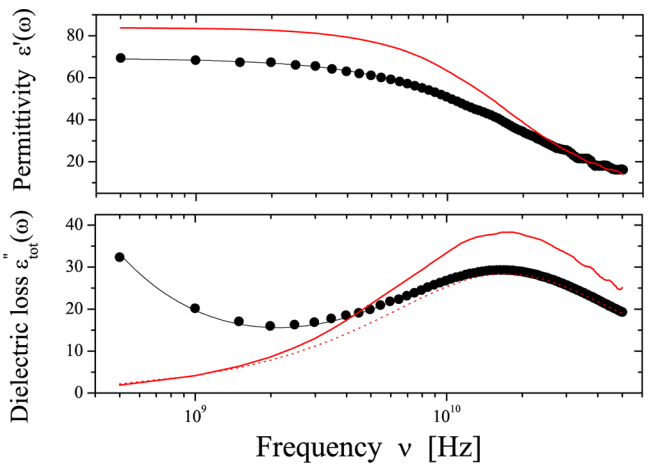

Figure 2. Upper panel: The permittivity $\epsilon'(\omega)$ of lysozyme aqueous solution (C = 200 mg/mL) as a function of frequency, at a temperature of 25 °C. The solid line represents the values of pure water at the temperature of the experiment. Bottom panel: The total dielectric loss $\epsilon''_{tot}(\omega)$ as a function of frequency. The solid line represents the dielectric loss of pure water. The dotted line represents the dielectric loss $\epsilon''_d(\omega)$, obtained from the total dielectric loss $\epsilon''_{tot}(\omega)$ by subtracting the conductivity loss $\epsilon''_{cond}(\omega) = \sigma/(\epsilon_0\omega)$.

The dielectric parameters $\Delta\epsilon_\gamma$, ν_γ, and α derived from the above quoted fitting procedure are shown in Figures 3 and 4. As can be seen, the dielectric strength $\Delta\epsilon_\gamma$ decreases with the protein concentration C, whereas the relaxation frequency ν_γ is practically independent of C with a value equal to the one of the pure aqueous phase. As far as the parameter α is concerned (inset of Figure 4), we observe a very small value in the low

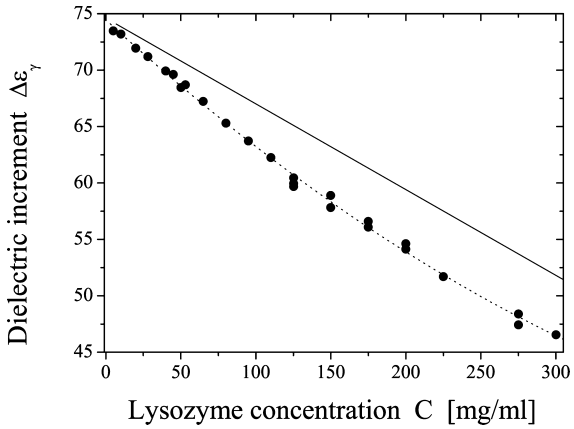

Figure 3. The dielectric increment $\Delta\epsilon_\gamma$ as a function of the lysozyme concentration C, up to 300 mg/mL at a temperature of 25 °C. The upper continuous line represents the values expected on the basis of the water concentration present in the system.

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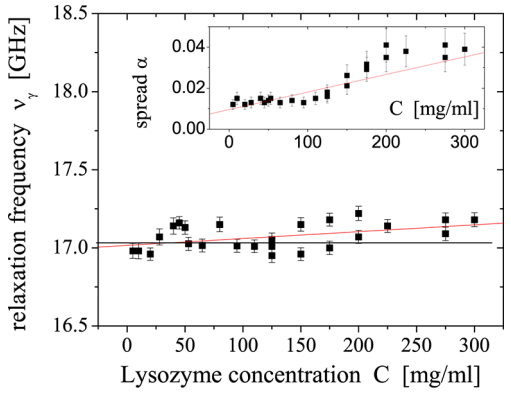

Figure 4. The relaxation frequency ν_γ as a function of the lysozyme concentration C, up to 300 mg/mL at a temperature of 25 °C. The continuous line represents the values expected for pure water. The inset shows the spread parameter α of the relaxation times as a function of the protein concentration C.

concentration interval, as expected for a single Debye relaxation process, and values a little bit higher as the concentration C is increased, probably indicative of the formation of small protein aggregates. However, over the whole concentration range investigated, the value of α is very small, and practically, no significative deviation from a single exponential decay occurs.

Finally, the electrical conductivity σ of the lysozyme aqueous solutions is shown in Figure 5 as a function of the protein concentration C.

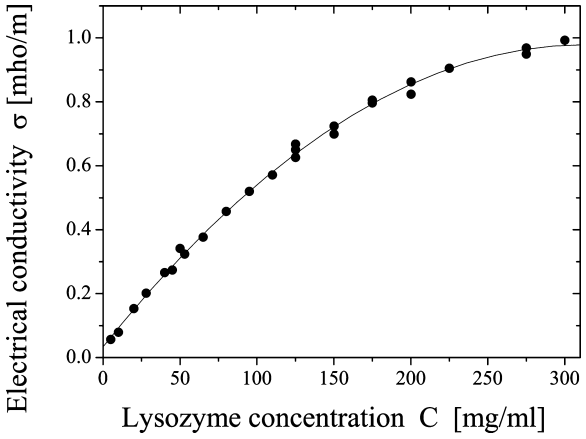

Figure 5. Electrical conductivity σ of lysozyme aqueous solutions as a function of the protein concentration C, at a temperature of 25 °C.

As far as the analysis of the dielectric relaxation is concerned, a final comment is in order. We performed a simultaneous fit of both the real and imaginary parts of the complex dielectric constant $\epsilon^*(\omega)$; namely, the same set of parameters ($\Delta\epsilon_\gamma$, ν_γ, α, and σ) account for both the permittivity and the dielectric loss. Moreover, the electrical conductivity σ derived from the fitting procedure is in very good agreement with the value determined experimentally at a very low frequency, well below the ones of the dielectric relaxations.

3. RESULTS AND DISCUSSION

The number of water molecules affected by each protein molecule can be deduced from the amplitude $\Delta\epsilon_\gamma$ of the γ-relaxation. In the presence of a hydration layer, where water molecules present a retarded motion, $\Delta\epsilon_\gamma$ decreases with the protein concentration C more rapidly than is expected for the presence of the bare protein (dilution effect, i.e., the trivial substitution of water by protein molecules in the solution), allowing the calculation of the hydration number N_{hyd}.

On the assumption that the dielectric increment $\Delta\epsilon_\gamma$ is proportional to the molar concentration C_w of free water in the system, the molar concentration of hydration water C_w^h is given by

$$C_w^h = C_w - C_w^0 \frac{\Delta\epsilon_\gamma}{\Delta\epsilon_\gamma^0} \tag{4}$$

where the index "0" denotes pure water. Consequently, the number N_{hyd} of water molecules affected per protein molecule is given by

$$N_{hyd} = \frac{C_w^h}{C_p} = \frac{\rho - C - \rho_w \frac{\Delta\epsilon_\gamma}{\Delta\epsilon_\gamma^0}}{C} \frac{M_p}{M_w} \tag{5}$$

where C_p is the molar concentration of protein and ρ is the density of the solution.

Obviously, the effect of the hydration water is reflected also in the δ-dispersion, which falls between the β- and γ-dispersion. However, due to the complexity of protein structure, it is still controversial if the δ-dispersion (which could be bimodal) can be solely assigned to the hydration water[29] or additional effects should be taken into account.[30]

Within the above stated framework, the hydration number N_{hydr} depends solely on the dielectric increment of pure water $\Delta\epsilon_\gamma^0$ and on the density ρ of the solutions at the different protein concentrations C investigated, besides the stoichiometric molar concentration C_w^0 of water in the system.

As far as the density of the protein solution is concerned, we used values measured by Millero et al.,[31] who found that the data could be represented by a linear function of the protein weight fraction W according to the relationship $\rho = \rho_w + AW$, with ρ_w = 0.997075 g/mL and A = 0.25963 g/mL, at a temperature of 25 °C.

The dielectric relaxation of pure water has been critically reviewed by Kaatze and Uhlendorf[32] who, on the basis of measurements in the frequency range from 1 to 60 GHz, together with results from literature in the frequency range up to 114 GHz, concluded that the dielectric process can be accounted for by a single Debye-type relaxation characterized by the parameters ϵ_s = 78.36 ± 0.05, ϵ_∞ = 5.16 ± 0.08, and τ = 8.27 ± 0.02 ps (at a temperature of T = 25 °C). Moreover, they concluded that, if deviations from the Debye relaxation behavior are present, these are too small to be confirmed by the available experimental results. These findings are confirmed by our measurements which yield a relaxation frequency for pure water of ν_γ of 17 GHz, a dielectric increment $\Delta\epsilon_\gamma$ of 73.8, and a value of α in the range 0.01−0.04.

The number N_{hydr} of water molecules per protein (eq 5) at the protein interface with an orientational polarization different from the one in bulk water (the total hydration water, as revealed by the dielectric spectroscopy measurements) is shown in Figure 6 as a function of the lysozyme concentration C. As can be seen, the hydration number is approximately constant in the low range of the protein concentration (up to values of the order of 100−150 mg/mL), but it shows a marked, approximately linear, decrease as the protein concentration is further increased.

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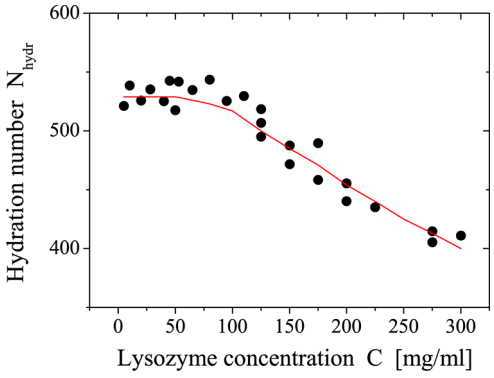

Figure 6. The number of water molecule hydration N_{hydr} as a function of the lysozyme concentration C, deduced from the dielectric increment $\Delta\epsilon_\gamma$. The full line represents the values calculated according to eq 9 with ΔR = 1.2 Å.

In our previous work,[20] due to the limited range of protein concentrations investigated (up to 120 mg/mL), we were induced to consider a hydration number independent of the protein concentration. Now, the extension of the investigation over a much wider concentration range, up to 300 mg/mL, evidences a different dependence with an approximately linear decrease of N_{hydr} with the increase of the concentration C.

This behavior can be justified, at least qualitatively, by the formation, with the increase of the concentration C, of small equilibrium clusters with a concentration dependent aggregation number. As in any colloidal suspension of charged mesoscopic particles, weakly charged globular proteins self-assemble into equilibrium clusters, with interactions being governed by a delicate balance between strong short-range attraction and weak long-range electrostatic repulsion.[33−35]

When the concentration is low, the protein molecules are rather monodisperse, the average distance between monomers is much larger than the hydration layer thickness, and consequently the hydration number N_{hydr} is, at least to a first approximation, independent of concentration. With the increase of the protein concentration, proteins aggregate in small equilibrium clusters, favoring the decrease of the whole hydration water.

The formation of lysozyme clusters in concentrated aqueous solutions was observed by Stradner et al.[1] by means of small-angle X-ray (SAXS) and neutron (SANS) scattering measurements. These authors found that the effective structure factor $S_{eff}(q)$ shows two different peaks assigned to cluster−cluster correlation and to the correlation of monomers within the single cluster, respectively. This latter peak exhibits a concentration independent value ($qR \approx 3.8$), indicating a packing density within the cluster of approximately Φ_c = 0.60 by volume.

Although these measurements have been recently questioned by Shukla et al.,[36] the existence of equilibrium clusters in concentrated lysozyme protein solutions has definitely been demonstrated by Kowalczyk et al.[37] using molecular simulations and experimental SAXS effective potentials and by Barhoum et al.,[38] who indicated, on the basis of NMR results, the coexistence of individual proteins and clusters at large protein concentration. Similar conclusions were reached by Cardinaux et al.,[39] who, combining an experimental and numerical study, showed that a cluster phase emerges as a result of a competition between a long-range screened Coulomb repulsion and a short-range attraction.

Within the framework of the results presented by Stradner et al.,[1] the average center-to-center distance of monomeric proteins within each cluster is, to a first approximation, given by

$$d \simeq \sqrt[3]{\frac{4\pi {R_H}^3}{3\Phi_c}} \tag{6}$$

where R_H is the hydrodynamic radius of the lysozyme.

The infinite-dilution hydrodynamic radius R_H of lysozyme deduced from dynamic light scattering measurements through the Stokes−Einstein relationship, at pH 4, is R_H = 18.0 ± 0.5 Å.[40] However, the crystal structure of hen egg-white lysozyme shows that the unhydrated monomer can be modeled by a prolate ellipsoid with major semiaxis of 22.5 Å and minor semiaxis of 15 Å. These values yield an equivalent spherical radius of the unhydrated monomer of R_{bare} = 17.2 Å, with an uncertainty of about 0.1 Å.[41] The difference between the hydrodynamic radius R_H and the crystal structure effective radius R_{bare} accounts for water hydration in the Stern layer around the protein surface. We found that d is merely twice the radius R_H, suggesting that, at least to a first approximation, the total water component within each cluster behaves as hydration water.

Moreover, Stradner et al.[1] have shown that, at least in the high protein concentration interval, the aggregation number n_c, i.e., the number of protein molecules (monomers) within each cluster, increases linearly with the fractional volume Φ of the protein in the solution, according to

$$n_c = K\Phi = K\frac{C}{\rho_0} \tag{7}$$

where the factor K is of the order of K = 25 and ρ_0 is the density of the lysozyme (ρ_0 = 1.35 g/cm^3). The average size of each cluster is given by

$$R_c = \left(\frac{n_c R_H}{\Phi_c}\right)^{1/3} \tag{8}$$

Within this scenario, the total hydration number N_{hydr} (the total hydration water per protein molecule) can be written as

$$N_{hydr} = \frac{(1-\Phi_c)}{n_c}\frac{V_c}{v_0} + \frac{4\pi}{n_c v_0}{R_c}^2\Delta R \tag{9}$$

where v_0 is the volume of a water molecule ($v_0 = 3 \times 10^{-23}$ cm^3). In eq 9, the first term takes into account the contribution of the hydration water inside the cluster and the second term models the contribution of the hydration water at the cluster interface. ΔR is the thickness of this hydration layer.

Equation 9 is in very good agreement with the values of the hydration number N_{hydr} derived from the decrement of the dielectric strength $\Delta\epsilon_\gamma$ as a function of the protein concentration C (see Figure 6). It is worth noting that eq 9 does not contain any adjustable parameter, the only exception being the parameter ΔR that controls the thickness of the hydration layer at the cluster interfaces.

Our results, as a whole, furnish a quantitative estimation of the *total* hydration water in lysozyme aqueous solutions, evidencing how the formation of small equilibrium clusters, as the protein concentration increases, induces a progressive decrease of its amount.

In the dilute condition, a hydration number N_{hydr} of the order of 500−550 (as shown in Figure 6) roughly means that

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the whole hydration layer (*bound* water and *loosely bound* water) is of the order of 4×10^{-8} cm, which is larger than the value we have estimated from the distance between proteins when they aggregate to form a cluster (the fractional volume Φ_c is of the order of 0.6, as reported by Stradner et al.,[1] and consequently the water component has a volume fraction of $1 - \Phi_c$). This means that, within each cluster, the hydration water preferably behaves as *bound* water and that the aggregation process tends to reduce the more external water layer that represents the *loosely bound* water contribution.

A final comment is in order. The evaluation of the hydration water N_{hydr} is based on the assumption that the strength $\Delta\epsilon_\gamma$ of the γ-dielectric relaxation is proportional to the amount of free water present in the system, i.e., water unaffected by the protein. While this assumption is reasonably correct in the case of relatively high water content as in the present case (highest protein concentration investigated $C = 300$ mg/mL), a different scenario appears when the total amount of water is low and protein molecules could impart a severe confinement even for the free water fraction. In this case, anisotropies and correlated motions in the free water component may become important, breaking down the proportionality between $\Delta\epsilon_\gamma$ and N_{hydr}. The dynamics of confined water in these strong confinement conditions has been extensively investigated in the past few years, showing that water exhibits dynamics over many time scales, depending on the environment. These effects are particularly relevant for hydration levels equal or less than $h = 0.4$ (i.e., 0.4 g of H_2O/g of dry lysozyme), where the coupling of protein and hydration water dynamics induces structural rearrangements of the water component on a macroscopic scale.[42−47] In the present case, at the maximum protein concentration investigated, the hydration level h is of the order of $h = 2.5$ and effects due to a strong water confinement can be, to a first approximation, neglected.

4. CONCLUSIONS

The amount of hydration water of lysozyme in concentrated aqueous solutions (up to 300 mg/mL) has been evaluated by means of dielectric relaxation measurements in the frequency range where the γ-dispersion dominates, by comparing the dielectric response of bulk water in the protein solution to the one expected in the presence of bare proteins, at the same stoichiometric concentration. This approach has the advantage of being, to a large extent, independent of a particular model, even if it does not discriminate between *bound* water and *loosely bound* water, taking into account the *total* amount of water, whose dynamics occurs on a time scale different from the one of bulk water.

With these limitations in mind, we have found that the amount of hydration water maintains constant at low protein concentration, when proteins, in the absence of aggregates, are rather monodisperse. Under these conditions, the hydration number N_{hydr} is of the order of 520, consisting of an inner hydration shell (*bound* water) followed by an external shell (*loosely bound* water). As the protein concentration is further increased, proteins aggregate in small equilibrium structures (protein clusters), as revealed by SAXS and SANS measurements,[1] with an aggregation number that increases linearly with the protein concentration C. In these conditions, we find that the amount of hydration water decreases roughly linearly with the protein concentration. On the basis of a simple stoichiometry, this decrease can be justified considering that, within each cluster, *bound* hydration water prevails, the average distance between protein molecules fitting with the thickness of the inner hydration layer. The aggregation process forming protein clusters tends to remove the *loosely bound* water and only *bound* water persists, having a longer residence time than the one at larger distance from the protein surface. Once formed, the equilibrium protein clusters do not interact, at least for concentrations up to 300 mg/mL, and, at their outer surface, hydration includes both *bound* hydration water and *loosely bound* water components. The two contributions, *bound* hydration water within each cluster and *loosely bound* hydration water at its external surface, depend on the number of aggregation (determined experimentally from SAXS and SANS measurements[1]) in a way to account for the observed dependence of N_{hydr} on the protein concentration C.

A quantitative understanding of water dynamics near the protein surface remains a central role in the structure and functionality of water-soluble biomolecules, and these findings could provide useful suggestions for investigation of biological water close to a protein.

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Notes

The authors declare no competing financial interest.

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