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Specific features of the temperature behavior of lysozyme diffusivity in solutions with different protein concentrations

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ABSTRACT

The paper presents dynamic light scattering investigations of the temperature behavior of lysozyme diffusivity in solutions with different protein concentrations. The objects are lysozyme solutions with concentrations ranging from 10 mg/ml up to 250 mg/ml; each sample has been studied at temperatures increasing from 15° to 55 °C. The analysis of the autocorrelation functions obtained by DLS for all the solutions has revealed two types of motion with different relaxation times: a fast mode that corresponds to the diffusion of lysozyme molecules and a slow mode associated with the motion of clusters, i.e., complexes consisting of several protein molecules. It is shown that the temperature dependences of the diffusion coefficient of lysozyme in all the solutions are well described by the Arrhenius law. The temperature behavior of the relaxation time of the slow mode has been found to be different for the low- and high-concentration lysozyme solutions. The low-concentration protein solution is characterized by a monotonous decrease in the slow mode relaxation time with increasing temperature. As the lysozyme concentration is increased to 100 mg/ml, a considerable change is observed: a minimum in the temperature dependence of the relaxation time of the slow mode appears. Possible reasons for such a behavior of the lysozyme diffusivity are discussed.

1. Introduction

Studies of protein diffusion in solutions and its behavior under the influence of different factors, such as temperature [1], ionic strength [2,3], denaturants [4–6], and others attract considerable attention of researchers because they shed light on intrinsic properties of proteins (for example, protein–protein interactions, protein stability, etc.). Of particular interest is protein diffusion in diluted solutions in which intermolecular interactions are negligibly weak, and thus valuable information on properties of individual protein molecules can be obtained. However, investigations of solutions with high protein concentrations are also extremely important. Because of the high protein concentrations in cells of living organisms the volume fraction of macromolecules in them reaches 0.3 [7] and, therefore, many processes are governed by the diffusion of macromolecules [8,9]. For instance, oxygen transport in tissues and cells is directly related to the diffusion of hemoglobin and myoglobin [7]. Diffusion of macromolecules in living cells having high protein concentrations leads to formation of protein complexes responsible for many biological processes. It can be supposed that mechanisms of protein complex formation can be similar to those of equilibrium cluster formation in solutions with high protein concentrations because of liquid–liquid phase separation. Thus, studies of the diffusion of macromolecules under the crowded conditions lead to a deeper understanding of such processes as a diffusion-controlled macromolecular reaction or the onset of a critical behavior and phase transition which are relevant physiologically.

To investigate the diffusion of proteins and other biopolymers, such techniques as neutron spectroscopy [10–12], NMR [13–15], polarized and depolarized light scattering [16–18], etc. are widely used, but the key technique is dynamic light scattering (DLS) [19,20]. This is because short-time fluctuations of the scattered light intensity measured in DLS experiments are related to the scattered particle motions and manifest themselves as modes with corresponding relaxation times. An important advantage offered by DLS is that it gives information not only on diffusion of individual protein molecules but also on their complexes or aggregates if they

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appear in a solution. For example, DLS studies of lysozyme denaturation by ethanol have shown that a fast and/or slow mode is observed depending on the ethanol concentration, i.e., on the protein denaturation and aggregation [4]. The intermolecular protein interactions at crystallization accompanied by the formation of protein complexes are widely studied by DLS [21–24].

Recent neutron spin echo and small angle neutron scattering (SANS) and DLS investigations have shown that clusters containing several protein molecules can be formed in high-concentration lysozyme solutions [11,12,25] and in solutions of other proteins, such as lumazine synthase [26], sickle-cell hemoglobin [27] and some small-molecular substances. It is supposed that small equilibrium clusters in lysozyme solutions with a low ionic force are formed due to a balance between short-range attraction and weakly screened long-range electrostatic repulsion. The clusters formed are a dense liquid phase that has a high molecular density comparable to that in the solid state and high mobility of protein molecules [12,26,27]. It is metastable with respect to the low-concentration protein solution. Such clusters have the sizes lying in the limits from 100 nm to several thousands of nm and include about 10^6 protein molecules [25]. The protein molecules move collectively together inside a cluster during a short period, but escape from the cluster after a certain time [11]. Cluster sizes have been found to increase with decreasing temperature [11]. To gain understanding of specific features of protein diffusivity in solutions with a high volume fraction of protein, we have studied lysozyme solutions with protein concentrations ranging from 10 to 250 mg/ml at temperatures increasing from 15 °C to 50 °C by dynamic light scattering.

2. Material and methods

2.1. Lysozyme solution preparation

Hen egg white lysozyme was purchased from Fluka (cat. No. 62970, Steinheim, Switzerland). All other chemicals were obtained from Merck (Rahway, NJ). Lysozyme solutions with concentrations 10 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml, and 250 mg/ml were prepared on the basis of 0.1 M sodium acetate buffer with pH 4.6. In order to reveal specific features of diffusion motion of protein molecules under crowded conditions, data for the high-concentration lysozyme solutions (100–250 mg/ml) were compared with the data for the lysozyme solution with a concentration of 10 mg/ml. To prepare the solutions, distilled water from a Milli-Q water purification system (Millipore, Billerica, MO) was used. The protein concentration was determined by measuring absorbance at 280 nm and 25 °C by a Jasco-V550 spectrophotometer (Jasco, Japan) with an extinction coefficient for lysozyme of 2.64 L/(g cm) [28]. Prior to the dynamic light scattering experiment each sample was filtered through a filter with 0.22-μm pores to remove dust.

2.2. Dynamic light scattering measurements

The DLS system included a solid-state laser (Coherent Inc., Santa Clara, CA, U.S.A.) with a wavelength 532 nm and a Brookhaven light scattering instrument (BI-200SM motor-driven goniometer) with a BI-9025AT photon counter and a BI-9000AT digital autocorrelator. The glass tube with the sample under study was placed into decalin (Aldrich 29477-2, CAS [91-17-8], refractive index $n=1.47$) which was used as an index-matching liquid to reduce flares at the glass–liquid interface. Decalin was recirculated through a 0.22 μm pore size hydrophobic membrane filter (Millipore, Billerica, MA, U.S.A.) until all visible dust was removed. In the DLS experiment the sample was heated from 15° to 50 °C and the temperature in the experimental chamber was maintained within ±0.1 °C with the help of a recirculating water bath (FIRSTEK, Taiwan). Each sample was kept for 15 min at each temperature before each measurement. Our DLS equipment did not have an analyzer in front of photo detector. Therefore the total intensity of scattered light was measured.

In DLS experiment, the intensity–intensity autocorrelation functions $g^{(2)}(\tau)$ was measured as

$$g^{(2)}(\tau)=\frac{\langle I(\tau)\bullet I(t+\tau)\rangle}{\langle I(t)\rangle^2} \tag{1}$$

The autocorrelation function $g^{(2)}(\tau)$ is related to the dynamic structure factor $g^{(1)}(\tau,q)$ of the sample by the relation

$$g^{(2)}(\tau)=B+k\left[g^{(1)}(\tau,q)\right]^2 \tag{2}$$

where B is a baseline, k is a contrast factor that determines the signal-to-noise ratio, and q is a magnitude of the scattering wave-vector. All the experiments were carried out at a scattering angle θ of 90° and, therefore, we will operate further with $g^{(1)}(\tau)$.

In the case of diluted protein solutions the decay of the function $g^{(1)}(\tau)$ defines the characteristic time τ for fluctuations of concentration of protein molecules in the solution. When several modes, such as translational diffusion, internal motion of scatterers, or others, exist in a solution, $g^{(1)}(\tau)$ is a sum of decays corresponding to each mode. Earlier studies of high-concentration lysozyme solutions revealed modes with different relaxation times and corresponding diffusion coefficients which are due to the diffusion of lysozyme molecules and the existence of clusters in the solution [25,29]. Our DLS experiments also revealed two modes. The analysis of the data obtained has shown that one of them is a fast mode with the relaxation time τ_f that corresponds to the lysozyme molecule diffusion, and that the other is a slow mode with relaxation time τ_s which characterizes the behavior of protein clusters formed at high protein concentrations [11,25]. In this case the function $g^{(1)}(\tau)$ can be presented as

$$g^{(1)}(\tau)=a_1\exp\left(-t/\tau_f\right)+a_2\exp\left[-(t/\tau_s)^{\beta}\right] \tag{3}$$

where a_1 and a_2 are the relative amplitudes and $a_1+a_2=1$, β is the stretching exponent that describes the deviation (broadening) from the exponential function. Values of β can vary with limits $0<\beta\leq 1$, and $\beta=1$ means an exponential decay. The mode described by the stretched exponent (with $\beta<1$) is typically attributed to a distribution of different-sized particles [4,17], which can occur when complexes are formed from several protein molecules (for example, dimers, trimers, etc.).

The relaxation time τ_f characterizes the diffusive motion of individual molecules. The relaxation time τ_f obtained in our experiments is related to the average apparent diffusion coefficient D_{app} as

$$D_{app}=\frac{1}{q^2\tau_f} \tag{4}$$

where q is the scattering wave-vector magnitude. It should be noted that D_{app} obtained for lysozyme in our experiments is in good agreement with the literature data [2,4,15,20]. To calculate the diffusion coefficient D_{app} of lysozyme in solutions with different concentrations, the scattering wave-vector magnitude q was calculated as

$$q=\frac{4\pi n}{\lambda}Sin\left(\theta/2\right) \tag{5}$$

where n is the refractive index of the solution, and λ is the laser light wavelength. All the experiments were carried out at a scattering angle θ of 90°. In order to calculate wave vectors for the solutions with different concentrations in the entire temperature interval studied, the temperature dependence of the refractive index n for each sample was measured. The measurements were performed by an Abbemat multiwavelength refractometer (Anton Paar, Austria). The values of

the index of refraction at 515 nm were used to calculate the temperature dependences of the apparent translational diffusion coefficient of lysozyme by formulas (4) and (5).

3. Results and discussion

Fig. 1(a) shows the experimental intensity–intensity autocorrelation functions $g(\tau)=g^{(2)}(\tau)-1$ for the lysozyme solution with concentration 10 mg/ml at 20 °C and 46 °C and also results of fitting of experimental autocorrelation functions by Eq. (3). It can be seen that the decay of function (4) describes well the experimental $g(\tau)$. As temperature increases, $g(\tau)$ shifts somewhat towards faster times, but the character of its decay remains nearly unchanged. At a higher lysozyme concentration in the solution, 250 mg/ml (Fig. 1(b)), the contributions of two relaxation processes become pronounced. A fast mode with relaxation time τ_f (~10^{-5} s) and a slow mode with relaxation time τ_s (10^{-4}–10^{-3} s) manifest themselves in the autocorrelation function. It is clear that heating causes the slow mode amplitude increase and relaxation time decrease. Attention should be paid to the small correlation amplitude of experimental autocorrelation function of about 0.2 at $\tau\to 0$ in Fig. 1. This is a consequence of a low coherency of $I(q)$ because the apertures in front of the photodetector had to be sufficiently opened to compensate for low $I(q)$.

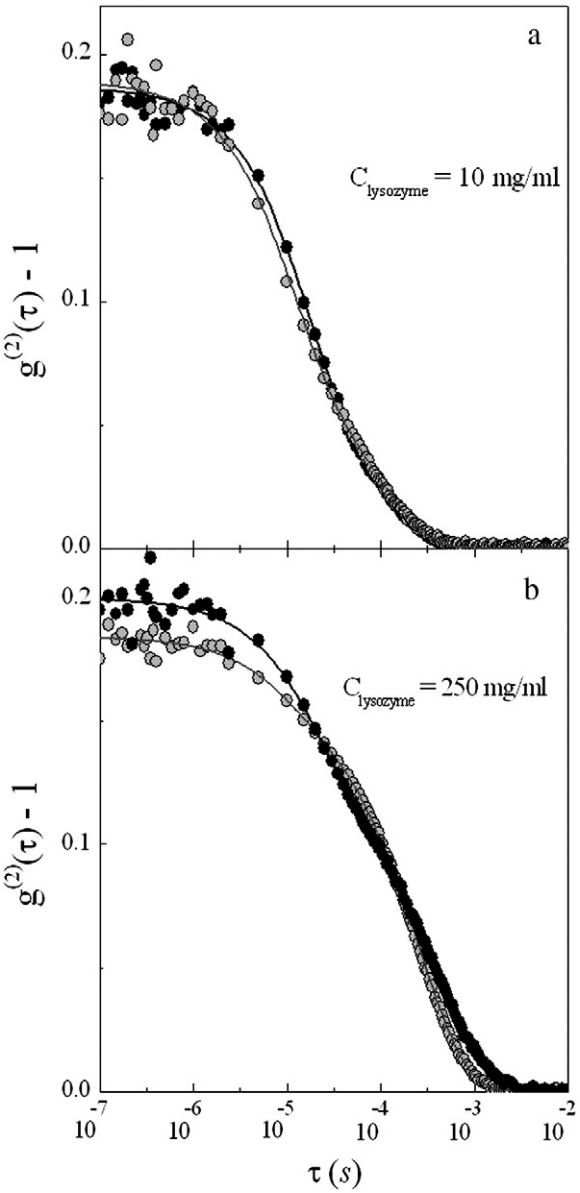

Fig. 1. Experimental intensity/intensity autocorrelation functions obtained in the DLS experiment for the lysozyme solutions having concentrations (a) 10 mg/ml and (b) 250 mg/ml at 20 °C (black circles) and 46 °C (open circles). The circles show the experimental results, the solid lines show the results of fitting by Eq. (3).

Let us now consider the behavior of τ_f with increasing lysozyme content in the solution. At room temperature $\tau_f=(2.2\pm 0.7)\times 10^{-5}s$ is the same within experimental errors for all the lysozyme solutions, both with a low (10 mg/ml) and high (100, 150, 200, and 250 mg/ml) protein concentrations. This relaxation time corresponds to the lysozyme apparent diffusion coefficient $D_{app}=(6.9\pm 0.8)\times 10^{-7}\ cm^2/s$. The magnitude of D_{app} obtained in our study agrees well with the literature data [2,4,15,20]. The same D_{app} for the solutions with different protein concentrations can be explained by the fact that each lysozyme molecule in 0.1 M acetate buffer, pH 4.6, has a positive surface charge equal to about ten electronic charges [17]. The protein surface charge can be partially screened by the salts presented in the solution. However, as our earlier small angle neutron scattering (SANS) studies showed, repulsive interactions between lysozyme molecules dominate under the experimental conditions used [12,30,31]. To be more exact, the scattering curve I(q) obtained in the SANS experiments demonstrated that intensity decreased at $q\to 0$. At the same time a pronounced peak at a certain q evolved indicating strong positional correlations between the protein molecules. This peak could appear as a result of cluster formation in the solution, consistent with the data given in [12].

The results of the fit of the autocorrelation functions obtained in DLS experiments for lysozyme solutions with different concentrations were used to plot the temperature dependences of the fast τ_f (Fig. 2) and slow relaxation times τ_s (Fig. 3). It is important to note that the error bars presented in Figs. 2 and 3 were obtained from fitting of the autocorrelation function by Eq. (3). Let us at first consider the temperature behavior of τ_f. For solutions with high protein concentrations (100, 150, 200, and 250 mg/ml) τ_f monotonously decreases from $(2.2\pm 0.7)\times 10^{-5}s$ to $(0.9\pm 0.5)\times 10^{-5}s$ when the sample temperature is increased. The temperature dependences of τ_f for these solutions are similar. The temperature dependence of τ_f for the 10 mg/ml lysozyme solution exhibits the same behavior in the vicinity of room temperature, but deviates from the other dependences in the region of high temperatures. The relaxation times for the 10 mg/ml lysozyme solution in the high-temperature region are somewhat higher than those of the high-concentration

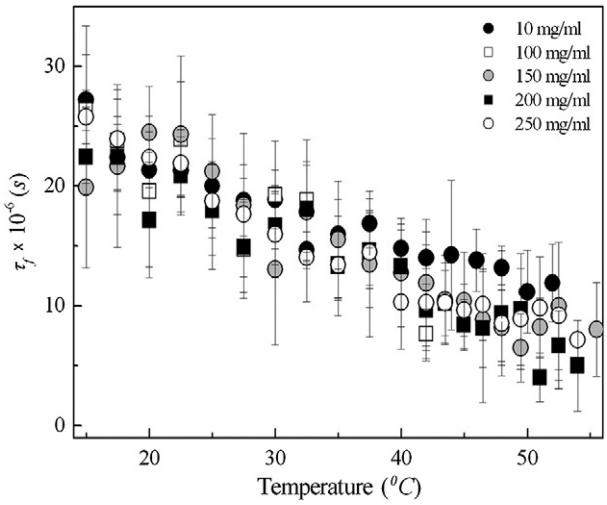

Fig. 2. Temperature dependences of relaxation times of the fast mode τ_f for the solutions with lysozyme concentrations: 10 mg/ml (black circles), 100 mg/ml (open squares), 150 mg/ml (gray circles), 200 mg/ml (black squares), and 250 mg/ml (open circles).

solutions. This is attributable to different activation energies for the diffusion of molecules in the solutions with different protein concentrations. Since the relaxation times τ_f correspond to the diffusion of lysozyme molecules, the apparent diffusion coefficient can be calculated for the entire temperature interval by formulas (4) and (5) [19,20]. Fig. 4 shows D_{app} for lysozyme in the logarithmic scale as a function of reciprocal temperature $1000/T$ for two protein concentrations (10 mg/ml and 250 mg/ml). It can be seen that the dependences for both samples in Fig. 4 are linear. The temperature behavior of the lysozyme diffusion coefficient in all the solutions is well described by the Arrhenius equation

$$D = D_0 \exp\left(-\frac{\Delta E_a}{RT}\right) \tag{6}$$

where D_0 is the preexponential coefficient, ΔE_a is the activation energy for the diffusion of lysozyme molecules, and R is the gas constant. It can be seen from Fig. 4 that the lysozyme solutions with low and high protein concentrations have different activation energies, which is evidenced by different slopes of linear dependences of D_{app} on temperature in the Arrhenius plot. The activation energies ΔE_a for all the samples are summarized in Table 1. For the lysozyme solutions ΔE_a lies in the range from 1.35 to $2.39 \pm 0.16 kcal/mol$. The energies we obtained are well below those of bovine serum albumin diffusion (ΔE_a ~ 4.5–5.2 kcal/mol) [1] determined mainly by rearrangements in the hydrogen bond network [32,33]. It is likely that the lysozyme molecule diffusion has lower energies because of short-range attractive interactions which play an important role in cluster formation [12].

Let us now consider the slow mode for the solutions with different protein concentrations (Fig. 3). It is interesting to note that the relaxation times characteristic of the slow mode τ_s are longer by two order of magnitude than those of the fast mode τ_f. While τ_f characterizes the diffusion motion of molecules, τ_s is hardly to be related to the behavior of clusters. Since formation of aggregates is hardly probable because of repulsive interactions between protein molecules [28,29,33], the slow mode is attributable to formation of clusters in solutions with high protein concentrations [11,12]. Mechanisms of formation of such metastable clusters that are a dense liquid phase were described in [25–27].

Fig. 3 shows the temperature dependencies of the relaxation time of the slow mode τ_s for the solutions with different concentrations. It is evident that as the protein content in the solution grows, the relaxation time τ_s increases, which is observed in the entire temperature interval studied. This indicates that the average cluster sizes increase with increasing protein concentration. The temperature behavior of τ_s for the solutions with low and high volume fractions of protein appreciably differs. For the sample with a lysozyme concentration of 10 mg/ml the relaxation time τ_s monotonically decreases with increasing temperature. Both the cluster sizes and their mean lifetimes gradually decrease at heating, which is due to a more intense thermal motion of molecules. This agrees well with the data of other authors [12]. The lysozyme diffusion coefficient increases (see Fig. 4) with increasing temperature and therefore the mean time during which molecules move inside the clusters decreases. For the protein concentrations of 100 mg/ml, τ_s at first decreases as temperature rises to ~40 °C, and then increases at higher-temperatures. The minimum at T_m in the temperature

Table 1
Activation energies ΔE_a for translational lysozyme diffusion in solutions with different protein concentrations.

Lysozyme concentration (mg/ml)	Activation energy (kcal/mol)
10	2.39 ± 0.16
100	1.42 ± 0.27
150	1.35 ± 0.13
200	1.43 ± 0.17
250	1.55 ± 0.07

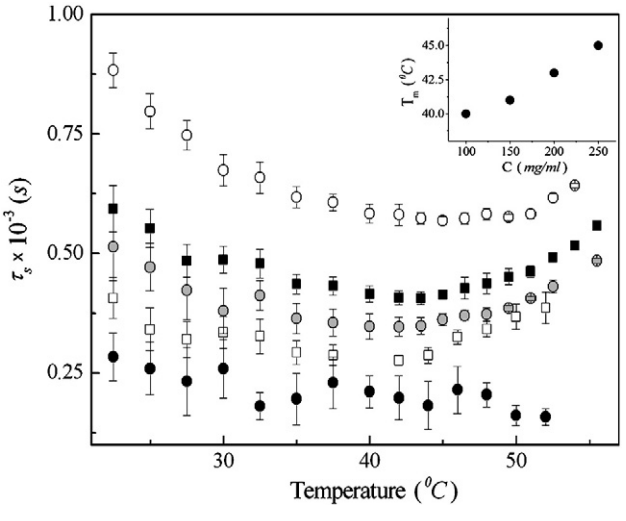

Fig. 3. Temperature dependences of relaxation times of the slow mode τ_s for solutions with lysozyme concentrations: 10 mg/ml (black circles), 100 mg/ml (open squares), 150 mg/ml (gray circles), 200 mg/ml (black squares), and 250 mg/ml (open circles). The inset shows how the temperature of minimum T_m at the temperature dependence of τ_s varies with protein concentration in the solution.

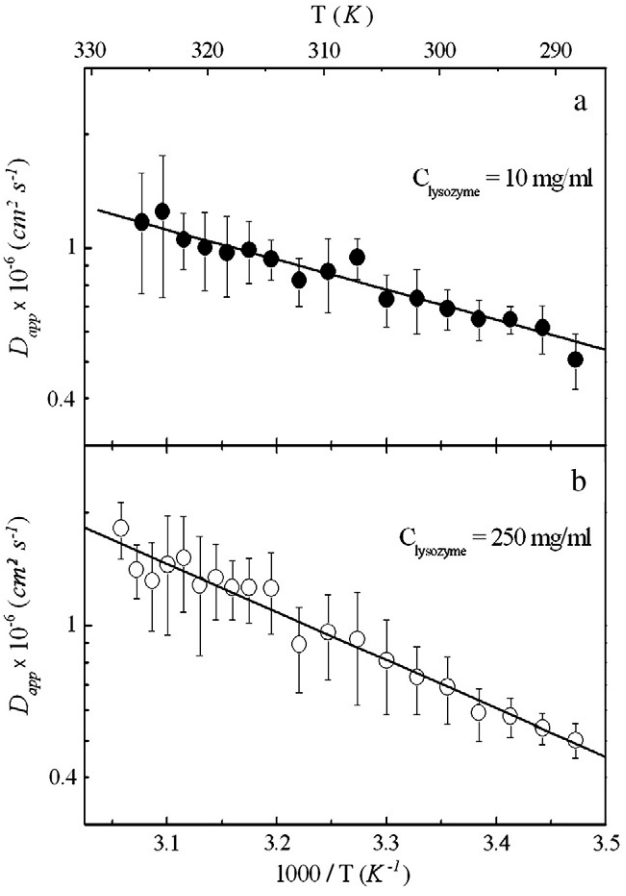

Fig. 4. Temperature dependences of apparent diffusion coefficient D_{app} for lysozyme solutions with concentrations (a) 10 mg/ml and (b) 250 mg/ml presented as an Arrhenius plot. The black lines show results of fitting of the dependences by Eq. (6).

dependence indicates that cluster sizes begin to increase above some critical temperature. It is interesting that minima are also observed in the temperature dependences of τ_s for the solutions with lysozyme concentrations 150, 200, and 250 mg/ml. This minimum shifts to a higher-temperature region as the protein concentration increases (see the inset to Fig. 3). It is important to note that the protein concentration growth leads to a considerable increase in the slow mode relaxation time τ_s in the entire temperature interval studied. This suggests that, as protein concentration increases, the mean sizes of clusters also increase. In this case a changeover from the regime of cluster size decrease with increasing temperature (below T_m) to the cluster size increase regime (above T_m) occurs at a higher temperature.

The sizes of individual clusters in a solution, and, hence, their relaxation times can be different, and, therefore, τ_s should be regarded as an averaged value for all the clusters. The information on the distribution of relaxation times τ_s of clusters in each solution can be gained from the magnitude of β which was obtained by the fitting of the experimental function $g(\tau)$ (see Eq. (3)). For the solutions with high protein concentrations at room temperature β is 0.71 ± 0.05 and increases linearly to 0.89 ± 0.05 as temperature increases to 55 °C. This means that the distribution of relaxation times τ_s at increasing temperature becomes narrower.

4. Conclusions

The DLS study of lysozyme solutions with different protein contents has shown that the autocorrelation functions obtained in the experiments are determined by the contributions of two relaxation modes with different relaxation times. The fast mode with relaxation time τ_f characterizes the diffusion of lysozyme molecules, whereas the slow mode with relaxation time τ_s is associated with a correlated motion of lysozyme molecules within a cluster. The analysis of the temperature dependences of D_{app} has shown that the activation energies for the diffusion of macromolecules decrease with increasing protein concentration in the solution. This can be explained by the fact that under crowded conditions the diffusion of molecules is affected by intermolecular interactions. The temperature behavior of the relaxation time τ_s differs appreciably for solutions with low and high protein concentrations: for the 10 mg/ml lysozyme solution τ_s monotonically decreases at heating and for the high-concentration solutions a minimum appears at temperature T_m. The minimum shifts to the higher-temperature region as the protein content increases. Such a behavior of τ_s (T) is likely to be associated with a changeover from the regime of cluster size decrease to the regime of cluster size increase at heating.

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