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Mechanism of suppression of dithiothreitol-induced aggregation of bovine α-lactalbumin by α-crystallin

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ABSTRACT

The kinetics of dithiothreitol (DTT)-induced aggregation of α-lactalbumin from bovine milk has been studied using dynamic light-scattering technique. Analysis of the distribution of the particles formed in the solution of α-lactalbumin after the addition of DTT by size showed that the initial stage of the aggregation process was the stage of formation of the start aggregates with the hydrodynamic radius (R_h) of 80–100 nm. Further growth of the protein aggregates proceeds as a result of sticking of the start aggregates. Suppression of α-lactalbumin aggregation by α-crystallin is mainly due to the increase in the duration of the lag period on the kinetic curves of aggregation. It is assumed that the initially formed complexes of unfolded α-lactalbumin with α-crystallin were transformed to the primary clusters prone to aggregation as a result of the redistribution of the denatured protein molecules on the surface of the α-crystallin particles.

1. Introduction

When studying the kinetics of thermal aggregation of proteins using dynamic light-scattering (DLS), it was demonstrated that the initial stage of the aggregation process was formation of the start aggregates [1,2]. Each start aggregate contains hundreds of denatured protein molecules. DLS measurements provide a simultaneous registration of the native forms of protein substrates and protein aggregates. The intermediate states between the native enzyme forms and start aggregates were not detected. The hydrodynamic radius of the start aggregates was tens of nanometers. The start aggregate formation was revealed in the process of thermal aggregation of the following model substrates: β_L-crystallin from bovine lens [1], glyceraldehyde-3-phosphate dehydrogenase and glycogen phosphorylase *b* from rabbit skeletal muscle [2–6], aspartate aminotransferase from pig heart mitochondria [7] and tobacco mosaic virus coat protein [8].

α-Crystallin, a member of small heat shock protein family, functions as a molecular chaperone by interacting with unfolded or misfolded proteins to prevent their aggregation [9]. It has a broad molecular mass distribution of 300 to 1000 kDa and forms large oligomer assemblies that display great heterogeneity [10]. In the presence of α-crystallin deceleration of thermal aggregation was observed accompanied by a decrease in the hydrodynamic radius of the start aggregates [1,7]. It should be noted that the chaperone-like activity of α-crystalline is enhanced at temperatures above 30 °C [11,12]. Therefore, to avoid complication at interpreting the results of thermal aggregation experiments, another way to denature model proteins should be used.

It is of special interest to elucidate whether the mechanism of aggregation involving the stage of formation of the start aggregates is fulfilled in the case of dithiothreitol (DTT)-induced aggregation of model substrates containing disulfide bonds, such as α-lactalbumin, insulin, and lysozyme. It should be noted that α-crystallin itself is not susceptible to DTT since it does not contain disulfide bonds.

A mammalian milk protein, α-lactalbumin, is a small (14.2 kDa) calcium-binding protein containing four disulfide bridges. It demonstrates a significantly different structural stability in its calcium-bound and calcium-free apo-form. Under stress conditions, the Ca^{2+}-depleted form of α-lactalbumin attains a classical molten globule state [13,14]. α-Lactalbumin partially denatured by reducing its disulfide bonds by DTT is widely used as a simple model substrate in folding studies [15–18]. A detailed study has been performed using DLS measurements to gain insight into the kinetics of DTT-induced aggregation of α-lactalbumin and to record size distribution of particles in the absence and presence of α-crystallin [19–21]. However, the mechanisms involved in aggregation of α-lactalbumin under stress conditions and in the interaction of α-crystallin with a denatured substrate thereby preventing its uncontrolled aggregation are far from being clear.

In the present work the kinetics of DTT-induced aggregation of α-lactalbumin was studied at various concentrations of the protein

Abbreviations: DLCA, diffusion-limited cluster–cluster aggregation; DLS, dynamic light scattering; DTT, dithiothreitol; RLCA, reaction-limited cluster–cluster aggregation.

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using DLS. Analysis of the distribution of the particles formed in the solution of α-lactalbumin after the addition of DTT by size allowed us to conclude that the aggregation proceeds through the stage of formation of the start aggregates with the hydrodynamic radius (R_h) of 80–100 nm. Character of the dependence of the hydrodynamic radius of the particles formed in the presence of α-crystallin indicated that immediately after the addition of DTT the complexes of unfolded α-lactalbumin with α-crystallin were formed in the system. It is assumed that the duration of the lag period on the kinetic curves of aggregation obtained in the presence of α-crystallin is determined by the transformation of the α-crystallin–unfolded α-lactalbumin complexes into the primary clusters (with $R_h \approx 20$ nm) prone to aggregation as a result of the redistribution of the unfolded α-lactalbumin molecules on the surface of the α-crystallin particles.

2. Materials and methods

2.1. Materials

Type I α-lactalbumin from bovine milk, DTT and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Sigma. All other chemicals were of reagent grade. All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA).

2.2. Isolation and purification of α-crystallin

Purification of α-crystallin from freshly excised lenses of 2-year-old steers was performed according to the procedure described earlier [22]. The α-crystallin concentration was determined from the absorbance at 280 nm using the extinction coefficient $A^{1\%}_{280}$ of 8.5 [23].

2.3. DTT-induced aggregation of α-lactalbumin

Aggregation of α-lactalbumin (0.2–1.6 mg mL^{-1}) in the absence or presence of varying concentrations of α-crystallin was studied in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA. Reduction of α-lactalbumin was initiated by adding DTT to 0.5 mL of the sample to a final concentration of 20 mM. The experiments were performed at 37 °C.

2.4. Dynamic light-scattering studies

DLS measurements were performed by a commercial instrument Photocor Complex (Photocor Instruments Inc., USA; www.photocor.com) as described in our previous works where DLS was used for the study of the kinetics of thermal aggregation of proteins [1,4,5,24]. An He–Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) was used as the light source. The temperature of sample cell was controlled by the proportional integral derivative (PID) temperature controller to within ±0.1 °C. The quasi-cross correlation photon counting system with two photomultiplier tubes was used to increase the accuracy of particle sizing in the range from 1.0 nm to 5.0 μm. DLS data have been accumulated and analyzed with multifunctional real-time correlator Photocor-FC. DynaLS software (Alango, Israel) was used for polydispersity analysis of the DLS data. The diffusion coefficient D of the particles is directly related to the decay rate τ_c of the time-dependent correlation function for the light-scattering intensity fluctuations: $D = 1/2\tau_c k^2$. In this equation k is the wavenumber of the scattered light, $k = (4\pi n/\lambda)\sin(\theta/2)$, where n is the refractive index of the solvent, λ is the wavelength of the incident light in vacuum and θ is the scattering angle. The mean hydrodynamic radius of the particles (R_h) can then be calculated according to the Stokes–Einstein equation: $D = k_B T/6\pi\eta R_h$, where k_B is Boltzmann's constant, T is the absolute temperature and η is the shear viscosity of the solvent.

To analyze the time-course of the increase in the light-scattering intensity (I) accompanying aggregation of α-lactalbumin, we used the approaches elaborated by us previously [25]. To calculate the duration of the lag period (t_2) on the dependences of the I value on time, we used the empiric equation:

$$I = [K_{LS}(t - t_2)]^2, \quad (1)$$

where K_{LS} is a constant with the dimension (counts/s)$^{1/2}$ min^{-1} (index LS means "Light Scattering"). It should be noted that in the present work the duration of the lag period was designated as t_2, in order to preserve the notation used by us earlier [2].

When studying the kinetics of thermal aggregation of proteins, we observed that the typical dependences of the hydrodynamic radius (R_h) of the protein aggregates on time were as follows. The initial parts of the dependences of R_h on time were linear. The following equation may be used for the description of these parts of the dependences of R_h on time [1]:

$$R_h = R_{h,0}\left[1 + \frac{t - t_3}{t_{2R}}\right], \quad (2)$$

where $R_{h,0}$ is the hydrodynamic radius of the protein aggregates at $t = t_3$, i.e., at the moment of time at which the hydrodynamic radius of the protein aggregates begins to increase (the notation proposed in [2] is used), and t_{2R} is the time interval over which the hydrodynamic radius increases from $R_{h,0}$ to $2R_{h,0}$. The reciprocal value of parameter t_{2R} characterizes the rate of aggregation. The higher the $1/t_{2R}$ value, the higher is the rate of aggregation.

Analysis of the full dependences of R_h of the protein aggregates for thermal aggregation of Ph*b* on time [1,3,5–8] showed that above the definite point of time designated as t^* the dependences of R_h on time followed the power law:

$$R_h = R_h^*[1 + K_1(t - t^*)]^{1/d_f}, \quad (3)$$

where R_h^* is the R_h value at $t = t^*$, K_1 is a constant and d_f is the fractal dimension of the aggregates. The d_f value was found to be close to 1.8. This value of d_f is typical of the regime of diffusion-limited cluster–cluster aggregation (DLCA), wherein each collision of the interacting particles results in their sticking [26–28].

In the presence of α-crystallin a decrease in the rate of aggregation of the protein substrates occurs, and the dependence of the hydrodynamic radius of the protein aggregates on time becomes exponential [1,5,7]:

$$R_h = R_{h,0}\left\{\exp\left[\frac{\ln 2}{t_{2R}}(t - t_3)\right]\right\}, \quad (4)$$

where t_3 is the moment of time at which the R_h value begins to increase and t_{2R} is the time interval over which the R_h value is doubled. Such a character of the dependence of R_h on time indicates that the aggregation process proceeds in the regime of reaction-limited cluster–cluster aggregation (RLCA), wherein the sticking probability for the colliding particles is lower than unity [28].

2.5. Fluorescence spectroscopy

The tryptophan fluorescence measurements were performed using a fluorescence spectrophotometer Cary eclipse (USA) with a temperature-controlled cuvette holder. Samples were excited at 297 nm and fluorescence emission spectra were recorded from 300 to 400 nm. The excitation and emission bandwidths were set at 5 nm. Before measurement, protein samples (α-crystallin and/or α-lactalbumin) were preincubated for 30 min at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA, in the absence or presence of 20 mM DTT. An aliquot of the incubation mixture was then added to 500 μL of the buffer to a final protein concentration of 0.1 mg mL^{-1}. The fluorescence measurements were performed using a quartz cuvette with a 1 cm path length. Each spectrum was the

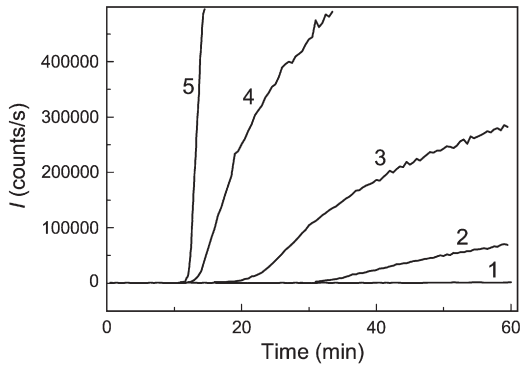

Fig. 1. Kinetics of DTT-induced aggregation of α-lactalbumin at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA. The final concentration of DTT was 20 mM. The dependences of the light-scattering intensity (I) on time. The concentrations of α-lactalbumin were as follows: 0.2 (1), 0.4 (2), 0.6 (3), 0.8 (4) and 1.6 (5) mg mL^{-1}.

average of 12 scans. In all experiments the time of sample exposition to illumination was minimized in order to avoid the UV-induced spectral effects as a result of reduction of the S–S bonds and the consequent structural rearrangements in the protein molecule.

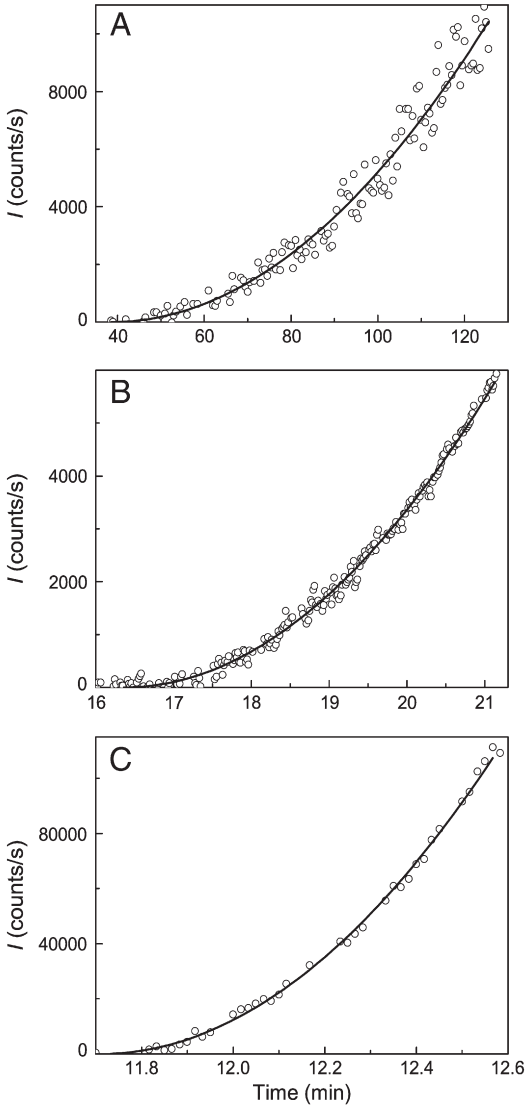

Fig. 2. Analysis of the initial parts of the dependences of the light-scattering intensity (I) on time for DTT-induced aggregation of α-lactalbumin. The final concentration of DTT is 20 mM. The concentrations of α-lactalbumin were as follows: 0.2 (A), 0.6 (B) and 1.6 (C) mg mL^{-1}. Points are the experimental data. Solid curves were calculated from Eq. (1).

2.6. Calculations

Origin 7.0 (OriginLab Corporation, USA) was used for the calculations.

3. Results

3.1. Kinetics of dithiothreitol-induced aggregation of α-lactalbumin

DLS technique allows measuring the light-scattering intensity and size of particles in the course of protein aggregation. Aggregation of α-lactalbumin was studied by DLS at 37 °C in 50 mM Na–phosphate buffer, pH 6.8, at various concentrations of the protein. The aggregation

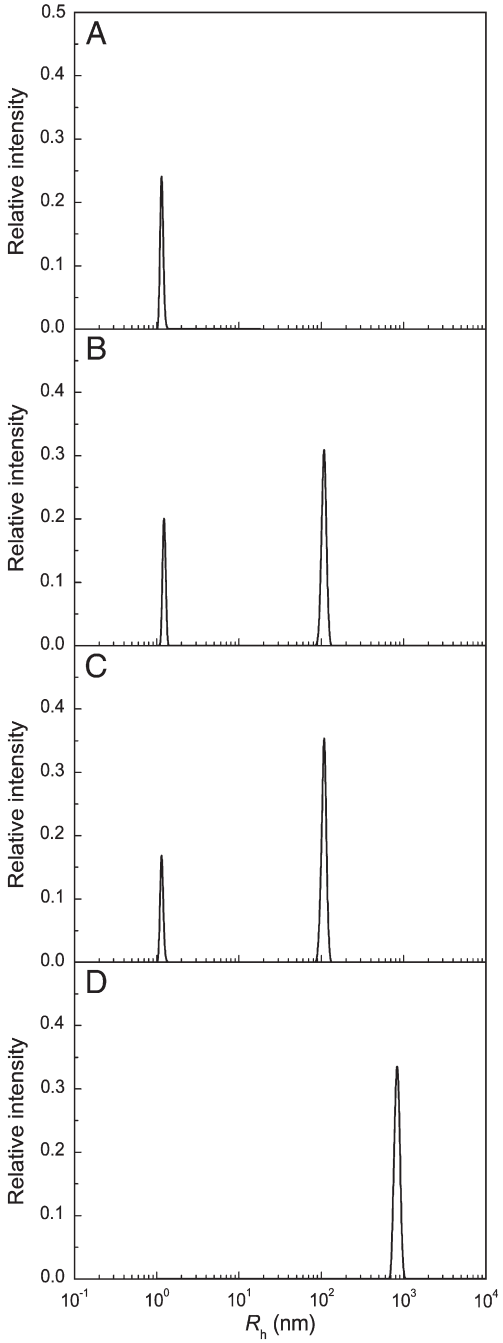

Fig. 3. The distribution of the particles by size for α-lactalbumin (0.6 mg mL^{-1}) incubated at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl, 1 mM EGTA and 20 mM DTT, for 5 (A), 18.5 (B), 23 (C) and 45 (D) min. Units of the ordinate axis correspond to the commonly used relative coherence of the measured correlation function, i.e., ratio of the correlated to uncorrelated part of the light-scattering intensity.

process was initiated by the addition of DTT to the final concentration of 20 mM (Figs. 1–3).

Fig. 1 demonstrates the increment of the light-scattering intensity (I) accompanying DTT-induced aggregation of α-lactalbumin. The increase in the concentration of α-lactalbumin in the interval from 0.2 to 1.6 mg mL^{-1} results in the increase in the rate of aggregation registered by the enhancement of the light-scattering intensity (curves 1–5). As can be seen from Fig. 1, the kinetic curves have well-defined lag periods. To estimate the duration of lag period, the initial parts of the dependences of I on time were analyzed using Eq. (1). As an example, Fig. 2 shows the fitting of the initial parts of the dependences of I on time obtained at the concentrations of α-lactalbumin of 0.2, 0.6 and 1.6 mg mL^{-1}. The values of the duration of lag period (t_2) and the values of parameter K_{LS} are given in Table 1. The value of t_2 decreases from 39 to 11.6 min, when the concentration of α-lactalbumin increases in the interval from 0.2 to 1.6 mg mL^{-1}. Parameter K_{LS} characterizing the rate of aggregation increases by a factor of 320 at eight-fold increase in the concentration of α-lactalbumin (from 0.2 to 1.6 mg mL^{-1}).

The measurements of the size of the particles formed in the solution of α-lactalbumin after the addition of DTT allow us to draw the conclusion regarding the mechanism of the formation of the protein aggregates. Consider as an example the time-course of aggregation in the solution of α-lactalbumin at the concentration of 0.6 mg mL^{-1} (Fig. 3). The distribution of the particles by size was measured at 30 s intervals. Over the first 18 min the single peak with $R_h = 1.2 \pm 0.1$ nm is registered in the system (Fig. 3A). It is evident that this peak corresponds to the non-aggregated form of α-lactalbumin. Aggregates with larger size obviously do not present in the solution during this stage. Sensitivity of the DLS method is considerably increasing with the growth of particles and they would be observed even at low concentration. At $t = 18.5$ min the start aggregates with $R_{h,0} = 92$ nm appear and two peaks are registered on the distribution of the particles by size (Fig. 3B). This moment in time is designated as t_1. It should be noted that intermediates between the peaks with $R_h =$ 1.2 nm and $R_h = 92$ nm are lacking. Thus, the formation of the start aggregates proceeds on the all-or-none principle.

In the interval from 18.5 to 23 min the position of the peak corresponding to the start aggregates remains unchangeable (Fig. 3C). Since the moment in time at which the start aggregates appear ($t_1 = 18.5$ min) is very close to the moment in time at which the light-scattering intensity begins to increase ($t_2 = 16.4 \pm 0.1$ min), one can conclude that the increment of the light-scattering intensity in the interval from 16.4 to 22.8 min is due to the accumulation of the start aggregates in the system, the size of the start aggregates remaining unchangeable. At $t > 23$ min the size of the start aggregates begins to increase.

Fig. 3D shows the distribution of the particles by size at $t = 45$ min. The hydrodynamic radius of the protein aggregates reaches the value of about 800 nm. The non-aggregated form of α-lactalbumin is not detected probably because the huge light-scattering from large aggregates masks weak scattering from small particles of α-lactalbumin on the final stage of the aggregation process. Unfortunately, the DLS method practically does not allow to estimate concentrations of each component of the particle size distribution. That is why the detailed mechanism of aggregate growth and variation of the number of particles in each component in the course of aggregation is out of discussion.

The time-course of the positions of the peaks on the distribution of the particles by size for aggregation of α-lactalbumin at the concentration of 0.6 mg mL^{-1} is demonstrated in Fig. 4A. The solid circles correspond to the non-aggregated form of α-lactalbumin, and the open circles correspond to the protein aggregates. Inset with expanded ordinate axis demonstrates the constancy of the R_h value for the start aggregates in the interval from 18.5 to 22.8 min (the average value of $R_{h,0}$ is equal to 92 ± 8 nm) and the increase in the R_h value at $t > 23$ min.

Fig. 4B–D shows the dependences of the R_h values for the non-aggregated form of α-lactalbumin and aggregates of α-lactalbumin at higher concentrations of the protein (0.8, 1.0 and 1.6 mg mL^{-1}, respectively). As can be seen from this figure, at each concentration of α-lactalbumin the start aggregates appear at definite point in time ($t = t_1$). It is worthy of note that no intermediates are detected between the non-aggregated form of α-lactalbumin and protein aggregates. Over several minutes the size of the start aggregates remains constant, and at the certain point in time the hydrodynamic radius of the protein aggregates begins to increase. The values of t_1 (the point in time at which the start aggregates appear) and the average value of the hydrodynamic radius of the start aggregates ($R_{h,0}$) calculated at various concentrations of α-lactalbumin are given in Table 1. The corresponding values obtained at the concentrations of α-lactalbumin of 0.2, 0.4 and 1.2 mg mL^{-1} are added. The t_1 value decreases from 31.0 to 11.2 min, when the concentration of α-lactalbumin increases in the interval from 0.2 to 1.6 mg mL^{-1}. The hydrodynamic radius of the start aggregates $R_{h,0}$ remains practically constant and the $R_{h,0}$ value falls in the interval from 79 to 103 nm.

Let us designate the point in time at which the hydrodynamic radius of the start aggregates begins to increase as t_3. To determine more precisely the t_3 value, the initial increment of the R_h value in time was quantitatively analyzed using Eq. (2) or Eq. (4). At rather low concentrations of α-lactalbumin (0.2–0.8 mg mL^{-1}) the dependences of R_h on time over several minutes are exponential suggesting that aggregation proceeds in the RLCA regime (Fig. 4A and B). With a knowledge of the $R_{h,0}$ value we determined parameters t_3 and t_{2R} (the time interval over which the R_h value is doubled) using Eq. (4). The t_3 values and the reciprocal values of parameter t_{2R} (notice that $1/t_{2R}$ characterizes the rate of aggregation) are given in Table 1. When the concentration of α-lactalbumin increases in the interval from 0.2 to 0.8 mg mL^{-1}, the t_3 value decreases from 73 ± 2 to 13.9 ± 0.2 min and the $1/t_{2R}$ value increases from 0.015 ± 0.004 to 0.44 ± 0.03 min^{-1}.

The reason for the appearance of a lag period on the dependence of the R_h value for the protein aggregates on time is the following. The increase in the R_h value at $t > t_3$ is due to sticking of the start

Table 1
Parameters of DTT-induced aggregation of α-lactalbumin at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA.

[α-Lactalbumin], mg mL^{-1}	t_1, min	t_2, min	K_{LS}, (counts/s)$^{1/2}$ min^{-1}	$R_{h,0}$, nm	t_3, min	$1/t_{2R}$, min^{-1}	t^*, min	d_f
0.2	31.0	39 ± 1	1.18 ± 0.03	79 ± 2	73 ± 2	0.015 ± 0.004		
0.4	19.5	20.5 ± 0.1	6.0 ± 0.1	103 ± 9	33 ± 1	0.096 ± 0.008		
0.6	18.5	16.4 ± 0.1	16.0 ± 0.5	92 ± 8	22.8 ± 0.3	0.34 ± 0.03	30.5	1.80 ± 0.01
0.8	12.5	12.4 ± 0.1	94 ± 4	88 ± 9	13.9 ± 0.2	0.44 ± 0.03	23.5	1.79 ± 0.02
1.0	12.0	11.9 ± 0.1	96 ± 5	90 ± 4	13.8 ± 0.4	0.69 ± 0.05	20.5	1.80 ± 0.01
1.2	11.5	11.8 ± 0.1	272 ± 9	96 ± 6	12.9 ± 0.1	1.2 ± 0.1	19.0	1.80 ± 0.01
1.6	11.2	11.6 ± 0.1	382 ± 7	100 ± 2	12.1 ± 0.1	2.6 ± 0.1	14.5	1.79 ± 0.02

t_1 is the time of the appearance of the start aggregates; t_2 is the duration of the lag period on the dependences of the light-scattering intensity on time (t_2 was calculated from Eq. (1)); K_{LS} is a constant calculated from Eq. (1); $R_{h,0}$ is the average value of the hydrodynamic radius of the start aggregates calculated for the interval ($t_1 - t_3$); t_3 is the point in time, at which the hydrodynamic radius of the start aggregates begins to increase (t_3 was calculated from Eq. (4) at the lactalbumin concentrations of 0.2, 0.4, 0.6, and 0.8 mg mL^{-1} and from Eq. (2) at the lactalbumin concentrations of 1.0, 1.2, and 1.6 mg mL^{-1}); t_{2R} is the time interval, over which the R_h value increases from $R_{h,0}$ to $2R_{h,0}$; t^* is the point in time, above which the dependences of R_h on time begin to follow the power law; and d_f is the fractal dimension of the aggregates (d_f was calculated from Eq. (3)).

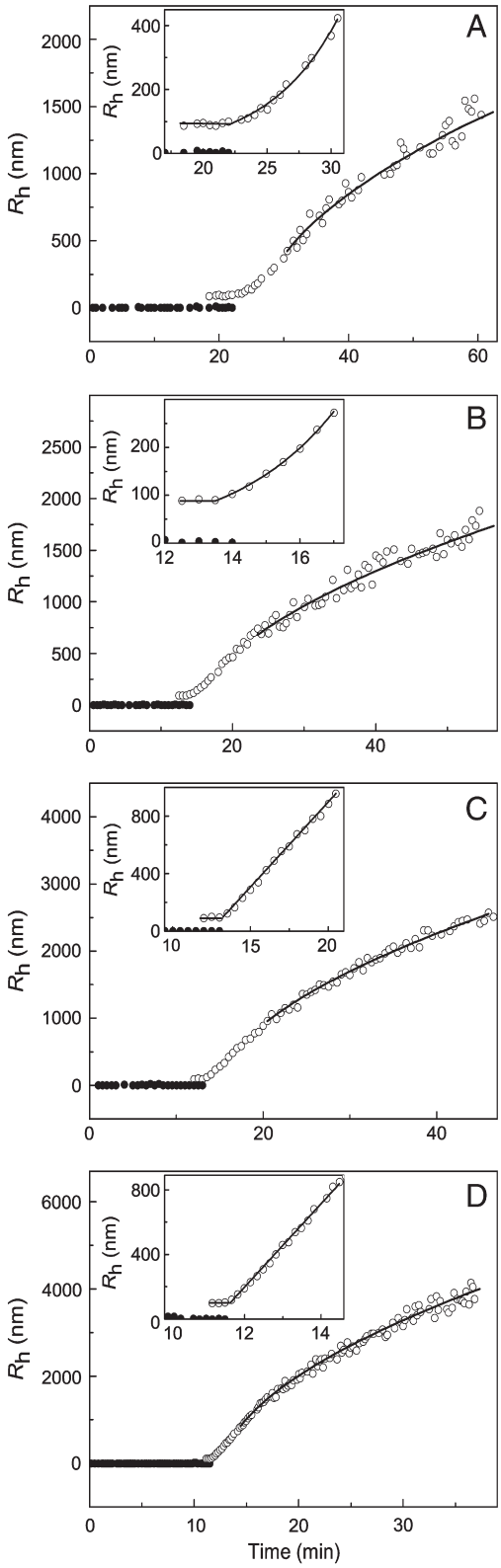

Fig. 4. Analysis of the size of the particles in the solution of α-lactalbumin after the addition of DTT (the final concentration of DTT was 20 mM). The dependences of the hydrodynamic radius (R_h) of the non-aggregated form of α-lactalbumin (solid circles) and protein aggregates (open circles) on time. The concentrations of α-lactalbumin were as follows: 0.6 (A), 0.8 (B), 1.0 (C) and 1.6 (D) mg mL^{-1}. The solid curves were calculated from Eq. (3). Insets show the dependences of R_h on time with the expanded ordinate axis. The horizontal lines correspond to the hydrodynamic radius of start aggregates ($R_{h,0}$). The solid curves were calculated from Eq. (4) (panels A and B) or Eq. (2) (panels C and D).

aggregates. The interaction of the start aggregates is the reaction of the second order. Therefore, at low concentrations of the start aggregates the rate of their sticking remains rather low, and the constant value of R_h is registered (the region $t_1 - t_3$). Accumulation of the start aggregates results in the sharp increase in the rate of their sticking and, as a consequence, in the appearance of the lag period on the dependence of R_h on time.

Above the definite value of time ($t > t^*$) the dependences of R_h on time become convex and may be described by Eq. (3) with the d_f value close to 1.8 (see Table 1). This part of the dependence of R_h on time corresponds to the DLCA regime of aggregation. The reason of the change in the kinetics of aggregation from the RLCA regime to the DLCA regime is that sticking probability of the colliding particles increases as the size of the protein aggregates increases [28].

When the concentration of α-lactalbumin is equal to 1.0 mg mL^{-1} and higher, the increment of the R_h value in time at $t > t_3$ is linear and may be described by Eq. (2) (Fig. 4C and D). With a knowledge of the $R_{h,0}$ value we can calculate parameters t_3 and t_{2R} (the time interval over which the R_h value increases from $R_{h,0}$ to $2R_{h,0}$). The values of t_3 and $1/t_{2R}$ are given in Table 1. When the concentration of α-lactalbumin increases in the interval from 1.0 to 1.6 mg mL^{-1}, the t_3 value slightly decreases (from 13.8 ± 0.4 to 12.1 ± 0.1 min) and 3.8-fold increase in the $1/t_{2R}$ value takes place. At $t > t^*$ the dependence of R_h on time follows the degree law, Eq. (3), with the d_f value close to 1.8 (Table 1). The linear dependence of the hydrodynamic radius of aggregates on time passing into the degree law is typical of the DLCA regime of aggregation of colloidal particles [29]. Thus, the increase in the concentration of α-lactalbumin results in the transition of the process of sticking of the start aggregates from the RLCA regime (the exponential dependence of R_h on time) to the DLCA regime (the linear dependence of R_h on time passing into the degree law).

3.2. The effect of α-crystallin on the kinetics of dithiothreitol-induced aggregation of α-lactalbumin

To characterize the effect of α-crystallin on the kinetics of DTT-induced aggregation of α-lactalbumin, we analyzed the time-course of the light-scattering intensity I and the hydrodynamic radius R_h values of the particles formed in the solution of α-lactalbumin (0.8 mg mL^{-1}) after the addition of DTT in the presence of various concentrations of α-crystallin in the interval from 0.02 to 0.08 mg mL^{-1}. As can be seen from Fig. 5, α-crystallin suppresses the increment of the light-scattering intensity in time. The higher the concentration of α-crystallin, the higher is the effect of suppression of aggregation. The analysis of the initial parts of the dependences of the light-scattering intensity in time gives additional information on the effect of α-crystallin on aggregation of α-lactalbumin. The application of Eq. (1) allowed us to calculate the duration of lag period (parameter

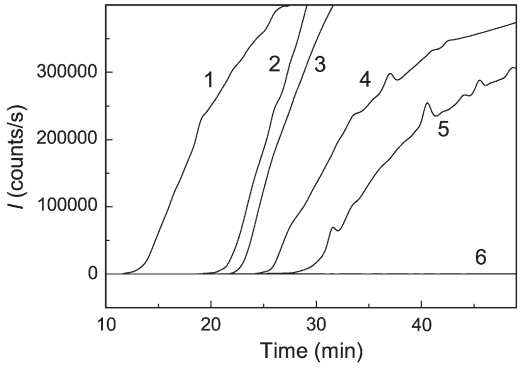

Fig. 5. The effect of α-crystallin on the kinetics of DTT-induced aggregation of α-lactalbumin (0.8 mg mL^{-1}). The final concentration of DTT was 20 mM. The dependences of the light-scattering intensity (I) on time at the following concentrations of α-crystallin: 0 (1), 0.02 (2), 0.04 (3), 0.06 (4), 0.08 (5) and 0.2 (6) mg mL^{-1}.

t_2) and parameter K_{LS} characterizing the rate of aggregation. Fig. 6 demonstrates the description of the initial parts of the dependences of the light-scattering intensity on time by Eq. (1) for the kinetic curves registered at the concentrations of α-crystallin of 0.02, 0.06 and 0.08 mg mL^{-1}. The values of parameters t_2 and K_{LS} obtained at various concentrations of α-crystallin are given in Table 2. The main effect of α-crystallin on the α-lactalbumin aggregation is the increase in the duration of the lag period. In the absence of α-crystallin t_2 is equal to 12.4 min. When the concentration of α-crystallin was 0.2 mg mL^{-1}, parameter t_2 reached the value of 173 ± 3 min. It is surprising that in the presence of rather low concentrations of α-crystallin the increase in parameter K_{LS} takes place. In the absence of α-crystallin K_{LS} is equal to 94 ± 4 (counts/s)$^{1/2}$ min^{-1}. However, when aggregation of α-lactalbumin is studied in the presence of α-crystallin at the concentration of 0.02 mg mL^{-1}, parameter K_{LS} increases to the value of 139 ± 2 (counts/s)$^{1/2}$ min^{-1}. The K_{LS} value remains rather high (K_{LS} = 29 ± 2 (counts/s)$^{1/2}$ min^{-1}) at the α-crystallin concentration of 0.06 mg mL^{-1}. Further increase in the concentration of α-crystallin results in the decrease in parameter K_{LS} to the values below the control value (K_{LS} = 94 (counts/s)$^{1/2}$ min^{-1}). For example, at the α-crystallin concentration of 0.2 mg mL^{-1} parameter K_{LS} is equal to 2.20 ± 0.05 (counts/s)$^{1/2}$ min^{-1}.

Fig. 7 shows the dependences of the hydrodynamic radius (R_h) of the particles formed in the solution of α-lactalbumin (0.8 mg mL^{-1}) after the addition of DTT in the presence of α-crystallin at the concentrations of 0.02, 0.06 and 0.08 mg mL^{-1}. Consider, for example, the time-course of R_h at the α-crystallin concentration of 0.02 mg mL^{-1} (Fig. 7A). Immediately after the addition of DTT the particles with R_h = 17.6 ± 0.9 nm are registered in the system. The value of R_h remains constant over 22 min. When interpreting this result, we should take into account that judging from our measurements the hydrodynamic radius of α-crystallin was 13.2 ± 0.06 nm. Besides, as shown in the fluorescence measurements (see the next section), the formation of the unfolded form of α-lactalbumin after the reduction of S–S bonds proceeded very quickly. Thus, one can assume that the particles with R_h = 17.6 nm are the complex of α-crystallin with the unfolded form of α-lactalbumin. Let us designate the hydrodynamic radius of this complex as $R_{h,complex}$. At definite point in time the hydrodynamic radius of the primary particles begins to increase. The increment of the R_h value in time follows the exponential law. The following variant of Eq. (4) may be used for the description of the dependence of R_h on time:

$$R_h = R_{h,complex}\left\{\exp\left[\frac{\ln 2}{t_{2R}}(t-t_3)\right]\right\}. \quad (5)$$

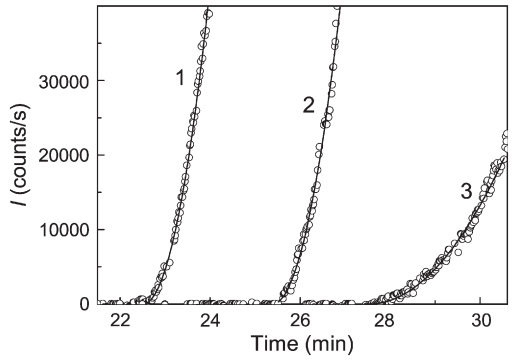

Fig. 6. The analysis of the initial parts of the dependences of the light-scattering intensity (I) on time for DTT-induced aggregation of α-lactalbumin (0.8 mg mL^{-1}). The final concentration of DTT was 20 mM. The concentrations of α-crystallin were as follows: 0.02 (1), 0.06 (2) and 0.08 (3) mg mL^{-1}. The solid curves are calculated from Eq. (1).

Table 2
Parameters of DTT-induced aggregation of α-lactalbumin (0.8 mg mL^{-1}) in the presence of various concentrations of α-crystallin.

[α-Crystallin], mg mL^{-1}	t_2, min	K_{LS}, (counts/s)$^{1/2}$ min^{-1}	$R_{h,complex}$, nm	t_3, min	$1/t_{2R}$, min^{-1}
0	12.4 ± 0.1	94 ± 4			
0.02	22.5 ± 0.1	139 ± 2	17.6 ± 0.9	22.3 ± 0.1	0.59 ± 0.02
0.04	23.7 ± 0.2	133 ± 2	18.1 ± 0.4	23.6 ± 0.2	0.73 ± 0.03
0.06	25.4 ± 0.2	129 ± 2	17.5 ± 0.8	25.2 ± 0.2	0.69 ± 0.02
0.08	27.5 ± 0.3	46 ± 1	20.9 ± 1.2	27.4 ± 0.2	0.25 ± 0.01
0.20	173 ± 3	2.20 ± 0.05	17.5 ± 0.9	169 ± 3	0.012 ± 0.006

t_2 is the duration of the lag period on the dependences of the light-scattering intensity on time (t_2 was calculated from Eq. (1)); K_{LS} is a constant calculated from Eq. (1); $R_{h,complex}$ is the hydrodynamic radius of the complex of α-crystallin with the unfolded form of α-lactalbumin; t_3 is the point in time, at which the R_h value begins to increase (t_3 was calculated from Eq. (5)); and t_{2R} is the time interval over which the R_h value is doubled.

With a knowledge of the $R_{h,complex}$, it is possible to determine parameters t_3 and t_{2R}. At the selected concentration of α-crystallin (0.02 mg mL^{-1}) the values of t_3 and t_{2R} were found to be 22.3 ± 0.1 and 1.69 ± 0.05 min.

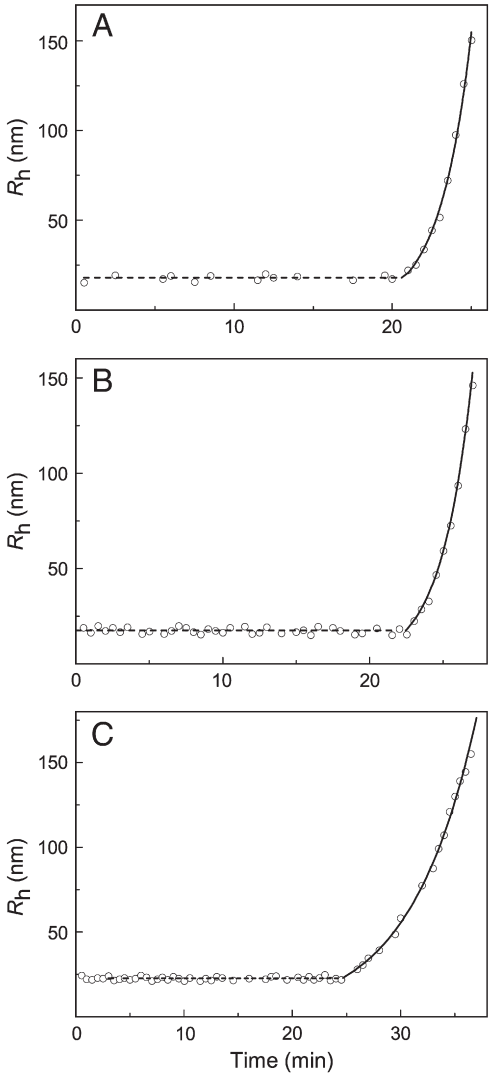

Fig. 7. The dependences of the hydrodynamic radius (R_h) of the particles formed in the solution of α-lactalbumin (0.8 mg mL^{-1}) after the addition of DTT in the presence of α-crystallin at the concentrations of 0.02 (A), 0.06 (B) and 0.08 (C). The solid curves are calculated from Eq. (5).

Obviously, it should be some intermediate smooth crossover from flat behavior of primary particles to exponential growth. However, accuracy of the measurements does not allow to reveal details of this crossover and, correspondingly, we are not able to suggest any theoretical description of this part of the curve.

The analogous character of the dependence of the hydrodynamic radius of the particles formed in the solution of α-lactalbumin after the addition of DTT is observed in the presence of other concentrations of α-crystallin. Fig. 7B and C show the dependences of R_h on time for aggregation of α-lactalbumin (0.8 mg mL^{-1}) in the presence of the α-crystallin concentrations of 0.06 and 0.08 mg mL^{-1}. The values of parameters $R_{h,complex}$, t_3 and $1/t_{2R}$ calculated at various concentrations of α-crystallin are given in Table 2. The hydrodynamic radius of the complex of α-crystallin with the unfolded form of α-lactalbumin ($R_{h,complex}$) remains practically constant at variation of the α-crystallin concentration, and the values of $R_{h,complex}$ fall in the interval 17–21 nm. As can be seen from Table 2, the values of t_2 coincide with the values of t_3. This means that the increase in the light-scattering intensity is due to sticking of the primary particles (the complexes of α-crystallin with the unfolded form of α-lactalbumin). It is interesting to analyze the change in the $1/t_{2R}$ value characterizing the rate of aggregation in the presence of α-crystallin. In the absence of α-crystallin the $1/t_{2R}$ value is equal to 0.44 ± 0.03 min^{-1}. At relatively low concentrations of α-crystallin (0.02–0.06 mg mL^{-1}) the increase in the $1/t_{2R}$ value takes place. For example, $1/t_{2R} = 0.59 \pm 0.02$ min^{-1} at the α-crystallin concentration of 0.02 mg mL^{-1}. High concentrations of α-crystallin induce the decrease in the $1/t_{2R}$ value in comparison with the control, i.e., the $1/t_{2R}$ value in the absence of α-crystallin. For example, $1/t_{2R} = 0.012 \pm 0.006$ min^{-1} at the α-crystallin concentration of 0.2 mg mL^{-1}.

3.3. Fluorescence studies of α-crystallin interaction with α-lactalbumin

The complex formation of the two proteins was also confirmed by the tryptophan fluorescence measurements (Fig. 8). Since both α-lactalbumin and α-crystallin contain tryptophan residues we used fluorescence spectroscopy to examine the tryptophan emission spectra of the individual proteins and the effect of binding of these components upon conformation of the proteins within the complex.

The incubation of protein samples (α-lactalbumin or α-crystallin) alone or a mixture of these proteins was performed either in the absence (A) or presence (B) of 20 mM DTT under the conditions presented above. Aliquots of the incubation mixture were then added to 500 μL of the buffer to a final protein concentration of 0.1 mg mL^{-1}.

In the absence of DTT, α-lactalbumin and α-crystallin taken alone give fluorescence spectra with emission maximum at 328 nm (A, curve 1) and 334 nm (curve 2), respectively. On reduction of disulfide bonds by DTT, unfolding α-lactalbumin can be monitored by a shift in the emission maximum of intrinsic fluorescence to 348 nm, as shown in Fig. 8B (curve 1). The results are consistent with those of others who have performed intrinsic tryptophan fluorescence measurements of conformational changes of α-lactalbumin induced by the stepwise or total reduction of its disulfide bridges [30–32].

In our experiments, the changes in tryptophan fluorescence of α-lactalbumin could be observed within 30 s after the start of the reduction process upon addition of DTT, the spectra being unchangeable at further incubation for at least 60 min. It should be noted that the average time constant for reduction of a disulfide bond itself and intramolecular reshuffling of disulfide bridges was estimated to be about 15 s [33]. Thus, the reduction of disulfide bonds followed by conformational changes in α-lactalbumin appears to be a very fast

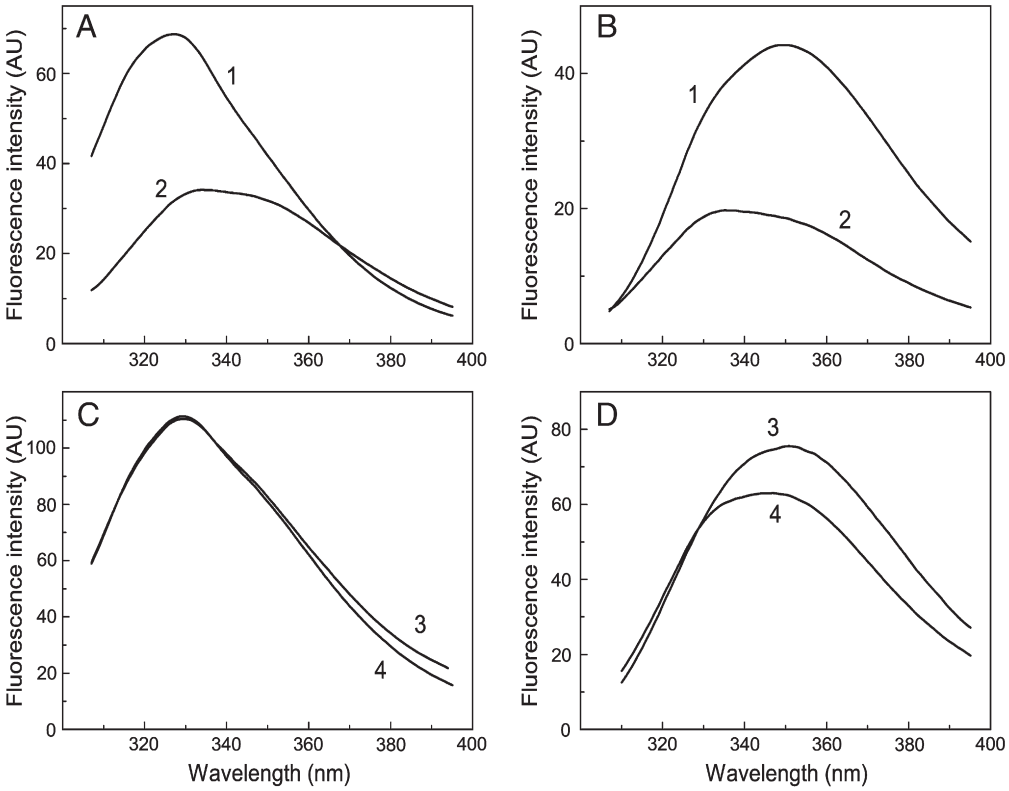

Fig. 8. The interaction between α-lactalbumin and α-crystallin monitored by tryptophan fluorescence. Intrinsic fluorescence emission spectra of α-lactalbumin (1) and α-crystallin (2) alone (A, B) and of their mixture (3) (C, D). The spectra are recorded upon excitation at 297 nm at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA obtained in the absence (A, C) or presence (B, D) of 20 mM dithiothreitol. The concentration of the proteins was 0.1 mg mL^{-1}. The relative fluorescence intensities (arbitrary units) were normalized to the final values by summation the individual spectra of α-crystallin and α-lactalbumin (4).

process. In contrast, bovine α-crystallin possesses no disulphide bonds and therefore its tryptophan emission spectrum did not change upon addition of DTT (B, curve 2). However, a quenching of tryptophan emission intensity by DTT was detected.

The results of the interaction experiments show that α-crystallin does not bind to the original form of α-lactalbumin (C). In the absence of DTT, the fluorescence intensity values and the wavelength of maximum fluorescence of the protein mixture (330 nm) are almost equal to those of the sum of the individual fluorescence emission spectra of α-lactalbumin and α-crystallin (C, curves 3 and 4, respectively), implying that there was little, if any, interaction between the two proteins.

Only the aggregation-prone reduced α-lactalbumin was found to form a complex with α-crystallin (D). A 351-nm emission maximum was recorded for the complex, whereas the 345-nm maximum represented the sum of the individual fluorescence spectra of the proteins (D, curves 3 and 4, respectively). The red shift in the wavelength of maximum fluorescence of the α-lactalbumin–α-crystallin complex of about 6 nm was revealed suggesting that the tertiary structure of the complex is less compact and has a more open configuration than the individual components. In addition, it is interesting to note that the maximum fluorescence intensity of the complex increased by 25% in comparison with control (D).

4. Discussion

The results of the study of the size of the particles formed in the solution of α-lactalbumin after the addition of DTT by DLS allow us to propose the following mechanism of the aggregation. A distinguishing feature of DTT-induced aggregation of α-lactalbumin is that aggregation proceeds through the stage of formation of the start aggregates. No intermediates between the non-aggregated form of α-lactalbumin and start aggregates are detected. This means that the concentrations of intermediates are negligibly low. Judging from the size of the start aggregates ($R_{h,0}$ = 80–100 nm), the start aggregate contains about 10^4 unfolded molecules of α-lactalbumin. It is reasonable to assume that formation of the start aggregates is preceded by generation of the clusters of lower size. To explain the appearance of lag period on the kinetic curves of aggregation, one can assume that the slow stage of the aggregation process is the stage of nucleation. Cooperative association of several molecules of unfolded α-lactalbumin (U) results in the formation of a nucleus (U_n). A start aggregate (A_{st}) is constructed from a definite number of nuclei. It should be noted that idea of nucleation is very popular in the attempts to explain the peculiarities of the kinetics of protein aggregation [25,34–40]. When the models of nucleation-dependent aggregation are used for description of the kinetics of protein aggregation, it is accepted that the growth of the aggregate proceeds as a result of the attachment of the unfolded protein molecule to the nucleus. The models of such a type predict that the aggregates of a limited size are formed when the pool of the unfolded protein molecules is exhausted. However, the analysis of the dependences of the hydrodynamic radius (R_h) of the protein aggregates on time for DTT-induced aggregation of α-lactalbumin showed that the R_h value did not tend to the limiting value at rather high values of time, and the increase in the R_h value in time followed the degree law ($R_h \sim t^{1/1.8} = t^{0.56}$). This fact suggests that the growth of the start aggregate proceeds as a result of sticking of the start aggregates rather than of the attachment of the unfolded α-lactalbumin molecule to a nucleus. The proposed mechanism of DTT-induced aggregation of α-lactalbumin is presented in Scheme 1.

Analogous mechanism of protein aggregation involving the stage of formation of the start aggregates and the stage of sticking of the start aggregates and the aggregates of higher order was proposed for thermal aggregation of proteins in our previous works [1–3,5,7,8]. In the case of DTT-induced aggregation of α-lactalbumin the size of the start aggregates remains practically unchangeable at the variation of the α-lactalbumin concentration. The same result was obtained for thermal aggregation of proteins [1,3,8].

1. Unfolding of the α-lactalbumin molecule (N and U are the native and unfolded states, respectively)

$$N \xrightarrow[fast]{DTT} U$$

2. The stage of nucleation (U_n is a nucleus)

$$nU \rightleftharpoons U_n$$

3. Formation of the start aggregates (A_{st})

$$mU_n \rightarrow A_{st}$$

4. Sticking of the start aggregates

$$A_{st} + A_{st} \rightarrow (A_{st})_2$$

$$(A_{st})_2 + A_{st} \rightarrow (A_{st})_3$$

. . . . . .

$$(A_{st})_k + A_{st} \rightarrow (A_{st})_{k+1}$$

. . . . . .

Scheme 1. Mechanism of DTT-induced aggregation of α-lactalbumin.

The fact that the size of the start aggregates is independent of the concentration of the protein involved in aggregation allows us to draw an analogy between the formation of the start aggregates and micelle formation. In the latter case the micelles of a definite size are formed when the critical micelle concentration is achieved. Such an analogy offers an explanation of why the formation of start aggregates proceeds according to the all-or-none principle.

The following explanation can be proposed for the change in the kinetic regime of DTT-induced aggregation of α-lactalbumin from the RLCA regime to the DLCA regime with increasing the α-lactalbumin concentration. The fulfillment of the exponential law for the initial increment of the R_h value in time at rather low concentrations of α-lactalbumin indicates that the aggregation process proceeds in the RLCA regime, wherein the sticking probability for the colliding particles is lower than unity. The increase in the size of the protein aggregates results in the enhancement of the contact region of the colliding particles and consequently in the increase in the sticking probability of the interacting particles. Thus, the transition to the DLCA regime at high concentrations of α-lactalbumin is simply due to the sharp decrease in the time interval over which the exponential law is fulfilled.

When interpreting the effect of α-crystallin on DTT-induced aggregation of α-lactalbumin, we should discuss first of all the following simplest mechanism of suppression of aggregation by α-crystallin. The unfolded α-lactalbumin forms a complex with α-crystallin, which is incapable of aggregation. After a certain period of time aggregation of unfolded α-lactalbumin remaining in a non-bound form is registered by the increase in light-scattering intensity and the increase in the hydrodynamic radius of the aggregates. In this case the values of parameters characterizing the rate of aggregation (K_{LS} and $1/t_{2R}$) should be lower than the corresponding values for the control (aggregation of α-lactalbumin in the absence of α-crystallin). The fact that the experimentally found values of parameters K_{LS} and $1/t_{2R}$ in the presence of α-crystallin at the concentrations of 0.02, 0.04 and 0.08 mg mL^{-1} (Table 2) is higher than the control values is indicative of an inadequacy of the mechanism under discussion. Besides, aggregation of the non-bound form of unfolded α-lactalbumin should proceed through the stage of formation of the start aggregates with $R_{h,0}$ = 80–100 nm. However, the hydrodynamic radius of the primary clusters participating in aggregation of α-lactalbumin in the presence of α-crystallin is essentially lower and equals to about 20 nm.

1. Unfolding of the α-lactalbumin molecule

$$N \xrightarrow[\text{fast}]{\text{DTT}} U$$

2. Complexation of the unfolded form of α-lactalbumin and α-crystallin (α)

$$iU + j\alpha \rightleftarrows U_i\alpha_j$$

3. Transformation of the complex $U_i\alpha_j$ into the primary clusters (C_{pr}) prone to aggregation

$$U_i\alpha_j \rightleftarrows C_{pr}$$

4. Sticking of the primary clusters

$$C_{pr} + C_{pr} \rightarrow (C_{pr})_2$$

$$(C_{pr})_2 + C_{pr} \rightarrow (C_{pr})_3$$

. . . . . .

$$(C_{pr})_k + C_{pr} \rightarrow (C_{pr})_{k+1}$$

. . . . . .

Scheme 2. Effect of α-crystallin on DTT-induced aggregation of α-lactalbumin.

Suppression of DTT-induced aggregation of α-lactalbumin by α-crystallin is revealed mainly as the increase in the duration of the lag period on the kinetic curves of aggregation. The character of the time-course of the hydrodynamic radius of the particles formed in the solution of α-lactalbumin after the addition of DTT in the presence of α-crystallin differs essentially from that for α-lactalbumin aggregation in the absence of α-crystallin. When α-lactalbumin aggregation is studied in the presence of α-crystallin, the formed complexes of the unfolded form of α-lactalbumin and α-crystallin are initially stable in time. To explain the sticking of the complexes at definite point in time ($t = t_3$), the following changes in the structure of these complexes can be postulated. In initially formed complexes the unfolded α-lactalbumin molecules are randomly distributed on the surface of the α-crystallin particles, and the complexes do not contain sticky sites for aggregation. The complexes of the unfolded form of α-lactalbumin and α-crystallin are dynamic structures, and therefore redistribution of the bound α-lactalbumin molecules can proceed through the stages of adsorption–desorption of the α-lactalbumin molecules. One can assume that such a redistribution of the bound α-lactalbumin molecules results in formation of a nucleus composed of the α-lactalbumin molecules on the surface of the complex. Formation of a nucleus is thermodynamically favorable owing to formation of the additional contacts between the α-lactalbumin molecules. The nuclei act as the aggregation sites, and the transformed complexes of the unfolded form of α-lactalbumin with α-crystallin play the role of the primary clusters (C_{pr}) prone to aggregation. The increase in the hydrodynamic radius of the particles in time is a result of sticking of the primary clusters. The proposed mechanism of the action of α-crystallin on DTT-induced aggregation of α-lactalbumin is represented in Scheme 2.

The obtained data show that at rather low concentrations of α-crystallin the sticking of the primary clusters (C_{pr}) proceeds with the higher rate than the sticking of the start aggregates (A_{st}). The possible reason of this unexpected result is the higher concentration of C_{pr} in comparison with the concentration of A_{st}.

In our previous works [1,5,7,41–43] the effect of α-crystallin on thermal aggregation of proteins was studied using DLS. It was shown that the addition of α-crystallin resulted in the increase in the duration of the lag period on the kinetic curves of aggregation. The hydrodynamic radius of the primary clusters formed in the presence of α-crystallin was in the range of 20–30 nm. Sticking of the primary clusters proceeded in the RLCA regime (the dependences of R_h on time followed the exponential law). Such peculiarities of the kinetics of thermal aggregation of proteins in the presence of α-crystallin may be explained in the frame of the mechanism represented in Scheme 2. Thermally denatured protein binds to the α-crystallin particles. Redistribution of the denatured protein molecules on the surface of the α-crystallin particles results in formation of nuclei combining several denatured molecules. Such nuclei provide aggregation of the α-crystallin–denatured protein complexes. The completion of the stage of nucleation for adsorbed denatured protein molecules determines the duration of the lag period on the kinetic curves of aggregation obtained in the presence of α-crystallin.

5. Conclusions

The results of the study of the DTT-induced aggregation of α-lactalbumin using DLS show that aggregation proceeds through the stage of formation of the start aggregates with the hydrodynamic radius of 80–100 nm. The mechanism of aggregation is essentially changed in the presence of α-crystallin. The initially formed complexes of unfolded α-lactalbumin with α-crystallin are transformed to the primary clusters (with $R_h \approx 20$ nm) prone to aggregation as a result of the redistribution of the denatured protein molecules on the surface of the α-crystallin particles.

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