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Effect of α-crystallin on thermal denaturation and aggregation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase

Helen A. Khanova [a], Kira A. Markossian [a,*], Sergey Yu. Kleimenov [a], Dmitrii I. Levitsky [a,b], Natalia A. Chebotareva [a], Nikolay V. Golub [a], Regina A. Asryants [b], Vladimir I. Muronetz [b,c], Luciano Saso [d], Igor K. Yudin [e], Konstantin O. Muranov [f], Michail A. Ostrovsky [f], Boris I. Kurganov [a,*]

[a] *Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia*
[b] *Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia*
[c] *Faculty of Bioengineering and Bioinformatics, Moscow State University, Moscow, Russia*
[d] *Department of Human Physiology and Pharmacology "Vittorio Erspamer", University of Rome "La Sapienza", Rome, Italy*
[e] *Oil and Gas Research Institute, Russian Academy of Sciences, Moscow, Russia*
[f] *Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, Moscow, Russia*

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Abstract

The study of thermal denaturation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of α-crystallin by differential scanning calorimetry (DSC) showed that the position of the maximum on the DSC profile (T_{max}) was shifted toward lower temperatures with increasing α-crystallin concentration. The diminishing GAPDH stability in the presence of α-crystallin has been explained assuming that heating of GAPDH induces dissociation of the tetrameric form of the enzyme into dimers interacting with α-crystallin. The dissociation of the enzyme tetramer was shown by sedimentation velocity at 45 °C. Suppression of thermal aggregation of GAPDH by α-crystallin was studied by dynamic light scattering under the conditions wherein temperature was elevated at a constant rate. The construction of the light scattering intensity versus the hydrodynamic radius (R_h) plots enabled estimating the hydrodynamic radius of the start aggregates ($R_{h,0}$). When aggregation of GAPDH was studied in the presence of α-crystallin, the start aggregates of lesser size were observed.

Keywords: α-Crystallin; Glyceraldehyde-3-phosphate dehydrogenase; Thermal aggregation; Thermal denaturation

1. Introduction

Thermal treatment of proteins results in destabilization of the protein compact structure which may be followed by protein aggregation and precipitation. α-crystallin, a member of a small heat shock protein (sHSP) superfamily, exhibits the chaperone-like activity and prevents thermally induced aggregation of proteins [1,2]. α-crystallin can recognize and bind unfolded proteins over a range of temperatures, and a direct correlation between temperature, monomer exchange and chaperone-like activity of α-crystallin has been demonstrated [3–5]. The ability of α-crystallin to suppress heat-induced aggregation of denatured proteins increases when α-crystallin is heated [6–8]. At 25 °C α-crystallin exhibits relatively poor chaperone-like activity [3,8–10], and a structural transition at 30–40 °C leads to enhanced chaperone activity by increasing or favorably reorganizing the hydrophobic substrate-binding surfaces [4,5,11–13]. The increased activity of aggregates of α-crystallin suggests that its chaperone-like activity depends in part on the packing parameters of the aggregate and on conformation of the subunit within the aggregate. α-Crystallin undergoes two structural transitions as a function of temperature: one around 30 °C and the other around 55 °C, resulting in the exposure of hydrophobic surfaces. The first transition involves subtle tertiary structural changes and quaternary structural reorganization in the heteroaggregates. [4,7,10]. The structure and the thermal stability of bovine α-crystallin studied by Fourier-transform infrared spectroscopy, circular dichroism, and

* Corresponding authors. Fax: +7 495 954 2732.
E-mail addresses: markossian@inbi.ras.ru (K.A. Markossian), boris@kurganov.com (B.I. Kurganov).

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differential scanning calorimetry show that α-crystallin undergoes a major thermotropic transition with a midpoint at 60–62 °C [7]. That results in partial unfolding of the protein, which then doubles in molecular weight and increases in size [7,8,10], but does not aggregate further [12]. Infrared spectroscopic data show that the secondary structure of α-crystallin is highly ordered and consists predominantly of beta-sheets [7] and Fourier-transform infrared spectra provide evidence that conformational transition at 60 °C is associated with a massive loss of the native beta-sheet structure [7]. Under physiological conditions the quaternary assembly of α-crystallin is characterized by a relatively fast subunit-exchange, indicating a certain degree of conformational flexibility of the α-crystallin protomers [8,11,14–17]. With increase in temperature the number of α-crystallin monomers per oligomer changes. This proceeds by temperature-dependent monomer exchange among individual oligomers [14].

Two main questions regarding the interaction of α-crystallin with target protein are in the focus of intense research: (1) what structural particularities of the target protein are important in the chaperone function of α-crystallin and (2) what are the protein structures which interact with α-crystallin [2,18,19]. It is known that a higher number of target protein binding sites become available at elevated temperatures [2]. The sites available at low temperatures are a subset of the total sites available at elevated temperatures.

Studies of thermal denaturation of carbonic anhydrase and α-lactalbumin show that only the aggregation-prone proteins in the molten globule state bind to α-crystallin [20]. Study of interaction of human recombinant αA- and αB-crystallin with early and late unfolding intermediates of citrate synthase at its thermal denaturation demonstrate that the chaperone activity of α-crystallin involves both transient and stable interactions depending on the nature of intermediates on the unfolding pathway; one type of interaction leads to reactivation of the enzyme activity while the other prevents aggregation [18].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), a tetrameric enzyme of 144 kDa, has been extensively studied for structural changes during denaturation [21–23]. It has been shown that the secondary structure of GAPDH is relatively heat stable, showing no appreciable change at 38.5 °C [23]. At this temperature at pH 8.5, the enzyme first dissociates within several minutes – probably into dimers – and with prolonged heating it becomes irreversibly aggregated. The dissociative mechanism of thermal denaturation of GAPDH was established in Ref. [24] using differential scanning calorimetry (DSC) and analytical ultracentrifugation. Aggregation and precipitation of rabbit muscle GAPDH at 55 °C is effectively inhibited in the presence of αB-crystallin [25].

The goal of the present work was to study thermal denaturation and aggregation of rabbit muscle GAPDH in the presence of α-crystallin. The results obtained by differential scanning calorimetry (DSC) showed that stability of the enzyme was reduced in the presence of α-crystallin. The study of kinetics of GAPDH aggregation using dynamic light scattering (DLS) allowed us to demonstrate that in the presence of α-crystallin the transition from the regime of diffusion-limited cluster–cluster aggregation (DLCA) to the regime of reaction-limited cluster–cluster aggregation (RLCA) takes place.

2. Materials and methods

2.1. Isolation and purification of GAPDH

GAPDH was isolated from rabbit muscles as described by Scopes and Stoter [26] with an additional purification step using gel-filtration on Sephadex G-100 (superfine). GAPDH concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A_{cm}^{1\%}$ of 10.6 [27].

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 [28,29]. α-Crystallin concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A_{cm}^{1\%}$ of 8.5 [8].

2.3. Calorimetric studies

Thermal denaturation of GAPDH and α-crystallin was studied by DSC. DSC experiments were performed using a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia). All measurements were performed in 10 mM Na-phosphate buffer, pH 7.5. The protein solution was heated at a constant rate of 1 K min^{-1} from 5 to 90 °C, and at a constant pressure of 2.2 atm. The reversibility of the thermal transition of GAPDH and α-crystallin was tested by checking the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling. Calorimetric traces of irreversibly denaturing proteins were corrected for instrumental background and for possible aggregation artifacts by subtracting the scans obtained from the second heating of the samples. The temperature dependence of the excess heat capacity was further analyzed and plotted using Origin software (OriginLab Corporation, USA).

2.4. DLS studies

For light scattering measurements a commercial instrument Photocor Complex was used (Photocor Instruments Inc., USA; www.photocor.com). This instrument allows measuring both dynamic and static light scattering at various scattering angles with stepper-motor controlled turntable [30]. An He–Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) has been used as a 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 (PMT) was used to increase the accuracy of particle sizing in the range of 0.5–10 nm. DLS data have been accumulated and analyzed with multifunctional real-time correlator Photocor-FC that has both logarithmic multiple-tau and linear time-scale modes.

DynaLS software (Alango, Israel) was used for polydisperse analysis of DLS data.

To obtain the DLS results with constant heating rate, as compared to DSC, a fast thermostat has been developed. Special design of this compact unit allowed simply replacing the standard sample cell holder of the main thermostat of the Photocor Complex setup by the fast thermostat. Fast platinum thermometers with time constant of 1 s have been used both for temperature control and for real-time monitoring of temperature directly in the sample cell. The fast thermostat is fully controlled with the existing PID controller through the macro procedure of the Photocor program. Temperature scanning rate can be assigned from 10 K min^{-1} to any slow value. Time of the DLS data accumulation is agreed with the rate of the temperature scanning to obtain correct results of particle sizing in the course of aggregation processes.

The kinetics of thermal aggregation of GAPDH were studied by DLS in 10 mM Na-phosphate buffer, pH 7.5. All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA). The buffer was placed in a cylindrical cell with the internal diameter of 6.3 mm and preincubated for 10 min at a certain temperature. Cells with stopper were used for incubation at high temperatures to avoid evaporation. The aggregation process was initiated by the addition of an aliquot of GAPDH to the final volume of 0.5 mL. To study the effect of α-crystallin on aggregation of GAPDH aliquots of both proteins were added into the cell simultaneously. When studying the kinetics of aggregation of GAPDH, the scattering light was collected at 90° scattering angle.

When studying the kinetics of thermal aggregation of GAPDH by DLS, the accumulation time of the autocorrelation function was 30 s. Because of the short time of accumulation, accuracy of the DLS measurements allows us to obtain only the mean size of particles, without details of distribution. Special experiments have been done to estimate needed time of accumulation to obtain real data about polydispersity of aggregates. In the beginning of aggregation for small particles, this time is within 30 min, that is not applicable for studying the aggregation kinetics.

When analyzing the dependences of the hydrodynamic radius on temperature, we observed that these dependences are identical in form to the dependences of the hydrodynamic radius on time which were obtained by us for aggregation of proteins registered at fixed temperature. As was shown in Refs. [24,28,31], the dependences of the hydrodynamic radius (R_h) on (t) time obtained in the absence of chaperones involves the initial portion described by the linear function:

$$R_h = R_{h,0}\left[1 + \frac{1}{t_{2R}}(t - t_0)\right], \quad (1)$$

where $R_{h,0}$ is the hydrodynamic radius of the start aggregates, t_0 is the duration of the latent phase leading to the formation of the start aggregates and t_{2R} is the time interval over which the hydrodynamic radius increases from $R_{h,0}$ to $2R_{h,0}$. Parameter t_{2R} characterizes the rate of aggregation. The lower the t_{2R} value, the higher the aggregation rate.

The linear increase in the R_h value is obeyed up to a certain point in time designated as t^*. From this time on, the dependence of R_h on time follows the power law:

$$R_h = R_h^*[1 + K_1(t - t^*)]^{1/d_f}, \quad (2)$$

where R_h^* is the value of R_h at $t=t^*$, K_1 is a constant and d_f is the fractal dimension. The fractal dimension is a structural characteristic of aggregates that are formed as a result of unordered interactions (random aggregation). The mass of an aggregate formed in such a way (M) is connected with its effective radius (R) by the following relationship: $M \sim R^{d_f}$.

Eq. (2) is equivalent to the dependence of R_h on time which is obeyed for diffusion-limited cluster–cluster aggregation (DLCA) [32,33]:

$$R_h = R_{h,0}(1 + K_1 t)^{1/d_f}, \quad (3)$$

where $R_{h,0}$ is the hydrodynamic radius of a seed particle. This regime of aggregation means that the sticking probability for the colliding particles is equal to unity. For the DLCA regime a universal fractal dimension of 1.8 is observed [32–34].

When aggregation of the protein substrate is studied in the presence of the protein possessing the chaperone-like activity (α-crystallin), the dependence of the hydrodynamic radius on time follows the exponential law [28,31]:

$$R_h = R_{h,0}\left\{\exp\left[\frac{\ln 2}{t_{2R}}(t - t_0)\right]\right\} \quad (4)$$

The exponential dependence of R_h on time is typical of reaction-limited cluster–cluster aggregation (RLCA) [32]. This regime of aggregation is realized when the sticking probability for the colliding particles is less than unity.

In the experiments on protein aggregation where temperature was elevated with a constant rate the following equations equivalent to Eqs. (1), (2) and (4) were used for description of the dependences of the hydrodynamic radius on temperature (T):

$$R_h = R_{h,0}\left[1 + \frac{1}{T_{2R}}(T - T_0)\right], \quad (5)$$

$$R_h = R_h^*[1 + K_1(T - T^*)]^{1/d_f}, \quad (6)$$

$$R_h = R_{h,0}\left\{\exp\left[\frac{\ln 2}{T_{2R}}(T - T_0)\right]\right\}, \quad (7)$$

where $R_{h,0}$ is the hydrodynamic radius of the start aggregates, T_0 is the temperature at which the start aggregates come to view and T_{2R} is the temperature interval over which the hydrodynamic radius increases from $R_{h,0}$ to doubled this value. Parameter T_{2R} characterizes the rate of aggregation. The lower the T_{2R} value, the higher the aggregation rate. R_h^* is the value of R_h at $T=T^*$. Eqs. (5) and (6) characterize protein aggregation in the absence of chaperone, whereas Eq. (7) characterizes protein aggregation in the presence of chaperone.

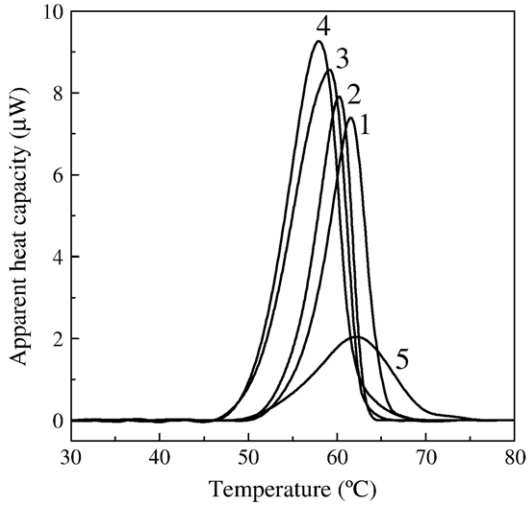

Fig. 1. Effect of α-crystallin on thermal denaturation of GAPDH (0.4 mg mL^{-1}, 10 mM Na-phosphate buffer, pH 7.5). DSC profiles were obtained in the absence (curve 1) and in the presence of α-crystallin (curves 2–4). The concentrations of α-crystallin were the following: (1) 0, (2) 0.1, (3) 0.2 and (4) 0.4 mg mL^{-1}. Curve 5 represents the DSC profile for α-crystallin (0.4 mg mL^{-1}). The heating rate was 1 K min^{-1}.

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

2.5. Analytical ultracentrifugation

Sedimentation velocity studies of the oligomeric state of GAPDH were carried out at 20 and 45 °C in a Model E analytical ultracentrifuge (Beckman), equipped with absorbance optics, a photoelectric scanner, a monochromator, and computer on line. A four-hole rotor An-F Ti and 12 mm double sector cells were used. The rotor was preheated at an appropriate temperature in the thermostat before the run. The sedimentation profiles of GAPDH were recorded by measuring the absorbance at 280 nm. The time interval between scans was 3 min. For digital data acquisitions La-n20-12 PC1 and La-1.5 PCI plates and software specially written by AG Zharov (www.ADClab.ru) were used. The sedimentation coefficients were estimated from the differential sedimentation coefficient distribution [$c(s)$ versus s] which were analyzed using SEDFIT programs [35,36]. The $c(s)$ analysis was performed with regularization at confidence levels of 0.68 and 0.95 and a floating frictional ratio, time independent (TI) noise, baseline, and meniscus position. The sedimentation coefficients were corrected to the standard conditions (a solvent with the density and viscosity of water at 20 °C) as described earlier [37].

3. Results

3.1. Effect of α-crystallin on thermal denaturation of GAPDH

The thermal unfolding of GAPDH (0.4 mg mL^{-1}) registered by DSC is characterized by sharp thermal transition with a maximum at 61.6 °C (Fig. 1). In the presence of α-crystallin the shift of the maximum position (T_{max}) toward lower temperatures is concentration-dependent. When concentration of α-crystallin was 0.4 mg mL^{-1}, the value of T_{max} decreased to 58.0 °C (Table 1). These data indicate that the stability of GAPDH is markedly reduced in the presence of α-crystallin.

Table 1
Position of the maximum (T_{max}) on the DSC profiles for denaturation of GAPDH and parameters of Eq. (4) used for description of the dependences of R_h on temperature for aggregation of GAPDH (0.4 mg mL^{-1}) in the absence and in the presence of α-crystallin

Conc. of α-crystallin, mg mL^{-1}	T_{max}, °C	$R_{h,0}$, nm	T_0, °C	T_{2R}, °C	T_{crit}, °C	$R_{h,crit}$, nm
0	61.6	22.5±0.8	45.4±0.2	0.79±0.04	–	–
0.1	60.2	19.1±0.2	51.15±0.17	3.47±0.13	58	76
0.2	59.1	16.1±0.3	55.2±0.3	2.7±0.16	61	75
0.4	58.0	13.2±0.2	50	30	–	–

3.2. Effect of α-crystallin on thermal aggregation of GAPDH

Before proceeding to study the effect of heating on the ability of GAPDH to aggregate, we measured the hydrodynamic radius of GAPDH at 20 °C. Fig. 2A shows the typical autocorrelation function at this temperature. The concentration of GAPDH was

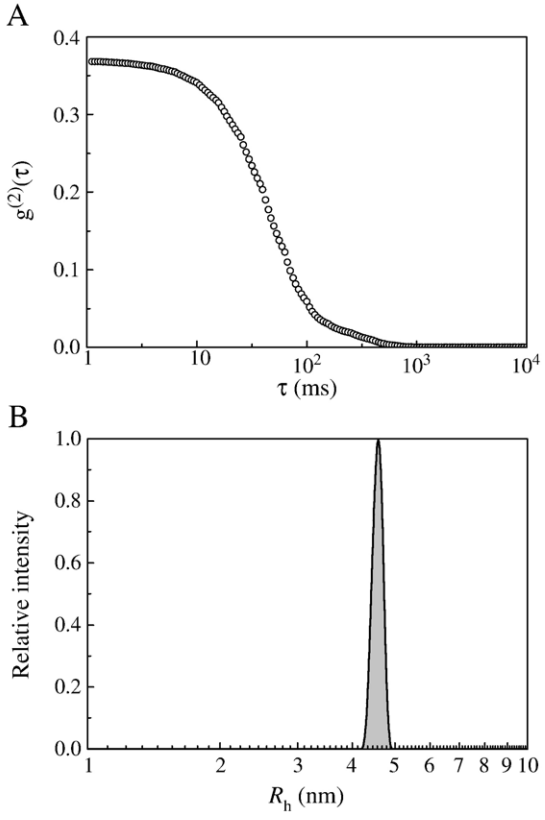

Fig. 2. Autocorrelation function (A) and hydrodynamic radius (B) of GAPDH (2 mg/mL; 10 mM Na-phosphate buffer, pH 7.5) at 20 °C. DLS measurements were carried out at scattering angle of 90°.

2 mg/mL. The mean hydrodynamic radius of GAPDH was found to be 4.3±0.1 nm (see Fig. 2B).

Thermal aggregation of GAPDH was studied by DLS under the regime wherein the temperature was elevated in the interval from 24 to 80 °C at a constant rate of 1 K min^{-1}. Fig. 3A shows the autocorrelation functions registered in the course of thermal aggregation of GAPDH (0.4 mg mL^{-1}) when the temperature reached certain values (50, 55, 57, 60, or 65 °C). It should be noted that at this concentration of GAPDH the reliable measurements of the size of native GAPDH are impossible. The distribution of aggregates by size remained unimodal in the course of aggregation, the mean value of hydrodynamic radius (R_h) increasing monotonously as the temperature was elevated. Fig. 3C and D show the autocorrelation functions and distribution of aggregates by size obtained for GAPDH aggregation in the presence of α-crystallin (0.2 mg mL^{-1}). As in the case with GAPDH aggregation in the absence of α-crystallin, the distribution of particles by size remained unimodal. Heating-induced aggregation of GAPDH was accompanied by the increase in the light scattering intensity (Fig. 4A). Light scattering intensity at high temperatures (higher than 65 °C) diminished due to precipitation of the large-sized aggregates. In the presence of α-crystallin the increment in the light scattering intensity became lower in magnitude, suggesting that α-crystallin suppressed GAPDH aggregation (curves 2–4 in Fig. 4A). It should be noted that in the presence of rather high concentrations of α-crystallin no precipitation of GAPDH aggregates at elevated temperatures occurred.

As seen from Fig. 4B, aggregates of lesser size are formed in the presence of α-crystallin. Since in our experiments the temperature (T) was varied with a constant rate ($T=T_{initial}+vt$; $T_{initial}$ is the initial temperature, t is the time and v is the rate of heating), we analyzed the dependences of R_h on temperature using approaches which we elaborated for analysis of the kinetics of protein aggregation registered at fixed temperature [24,28,31]. First of all, we constructed the light scattering intensity versus R_h plots for GAPDH aggregation kinetic curves obtained in the absence and in the presence of α-crystallin. The shape of these plots shows that rather large aggregates exist in the system at the instant the initial increase in the light scattering intensity is registered. These aggregates are entitled "start aggregates" [28,31]. The initial parts of the dependences of the light scattering intensity on R_h were linear. The length cut off on the abscissa axis by the straight line corresponds to the hydrodynamic radius of the start aggregates ($R_{h,0}$). When GAPDH aggregation proceeded in the absence of α-crystallin, the $R_{h,0}$ value was found to be 22.5±0.8 nm (Fig. 5A). In the presence of α-crystallin the lesser values of $R_{h,0}$ were obtained. When α-crystallin concentration was 0.1 mg mL^{-1}, $R_{h,0}$ was equal to 19.1±0.2 nm (Fig. 5B). The

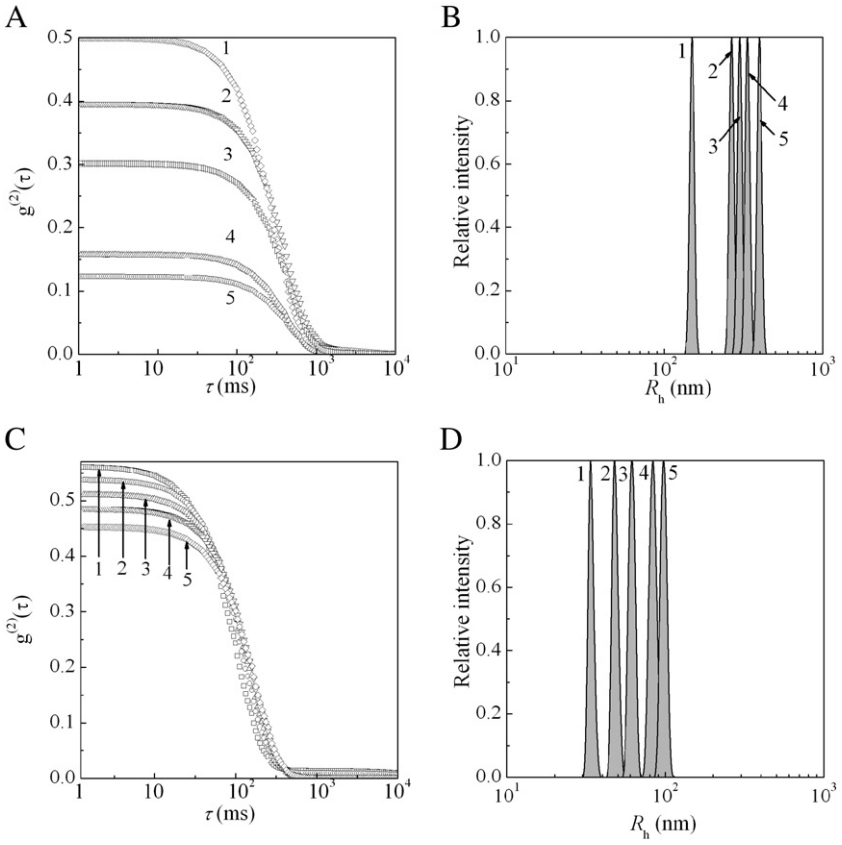

Fig. 3. Thermal aggregation of GAPDH (0.4 mg mL^{-1}) registered in the regime, wherein the temperature was elevated at a constant rate (1 K min^{-1}). Autocorrelation functions (A) and distributions of the particles by size (B) were registered at the following temperatures: (1) 50, (2) 55, (3) 57, (4) 60 and (5) 65 °C. Autocorrelation functions (C) and distributions of the particles by size (D) were registered in the presence of α-crystallin (0.2 mg mL^{-1}) at the following temperatures: (1) 57, (2) 59, (3) 60, (4) 65 and (5) at 70 °C.

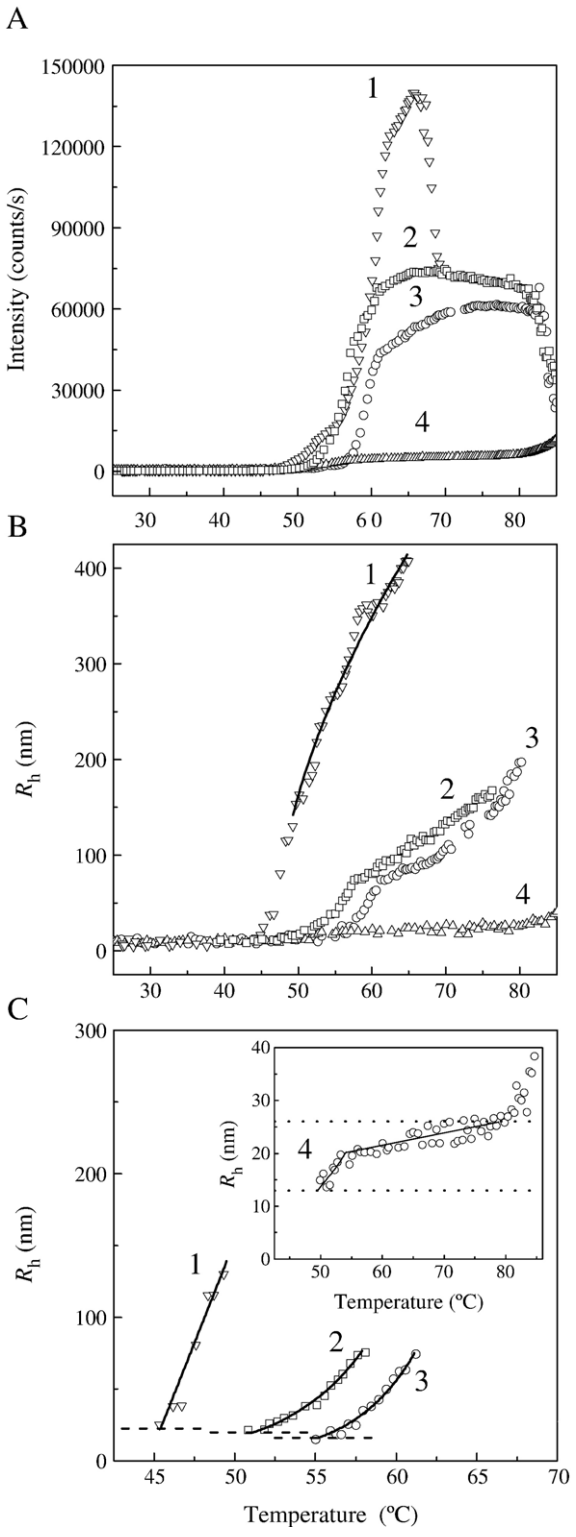

Fig. 4. Effect of α-crystallin on thermal aggregation of GAPDH (0.4 mg mL^{-1}). The dependences of the light scattering intensity (A) and hydrodynamic radius (R_h) on temperature. The concentrations of α-crystallin were the following: (1) 0, (2) 0.1, (3) 0.2 and (4) 0.4 mg mL^{-1}. The solid portion of the curve 1 in panel B was calculated from Eq. (2). (C) The initial parts of the curves 1, 2 and 3 presented in panel B. The horizontal dotted lines correspond to the $R_{h,0}$ values for the individual dependences of R_h on temperature. Solid curve 1 was calculated from Eq. (1). Solid curves 2 and 3 were calculated from Eq. (4). Inset in panel C shows curve 4 presented in panel B with the expanded ordinate axis. The lower horizontal dotted line corresponds to the $R_{h,0}$ value and the upper horizontal dotted line is drawn at the level $R_h=2R_{h,0}$.

$R_{h,0}$ value decreased to 13.1±0.2 nm, as the α-crystallin concentration rose to 0.4 mg mL^{-1} (Fig. 5D; Table 1).

The initial parts of the dependences of R_h on temperature are presented in Fig. 4C. The dotted horizontal lines correspond to the $R_{h,0}$ values. These dependences were analyzed using Eqs. (5) and (6). In the absence of α-crystallin R_h is a linear function of temperature with parameters $T_0=45.4\pm0.2$ °C and $T_{2R}=0.79\pm0.04$ °C (line 1 in Fig. 4C). The linear relationship between R_h and temperature is obeyed up to the temperature designated as T^*. Beyond this temperature the dependence of R_h on temperature follows the power law (6). Parameter d_f was found to be 1.79±0.10. The other parameters were the following: $T^*=49$ °C, $R_h^*=136$ nm, $K_1=0.41\pm0.06$ degree^{-1}.

The analysis of the initial parts of the dependences of R_h on temperature for GAPDH aggregation in the presence of α-crystallin at concentrations of 0.1 and 0.2 mg mL^{-1} (curves 2 and 3 in Fig. 4C) indicates that the exponential law (Eq. (7)) is obeyed. With the knowledge of parameter $R_{h,0}$ we can estimate parameters T_0 and T_{2R}. The values of these parameters are given in Table 1. The addition of α-crystallin resulted in the increase in T_0 and T_{2R} values.

A distinctive feature of the distribution of the particles by size obtained for GAPDH aggregation in the presence of α-crystallin (0.1 and 0.2 mg mL^{-1}) is the absence of a peak corresponding to free α-crystallin with $R_h=11.0\pm0.1$ nm (see Fig. 3D). This indicates that α-crystallin is completely incorporated in the start aggregates.

It should be noted that the exponential law is valid only up to a certain temperature designated as T_{crit}. At $T=T_{crit}$ a dramatic decrease in the rate of the change in the R_h value with temperature takes place. At α-crystallin concentrations of 0.1 and 0.2 mg mL^{-1} the T_{crit} values were 58 and 61 °C, respectively (see full curves 2 and 3 in Fig. 4B). The values of R_h corresponding to these T_{crit} values were practically the same ($R_{h,crit}=76$ and 75 nm, respectively; see Table 1).

Consider the dependence of R_h on temperature for aggregation of GAPDH (0.4 mg mL^{-1}) in the presence of relatively high concentration of α-crystallin (0.4 mg mL^{-1}; curve 4 in Fig. 4B). Inset in Fig. 3C shows this curve with the expanded ordinate axis. The initial part of this curve has a short length and it is impossible to characterize the functional dependence of R_h on time. However, with the knowledge of parameter $R_{h,0}$ we can estimate the parameter T_0 (50 °C). It is surprising that the parameter T_0 decreases as the α-crystallin concentration rises from 0.2 to 0.4 mg mL^{-1}. To estimate the parameter T_{2R} for curve 4 we have drawn a horizontal line at the level $R_h=2R_{h,0}$. This horizontal line and the dependence of R_h on temperature intersect at $T=80$ °C. Thus, the parameter T_{2R} is equal to 30 °C ($T_{2R}=80$ °C − T_0). The main reason for such a profound increase in the T_{2R} value is that already at $T=54$ °C the change in the regime of aggregation results in the drastic diminishing of the rate of the change in the R_h value with temperature.

3.3. Thermal denaturation and aggregation of α-crystallin

Since in the experiments on the influence of α-crystallin on GAPDH aggregation α-crystallin itself was also exposed to

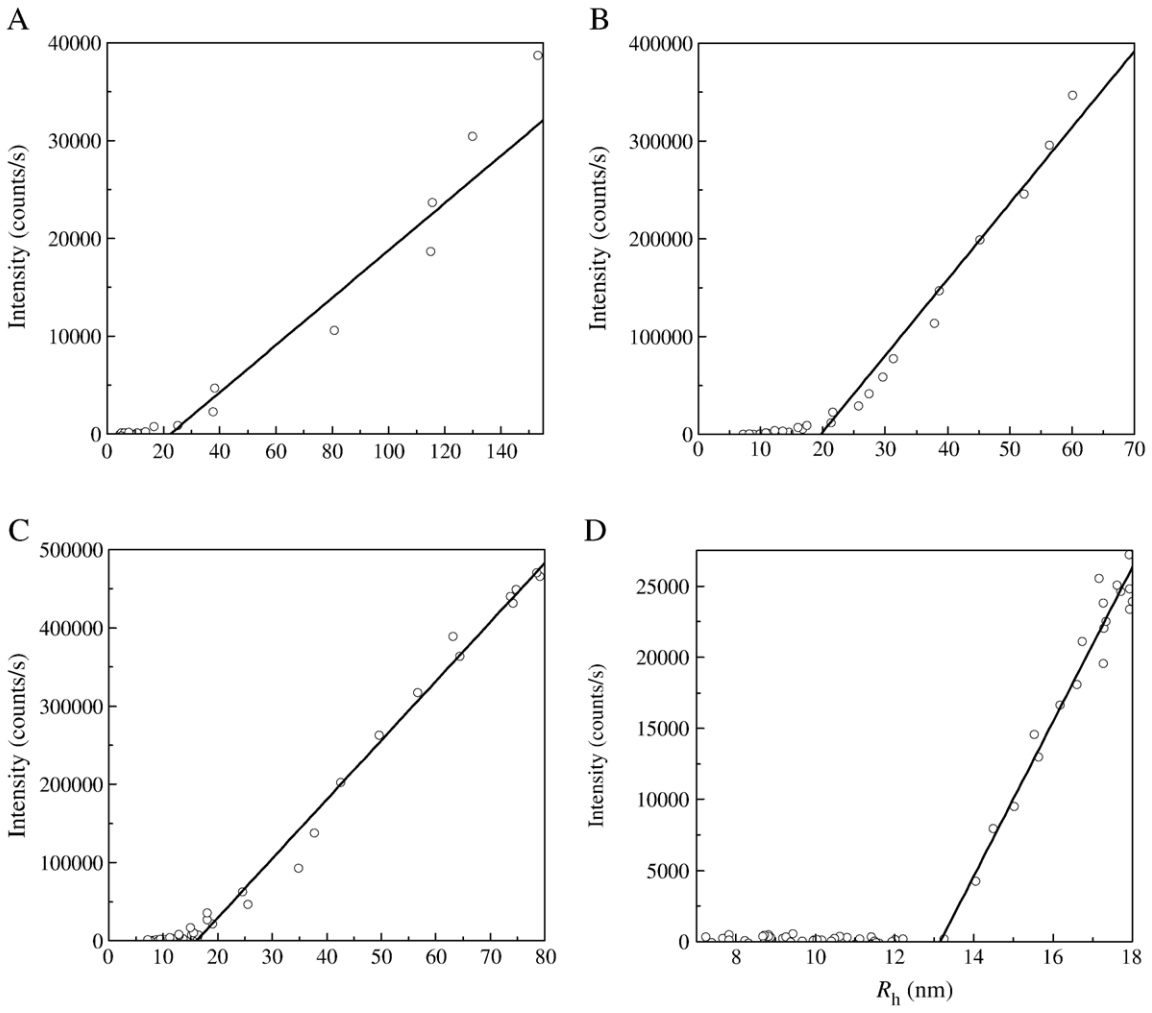

Fig. 5. The light scattering intensity versus the hydrodynamic radius plots constructed for GAPDH aggregation at various concentrations of α-crystallin: (A) 0, (B) 0.1, (C) 0.2 and (D) 0.4 mg mL^{-1}.

high temperatures, it was of special interest to define the size of α-crystallin particles under the regime of temperature elevation. Fig. 6A and B show the changes in the light scattering intensity and hydrodynamic radius for α-crystallin as the temperature was elevated in the interval from 24 to 75 °C at a rate of 1 K min^{-1}. At 24 °C the R_h value for α-crystallin was found to be 11.0±0.1 nm. The temperature increase resulted in a slight decrease in the light scattering intensity (curve 1 in Fig. 6A) accompanied by simultaneous diminishing of the R_h value (curve 1 in Fig. 6B). By this it meant that heating induces dissociation of α-crystallin into the oligomeric forms of lesser size. R_h reached the minimum value ($R_{h,min}$=8.7 nm) at 54 °C. Further temperature elevation results in the enhancement of the R_h value suggesting that aggregation of dissociated forms of α-crystallin takes place. At 75 °C the R_h value was 13.8 nm. Cooling from 75 to 26 °C over 80 min was accompanied by a slight increase in the light scattering intensity and enhancement of the R_h value to 15 nm.

The DSC data for α-crystallin obtained under the same conditions of heating showed that denaturation of α-crystallin started at temperatures higher than 48 °C (curve 1 in Fig. 6C). Excess heat capacity (C_p^{ex}) reached its maximum value at 63.7 °C. The second heating of protein sample immediately after cooling (curve 2 in Fig. 6C) indicated that denaturation of α-crystallin was to a large extent reversible. Judging from the ratio of the calorimetric heat of the first and second heatings, the degree of reversibility was 86%. Thus the presence of residual aggregates in the system after cooling (see Fig. 6A and B) was due to partially irreversible denaturation of α-crystallin.

3.4. Heating-induced dissociation of GAPDH

The displacement of the maximum position on the DSC profiles of GAPDH toward lower temperatures in the presence of α-crystallin suggests that α-crystallin interacts with the heating-induced intermediates of GAPDH unfolding. Since unfolding of GAPDH may include the formation of products of GAPDH dissociation, we studied the oligomeric state of the enzyme at elevated temperatures.

Fig. 7A shows the sedimentation behaviour of GAPDH at 20 °C. The major peak in the $c(s)$ distribution with $s_{20,w}$=8.2± 0.5 S corresponded to the tetrameric form of GAPDH. Incubation of GAPDH at 45 °C for 90 min induced dissociation of the enzyme. Fig. 7B demonstrates one major peak in the

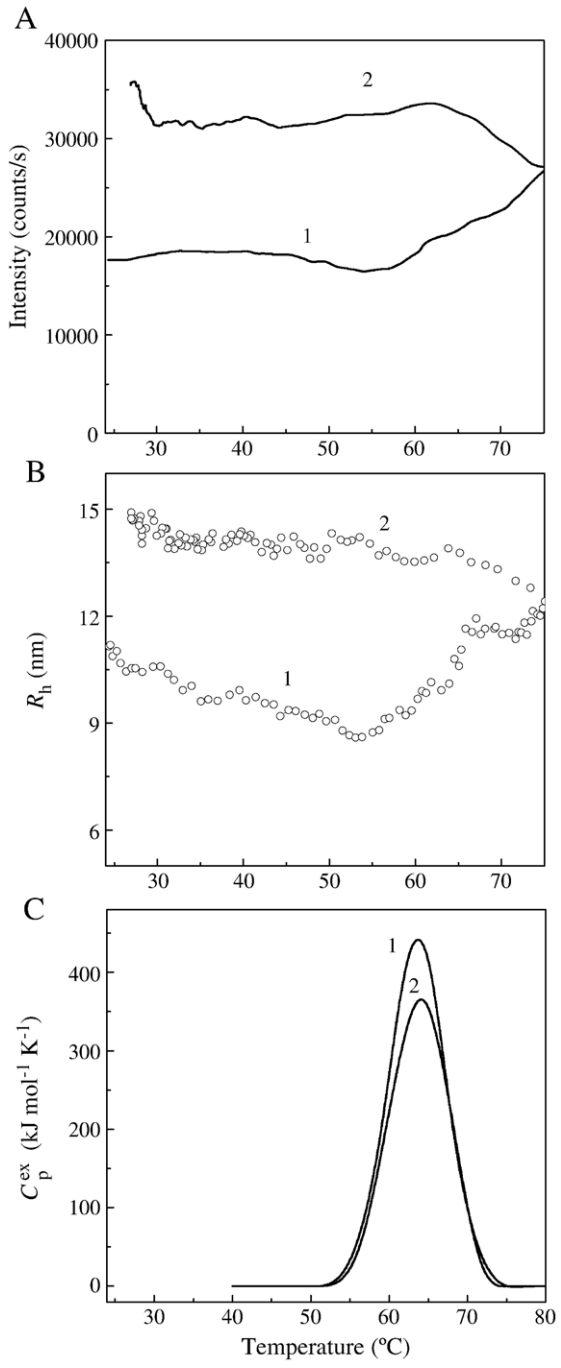

Fig. 6. Denaturation and aggregation of α-crystallin at elevated temperatures. The light scattering intensity (A) and hydrodynamic radius (B) of α-crystallin (1 mg mL^{-1}; 40 mM Na-phosphate buffer, containing 100 mM NaCl) are presented as functions of temperature. Curves 1 in panels A and B correspond to heating at a constant rate (1 K min^{-1}). Curves 2 correspond to cooling from 75 to 26 °C over 80 min. (C) The dependence of excess heat capacity (C_p^{ex}) on temperature for the original preparation of α-crystallin (1 mg mL^{-1}; 40 mM Na-phosphate buffer, containing 100 mM NaCl; curve 1). Curve 2 corresponds to second heating of α-crystallin immediately after cooling.

differential sedimentation coefficient distribution $c(s)$ at 45 °C. The sedimentation coefficient of the main species normalized to the standard conditions ($s_{20,w}=5.40\pm0.16$ S) corresponded to the dimeric form of GAPDH. Results obtained indicate that heating of GAPDH induces dissociation of the tetrameric form of the enzyme into dimers.

Since DLS allows estimating the size of protein oligomers, it was interesting to try to detect dissociation of GAPDH at elevated temperatures using this method. For this purpose we have studied the kinetics of GAPDH aggregation at 45 and 55 °C with GADPH concentrations higher than those usually used in similar experiments (see Section 3.2), namely with concentrations 1 and 2 mg/mL. At such protein concentrations we are able to keep track of the oligomeric state of native GAPDH for 50 min. The measurements of the size of native GAPDH at higher values of time are impossible due to the appearance of the protein aggregates. It has been found that the size of native GAPDH remained unchanged over 50 min ($R_h=4.3$ nm; the data are not shown). It is believed that the degree of GAPDH dissociation over this period of time remains relatively low and considerable changes in the oligomeric state of GAPDH occur at times higher than 50 min, where the measurements of the hydrodynamic radius of native GAPDH are no longer possible. Thus, DLS is unsuitable for registration of GAPDH dissociation at elevated temperatures.

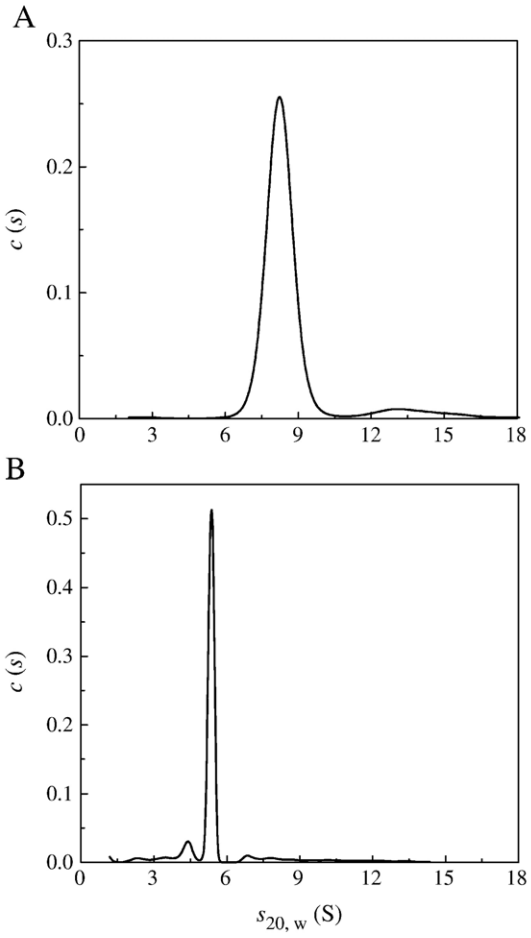

Fig. 7. Sedimentation behaviour of GAPDH (0.4 mg mL^{-1}; 10 mM Na-phosphate buffer, pH 7.5). (A) Differential sedimentation coefficient distribution $c(s)$ for GAPDH at 20 °C. (B) The $c(s)$ distribution for the enzyme preparation heated at 45 °C for 90 min. The $c(s)$ distribution was obtained at 45 °C and normalized to the standard conditions. Rotor speed was 40,000 rpm.

4. Discussion

When interpreting the effect of α-crystallin on thermal denaturation of GAPDH, we should take into account the changes in the oligomeric structure of both proteins at elevated temperatures. The data on analytical ultracentrifugation evidence heating-induced dissociation of the tetrameric form of GAPDH into dimers (Fig. 7B). The data presented in Fig. 6B and C allow us to assess the relationship between the dissociation and denaturation of α-crystallin. According to the DSC data, denaturation of α-crystallin is remarkable at temperatures higher than 48 °C. As seen from Fig. 6B, the decrease in the R_h value occurs at temperatures when denaturation is lacking, i.e. at the temperatures lower than 48 °C. It should be noted that dissociation of α-crystallin at 45 °C was shown by sedimentation velocity analysis [34]. Our results indicate that the initial stage of the temperature-induced changes in α-crystallin coincides with its dissociation into oligomeric forms of lesser size.

Rajaraman et al. [18] showed that apart from suppression of the thermal aggregation of citrate synthase, αA- and αB-crystallins also confer protection against heat-induced inactivation of this enzyme. The stabilizing effect of α-crystallin was explained to be determined by interaction of α-crystallins with unfolding intermediates of citrate synthase.

To explain the diminishing of GAPDH stability in the presence of α-crystallin, we assume that a mobile equilibrium tetramer ↔ 2 dimers exists for GAPDH at elevated temperatures, and α-crystallin displaces this equilibrium toward the formation of a dimeric form by complexing with dimers. In spite of the fact that such a complexation stabilizes the dimeric form in comparison with the free dimer, the stability of the bound dimer turned out to be less than that for the original tetrameric form. It is pertinent to note that according to the data on analytical ultracentrifugation of the solution containing the mixture of GAPDH and α-crystallin there is no interaction between α-crystallin and the tetrameric form of GAPDH at 20 °C (the data are not shown).

When analyzing the dependences of R_h on temperature for GAPDH aggregation in the absence and in the presence of α-crystallin, we came to a conclusion that these dependences were analogous to the dependences of R_h on time registered at fixed temperatures [28,34]. Taking into account that the initial temperature in the experiments with heating of GAPDH at a constant rate was 25 °C, parameter T_0 (the temperature of the appearance of the start aggregates) may be transformed to parameter t_0 (the duration of the latent phase resulting in the formation of the start aggregates): $t_0=(T_0-25\ °C)/v$ ($v=1$ K min^{-1}). In the absence of α-crystallin parameter t_0 was found to be 20.4±0.2 min. When aggregation of GAPDH (0.4 mg mL^{-1}) was studied in the presence of relatively low concentrations of α-crystallin, the higher values of parameter t_0 were obtained: $t_0=26.15\pm0.17$ and 30.3±0.3 min for α-crystallin concentrations of 0.1 and 0.2 mg mL^{-1}, respectively. Division of T_{2R} by v yields parameter t_{2R}, namely, the time interval over which the hydrodynamic radius increases from $R_{h,0}$ to doubled this value. This parameter characterizes the rate of aggregation. In the absence of α-crystallin the parameter t_{2R} was found to be 0.79± 0.04 min. The increase in the t_{2R} value was observed in the presence of α-crystallin ($t_{2R}=3.47\pm0.13$ min and 2.70± 0.16 min for α-crystallin concentrations of 0.1 and 0.2 mg mL^{-1}, respectively). Thus, the suppression of GAPDH aggregation by α-crystallin is due to decrease in the size of the start aggregates, the increase in the duration of the latent phase, resulting in the formation of the start aggregates, and the decrease in the rate of aggregation. The diminishing of the aggregation rate is due to incorporation of α-crystallin into the start aggregates. The sticking probability for the start aggregates containing α-crystallin is less than unity. This circumstance explains the transition of the kinetic regime of GAPDH aggregation from the regime of diffusion-limited cluster–cluster aggregation (where the dependence of R_h on time follows the power law) observed in the absence of α-crystallin to the regime of reaction-limited cluster–cluster aggregation (where the dependence of R_h on time follows the exponential law) observed in the presence of α-crystallin.

The inflexion points on the dependences of R_h on temperature (at $T=T_{crit}$) for GAPDH aggregation in the presence of α-crystallin may be due to the change of the regime of aggregation from reaction-limited aggregation to diffusion-limited aggregation [35,38].

At relatively high concentrations of α-crystallin (0.4 mg mL^{-1}) the character of the influence of α-crystallin on GAPDH aggregation is markedly changed. At this concentration of α-crystallin a decrease in the parameter T_0 (the temperature corresponding to the appearance of the start aggregates) is observed. This result, which was unexpected, indicates that α-crystallin facilitates initial stages of GAPDH aggregation. In our opinion, this effect of α-crystallin is due to the diminishing of GAPDH stability in the presence of α-crystallin. However, the initial stimulation of GAPDH aggregation by α-crystallin was completely abolished by the significant increase in the T_{2R} value ($T_{2R}=30$ °C; $t_{2R}=30$ min).

5. Conclusion

Results obtained in the present work testify that thermal denaturation of GAPDH proceeds by a dissociative mechanism, i.e. involves the stage of dissociation of original tetramer into the oligomeric forms of lesser size. This result coincides with the data obtained by us previously [24,39]. The capacity of GAPDH for dissociation at elevated temperatures may account for unusual effects of the protein possessing the chaperone-like activity (α-crystallin) on denaturation and aggregation of GAPDH. The effect of a decrease in protein stability in the presence of α-crystallin was not observed hitherto for other proteins. Undoubtedly, destabilization of GAPDH in the presence of α-crystallin indicates that α-crystallin interacts with the intermediates of GAPDH unfolding. It is reasonable to suggest that such intermediates are the products of dissociation of GAPDH tetramers. It should be noted that, when the denaturation process for oligomeric proteins does not include the kinetically significant stage of reversible dissociation of protein oligomer into subunits (as in the case of β-crystallin from bovine lens and alcohol dehydrogenase I from *Saccharomyces cerevisiae*), α-crystallin does not affect protein stability [28,34].

Since we set ourselves the task of elucidating the mechanism of suppression of aggregation of a model protein substrate (GAPDH) by one of the representatives of small heat shock proteins (α-crystallin), it was necessary that we should study the protective effect of α-crystallin in the wide range of the concentrations of this chaperone, but not only at high concentrations of α-crystallin close to concentrations which are found in crystalline lens. When α-crystallin is tested at relatively low concentrations, its effect on aggregation of GAPDH is identical to that for other protein substrates [28,34]: α-crystallin brings about the decrease in the size of the start aggregates, the increase in the duration of the latent phase, resulting in the formation of the start aggregates, the decrease in the rate of aggregation and the transition of the aggregation process from the regime of diffusion-limited cluster–cluster aggregation to the regime of reaction-limited cluster–cluster aggregation.

One can expect that at rather high concentrations of α-crystallin the mechanism of the suppression of aggregation of the protein substrates will be changed. Complexation of denatured molecules of the protein substrate with α-crystallin should prevent the formation of the start aggregates of the protein substrate. Such a situation is realized for suppression of aggregation of β-crystallin and alcohol dehydrogenase I by relatively high concentrations of α-crystallin [28,34]. However, when GAPDH is used as a protein substrate, there are some complications caused by the interaction of α-crystallin with the intermediates of GAPDH unfolding. This interaction results eventually to destabilization of the protein and sudden decrease in temperature at which aggregation begins (when aggregation is studied in the regime of heating with a constant rate). The decrease in the rate of aggregation takes place concurrently, and, if aggregation is assessed by the diminishing of the increment of the light scattering intensity, the general effect of α-crystallin will look like a significant suppression of aggregation.

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