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Kinetics of aggregation of UV-irradiated glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle. Effect of agents possessing chaperone-like activity

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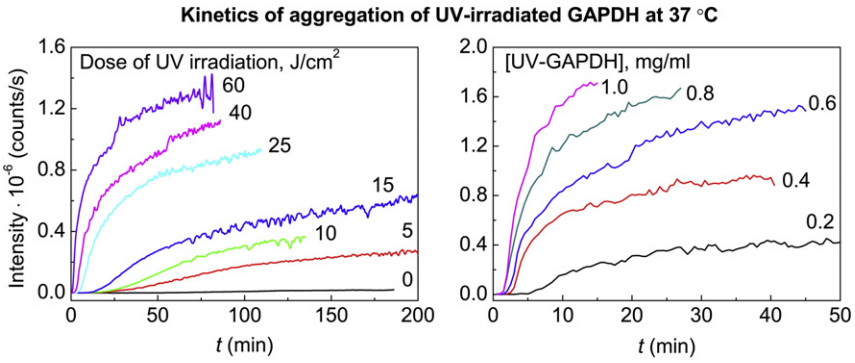
HIGHLIGHTS

- An aggregation test system based on aggregation of UV-irradiated GAPDH was proposed.
- The test system allows detecting the effects of various agents exclusively on the stage of aggregation of unfolded protein.
- The protective effect of α-crystallin and 2-hydroxypropyl-β-cyclodextrin on aggregation of UV-irradiated GAPDH was studied.

GRAPHICAL ABSTRACT



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ABSTRACT

An aggregation test system based on the aggregation of UV-irradiated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit skeletal muscle has been proposed. On the basis of the measurements of the enzyme activity and differential scanning calorimetry data a conclusion has been made that UV radiation results in formation of damaged protein molecules with lower thermostability. It was shown that the order of aggregation rate for UV-irradiated GAPDH with respect to the protein was close to 2. This means that such a test system allows detecting the effect of various agents exclusively on the stage of aggregation of unfolded protein molecules. The influence of α-crystallin and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) on aggregation of UV-irradiated GAPDH was studied. Despite the fact that HP-β-CD accelerates thermal aggregation of non-irradiated GAPDH, in the case of aggregation of UV-irradiated GAPDH HP-β-CD reveals a purely protective effect.

Abbreviations: DLCA, diffusion-limited cluster-cluster aggregation; DLS, dynamic light scattering; DSC, differential scanning calorimetry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; Phb, glycogen phosphorylase *b*; RLCA, reaction-limited cluster-cluster aggregation.

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1. Introduction

Mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is known as a glycolytic enzyme, but in fact GAPDH is a multifunctional protein involved in many cellular processes [1,2], such as DNA repair [3], tRNA export [4], membrane fusion and transport [5], cytoskeletal dynamics [6] and cell death [7]. In addition to the cytoplasm, where the majority of GAPDH is located, the protein is also found in the particulate fractions (nucleus, mitochondria and small vesicular fractions) [8]. When cells are exposed to various stress conditions, dynamic subcellular redistribution of GAPDH occurs. Tristan et al. [8], in the review on multifunctional properties of GAPDH, indicated that GAPDH was regulated by oligomerization, posttranslational modification and subcellular localization. GAPDH is involved in various pathological processes including age-related neurodegenerative disorders and cancer [9,10]. Besides, decrease in the enzymatic activity of GAPDH has been reported in human lens with age and in cataract [11,12].

GAPDH is a homotetramer composed of four identical subunits with molecular mass of 36 kDa. The polypeptide chain of GAPDH is folded in two domains, the NAD^+-binding and catalytic domains, which are relatively independent [13]. There is high interspecies conservation of the enzyme sequence and structure. The structure of rabbit muscle GAPDH shares 91% sequence identity with the human enzyme. GAPDH has an oxidation-sensitive SH-group at its active site. Revival of activity in cataract lenses by reducing agents suggests that a protein thiolation may occur at the active site, thus inhibiting the catalytic activity of GAPDH [14,15]. The inactivation of GAPDH during aging and cataract development may be caused in part by disulphide formation and in part by unfolding. The enzymatic activity of GAPDH can be recovered by reducing agents and α-crystallin [16].

Several in vitro studies have been devoted to unfolding, refolding, dissociation and aggregation of GAPDH [17–19]. GAPDH has been used as a target protein to examine the effect of some chaperones [20–24]. In previous works we studied the effect of α-crystallin and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) on thermal inactivation, denaturation and aggregation of GAPDH [22,24–26]. We showed that α-crystallin suppressed thermal aggregation of GAPDH. Although usually cyclodextrins act as artificial chaperones, which prevent protein aggregation [27–30], HP-β-CD accelerated GAPDH aggregation due to the fact that this agent induced destabilization of the protein molecule [26].

One of the factors provoking protein denaturation and aggregation is UV irradiation. UV light induces severe oxidative protein damage and may result in cataractogenesis. The most important UV light absorbers in proteins are the aromatic residues, Trp, Tyr and Phe as well as Cys and His [31]. UV irradiation can induce enzyme inactivation [32]. The mechanism of inactivation may involve ionization of residues of aromatic amino acids associated with an electron transfer mechanism and radical formation together with disruption of disulphide bridges [33,34]. There are few studies performed on UV-irradiated GAPDH. Voss et al. [35] tested the effect of UV-A (320–400 nm) and UV-B (290–320 nm) irradiation on GAPDH in vitro and analyzed the irradiation-induced enzyme activity loss, fragmentation, aggregation and quantified various oxidative amino acid modifications. It has been shown that UV-A and UV-B both selectively oxidize single amino acids, including cysteine, tyrosine and phenylalanine. The most dramatic changes were observed for the high-energy irradiation types, namely UV-B, for which aggregation and fragmentation, as well as amino acid modifications, were observed. Sulfur-containing (cysteine, methionine) and aromatic amino acids (tryptophan, tyrosine, phenylalanine) are especially vulnerable to oxidation.

In the present work we studied the effect of UV irradiation on the enzyme activity, tryptophan fluorescence, denaturation and aggregation of GAPDH. It has been demonstrated that UV-irradiated GAPDH may serve as a test system of a new type, which allows detecting the effect of the agents being tested exclusively on the aggregation stages. Using this test system we studied the anti-aggregation ability of α-crystallin and HP-β-CD.

2. Materials and methods

2.1. Materials

Na_2HPO_4 and NaH_2PO_4 were from Sigma-Aldrich, HP-β-CD (degree of substitution 3 ± 1) was from CycloLab LTD (Hungary). All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA).

2.2. Determination of refractive index, density and dynamic viscosity of HP-β-CD solutions

The values of the refractive index of the HP-β-CD solutions in 50 mM Na-phosphate buffer, pH 7.5, were determined in refractometer IRF-22 (Russia) at 37 °C. The HP-β-CD concentration was varied in the interval 0–114 mM. The density of HP-β-CD solutions at concentrations of 0–76 mM in 50 mM phosphate buffer, pH 7.5, was determined by the density meter DMA 4500 "Anton Paar" (Austria). Dynamic viscosity of 50 mM phosphate buffer, pH 7.5 in the presence of different HP-β-CD concentrations (0–76 mM) was determined by the automatic microviscosimeter "Anton Paar" (Austria) in system 1.6/1.500 mm at 37 °C. The obtained values of refractive index, density and dynamic viscosity are given in Table 1.

2.3. Isolation of GAPDH

GAPDH was isolated from rabbit muscles as described by Scopes and Stoter [36] with an additional purification step using gel-filtration on Sephadex G-100. GAPDH concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A_{cm}^{1\%}$ of 10.6 [37].

2.4. Isolation of α-crystallin

Purification of α-crystallin from freshly excised lenses of 2-year-old steers was performed according to the procedure described earlier [38]. α-Crystallin concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A_{cm}^{1\%}$ of 8.5 [39].

2.5. GAPDH assay

GAPDH activity was measured by monitoring an increase in the absorbance at 340 nm, caused by the formation of NADH, using a UV 1601 Schimadzu spectrophotometer (Japan). The enzymatic reaction was carried out at 25 °C and initiated by the addition of 2–6 μg of the enzyme to the reaction mixture (1 mL) containing 100 mM glycine, 100 mM NaH_2PO_4, pH 8.9, 1 mM NAD, 1 mM glyceraldehyde-3-phosphate and 5 mM EDTA. The specific activity of GAPDH preparation was 70 U mg^{-1} of the protein.

Table 1
Refractive index (n), density (ρ) and dynamic viscosity (η) of HP-β-CD solutions at 37 °C (50 mM Na-phosphate buffer, pH 7.5).

[HP-β-CD], mM	n	ρ, g/cm^3	η, mPa s
0	1.3320 ± 0.0002	0.99953 ± 0.00005	0.7036 ± 0.0002
4	1.3328 ± 0.0002	1.00105 ± 0.00005	0.7128 ± 0.0002
15	1.3350 ± 0.0002	1.00598 ± 0.00005	0.7453 ± 0.0003
38	1.3389 ± 0.0002	1.01501 ± 0.00005	0.8137 ± 0.0002
61	1.3430 ± 0.0002	–	–
76	1.3458 ± 0.0002	1.03052 ± 0.00005	0.9636 ± 0.0003
114	1.3528 ± 0.0002	–	–

2.6. UV irradiation of GAPDH

UV irradiation of GAPDH (2 mg mL^{-1}) was carried out in 1 cm path quartz cell. The volume of sample was 2 mL. UV irradiation was performed by means of a 1000 W high pressure Hg lamp (DRSH-1000, Russia) applied by 15 cm water filter and 282 nm interference filter with a half-width of 15 nm. The temperature of the samples in the cell was monitored with a miniature thermometer and did not exceed 10 °C. The UV output incident was measured with an optical spectrometer AvaSpec-2048. The summary incident power was calculated as an integral of 260–280 nm intervals and was equal to 6.2 ± 0.4 mW cm^{-2}. The failing UV energy was calculated as a summary incident power multiplied by the exposition time.

2.7. Fluorescence measurements

Fluorescence emission spectra of GAPDH were collected at 25 °C using a Shimadzu RF-5301PC fluorescence spectrophotometer equipped with a circulating water bath. Intrinsic tryptophan fluorescence emission spectra were measured in the range of 300–400 nm using an excitation wavelength of 295 nm. A protein concentration of 0.4 mg mL^{-1} was used for all experiments.

2.8. Calorimetric studies

Thermal denaturation of UV-irradiated GAPDH was studied by differential scanning calorimetry (DSC). DSC experiments were performed using a DASM-4 M differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) in 50 mM Na-phosphate buffer, pH 7.5. The protein solution was heated at a constant rate of 2 °C min^{-1} from 5 to 90 °C and at a constant pressure of 2.2 atm. The temperature dependence of the excess heat capacity was further analyzed and plotted using Origin software (OriginLab Corporation, USA).

2.9. Analytical ultracentrifugation

Sedimentation velocity experiments were performed at 20 °C in a Model E analytical ultracentrifuge (Beckman), equipped with absorbance optics, a photoelectric scanner, a monochromator and a computer on line. A four-hole rotor An-F Ti and 12 mm double sector cells were used. The sedimentation profiles of proteins were recorded by measuring the absorbance at 280 nm. All cells were scanned simultaneously. The time interval between scans was 2.5 min. The sedimentation coefficients were estimated from the differential sedimentation coefficient distribution [$c(s)$ versus s] using SEDFIT program [40]. Weight-average sedimentation coefficients were obtained by integration of the $c(s)$ distribution.

2.10. Dynamic light scattering studies

For light scattering measurements a commercial instrument Photocor Complex was used (Photocor Instruments Inc., USA; www.photocor.com). A He–Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) served as a light source. The temperature of sample cell was controlled by the proportional integral derivative temperature controller to within ±0.1 °C. A 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. Data on dynamic light scattering (DLS) have been accumulated and analyzed with a multifunctional real-time correlator. The time of the accumulation of the autocorrelation function was 30 s. DynaLS software (Alango, Israel) was used for polydispersity analysis of DLS data.

Aggregation of non-irradiated and UV-irradiated GAPDH was studied in 50 mM Na-phosphate buffer, pH 7.5, at 37 °C. For the experiments the buffer was placed in a cylindrical cell with an internal diameter of 6.3 mm and preincubated for 5 min at 37 °C. Cells with stopper were used to avoid evaporation. The aggregation process was initiated by the addition of an aliquot of UV-irradiated GAPDH to the final volume of 0.5 mL. To study the effect of α-crystallin and HP-β-CD on UV-irradiated GAPDH aggregation, we preincubated these chaperone-like agents with buffer in the cell for 5 min before adding an aliquot of protein. When studying the kinetics of protein aggregation, the scattering light was collected at 90° scattering angle.

To calculate the duration of the lag period (t_0) on the kinetic curves of UV-irradiated GAPDH aggregation, we used an empiric equation proposed in our earlier study [41]:

$$I = K_{agg}(t - t_0)^2, \quad (1)$$

where I is the light scattering intensity and K_{agg} is a constant with the dimension (counts s^{-1}) min^{-2}.

When analyzing the dependences of the hydrodynamic radius (R_h) of the protein aggregates on time, we took into account that the aggregation process might proceed in different kinetic regimes. If the sticking probability for the colliding particles is equal to unity (in other words, if each collision of the particles results in their sticking), the aggregation process proceeds in the regime of diffusion-limited cluster–cluster aggregation (DLCA) [42,43]. In this case the dependence of R_h on time from a definite point in time ($t = t^*$) follows the power law [19,44]:

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

where R_h^* is the R_h value at $t = t^*$, K_1 is a constant and d_f is a fractal dimension of the aggregates. When DLCA regime is fulfilled the value of d_f is equal to 1.8 [43].

If the sticking probability for the colliding particles is lower than unity, the reaction-limited cluster–cluster (RLCA) regime is fulfilled, and the dependences of R_h on time follows the exponential law [24,43–45]:

$$R_h = R_{h,0} \exp\left[\frac{\ln 2}{t_{2R}}(t - t_0)\right], \quad (3)$$

where $R_{h,0}$ is the hydrodynamic radius of the initial particles participating in the aggregation process, t_0 is the duration of the lag period for the aggregation process and t_{2R} is the time interval over which the R_h value of aggregates is doubled.

2.11. Calculations

Origin 8.0 (OriginLab Corporation, USA) software and Scientist (MicroMath, Inc., USA) software were used for the calculations. To determine the degree of agreement between the experimental data and calculated values, we used the coefficient of determination R^2 [46].

3. Results and discussion

3.1. Effect of UV irradiation on the enzymatic activity and thermostability of GAPDH

UV irradiation of GAPDH solutions results in the loss of the enzymatic activity. Fig. 1A shows the dependence of the residual activity (A/A_0) of GAPDH on the UV dose (D). The inactivation process follows the exponential law:

$$A/A_0 = \exp[-(\ln 2) D/D_{0.5}], \quad (4)$$

where A_0 and A are the enzymatic activities of native and UV-irradiated enzyme, respectively, and $D_{0.5}$ is the UV dose corresponding to 50% inactivation. The $D_{0.5}$ value was found to be 7.4 ± 0.6 J cm^{-2}.

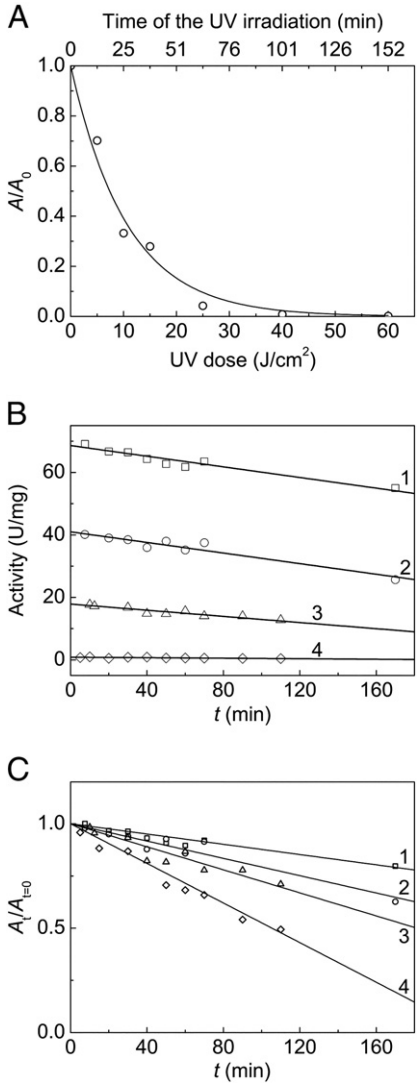

Fig. 1. Effect of UV irradiation on the enzyme activity and thermostability of GAPDH. (A) Dependence of the relative enzymatic activity (A/A_0) of irradiated GAPDH on the UV dose (bottom axis) and irradiation time (top axis). A_0 and A are the enzymatic activities of native and UV-irradiated enzyme, respectively. Solid curve was calculated from Eq. (4). (B and C) Dependences of the enzymatic activity and relative enzymatic activity ($A_t/A_{t=0}$) of UV-irradiated GAPDH (0.4 mg mL^{-1}) on incubation time, respectively, at 37 °C for the following UV doses: (1) 0, (2) 5, (3) 10 and (4) 25 J cm^{-2} (50 mM phosphate buffer, pH 7.5). A_t and $A_{t=0}$ are the current and initial enzymatic activity, respectively.

To reveal the effect of UV irradiation on GAPDH thermostability, we studied the kinetics of inactivation of the UV-irradiated enzyme at 37 °C. Fig. 1B shows the diminution of the enzyme activity with time for non-irradiated GAPDH and for the enzyme irradiated with the dose of 5, 10 and 25 J cm^{-2}. To compare these kinetic curves, we have constructed the $A_t/A_{t=0}$ vs. time plots (Fig. 1C), where A_t is the current value of the enzymatic activity and $A_{t=0}$ is the corresponding value for each kinetic curve at $t=0$. Over the first two hours the dependences of $A_t/A_{t=0}$ on time were linear, and the slope of these linear parts may be considered as a relative initial rate of thermoinactivation. As one can see in Fig. 1C, the initial rate of thermoinactivation increases with increasing the UV dose. When the UV dose was 25 J cm^{-2}, a 3.9-fold increase in the rate of inactivation took place.

DSC makes it possible to control the portion of the protein remaining in the native state in UV-irradiated preparation. This approach was first used in our previous work [47]. Fig. 2 shows the DSC profiles for native GAPDH and the enzyme irradiated by various UV doses. The

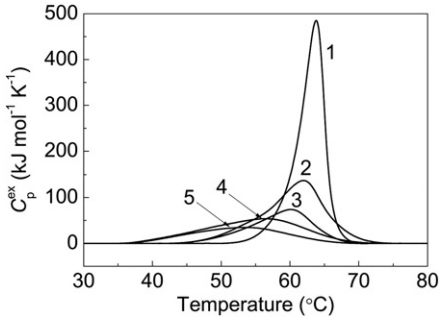

Fig. 2. Thermal denaturation of UV-irradiated GAPDH studied by DSC. Temperature dependences of the excess heat capacity (C_p^{ex}) of GAPDH irradiated by various doses of UV light: (1) 0, (2) 15, (3) 25, (4) 40 and (5) 60 J cm^{-2}. The initial concentration of GAPDH was 2 mg mL^{-1}. C_p^{ex} was calculated per GAPDH tetramer. Conditions: 50 mM phosphate buffer, pH 7.5. The heating rate was 2 °C min^{-1}.

area under the curve gives the total denaturation heat of the protein and is proportional to the amount of the native protein. As it is evident from Fig. 2, UV irradiation results in the decrease in the area under the curve suggesting that UV irradiation causes the diminution of the portion of the native protein. The decrease in the area under the curve with increasing UV dose is accompanied by the shift of the position of the maximum (T_{max}) on the DSC profile toward lower temperatures. The T_{max} value for non-irradiated GAPDH was 63.8 °C. When the UV dose was 60 J cm^{-2}, the position of T_{max} displaced to 53.5 °C. Thus, DSC data are in good agreement with the results of the enzyme activity measurements, which demonstrated the diminution of GAPDH thermostability under UV irradiation.

The comparison of the DSC profiles for non-irradiated and UV-irradiated protein is essential for verification of the one-hit model, in which inactivation (denaturation) of the protein molecule proceeds in line with the all-or-none principle as a result of the absorption of one resulting photon [33,48,49]. If the one-hit model is operative, the position of T_{max} should remain the same in the course of irradiation. The shift of the T_{max} position toward lower temperatures observed for UV-irradiated GAPDH means that a simple one-hit model is not valid, and consequently UV irradiation results in the formation of damaged protein molecules with lower thermostability. As can be seen from Fig. 2, the decrease in the area under DSC curve is strongest at low UV doses, whereas T_{max} shift becomes more pronounced at higher doses. Such a behavior of GAPDH during UV exposure may indicate that UV-induced denaturation of the GAPDH molecule is a multistage process where more labile states are formed at higher irradiation time. An analogous picture was observed for UV irradiation of dimeric glycogen phosphorylase *b* (Ph*b*; EC 2.4.1.1) from rabbit skeletal muscle [50].

3.2. Tryptophan fluorescence spectra of UV-irradiated GAPDH

UV irradiation results in the decrease in tryptophan fluorescence of GAPDH because of tryptophan destruction. The position of the maximum of tryptophan fluorescence did not change ($\lambda_{max}=336$ nm; data not shown). When studying the effect of UV radiation on β_L-crystallin, we also observed that the position of the maximum of tryptophan fluorescence for the UV-irradiated protein remained unchanged [47].

3.3. SDS-PAGE analysis of UV-irradiated GAPDH

As one can see in Fig. 3, where the results of SDS-PAGE analysis of UV-irradiated GAPDH are represented, UV irradiation of GAPDH is accompanied by the appearance of small amounts of fragments of the polypeptide chain and high mass aggregates with simultaneous diminution of the intensity of the band corresponding to the original GAPDH subunit.

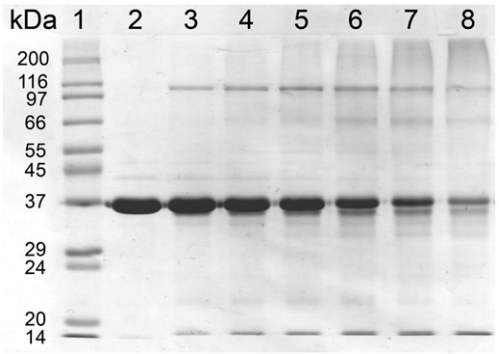

Fig. 3. SDS-PAGE analysis of UV-irradiated GAPDH. Lanes: (1) molecular weight markers, (2–8) GAPDH irradiated by UV dose of 0, 5, 10, 15, 25, 40 and 60 J cm^{-2}, respectively.

3.4. The sedimentation velocity analysis

Fig. 4 shows the differential sedimentation coefficient distributions $c(s)$ for native (A) and UV-irradiated GAPDH (B) at 20 °C. The main peak with $s_{20,w} = 8.18$ S (standard deviation, σ, 0.13 S) in panel A corresponds to the tetramer of GAPDH. As it can be seen, the distributions for UV-irradiated GAPDH (panel B) are rather wide and polydisperse, the weight-average sedimentation coefficient value (s_{av}) being 8.6 S (σ = 2.6 S) and 9.0 S (σ = 2.6 S) for GAPDH concentrations of 0.8 and 0.2 mg mL^{-1}, respectively. The reason of the relatively high degree of polydispersity of UV-irradiated GAPDH may be the following. UV irradiation causes not only denaturation, but also dissociation of the tetrameric enzyme and aggregation of the denatured forms. A minor peak with sedimentation coefficient of 5.0 S in $c(s)$ distributions is indicative of the presence of the dissociated forms. The main peaks in $c(s)$ distributions with values 8.06 and 8.13 S (panel B) do not differ too much from those for native GAPDH (8.18 S). One may suggest that a part of UV-irradiated GAPDH exists in the tetrameric form.

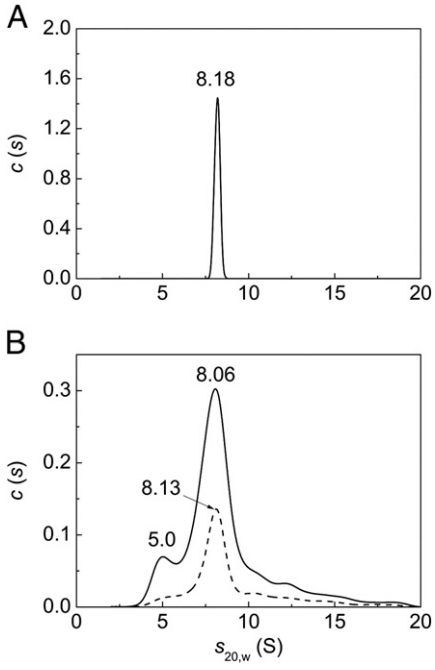

Fig. 4. The sedimentation velocity analysis of native GAPDH and the enzyme irradiated by UV dose of 40 J cm^{-2} enzyme at 20 °C. The differential sedimentation coefficient distributions $c(s)$ for non-irradiated GAPDH (A) and UV-irradiated enzyme (B). The concentration of native GAPDH was 0.4 mg mL^{-1} and concentrations of UV-irradiated GAPDH were 0.8 (solid line) and 0.2 (dash line) mg mL^{-1}. Rotor speed was 48,000 rpm.

3.5. Kinetics of aggregation of UV-irradiated GAPDH

Aggregation of UV-irradiated GAPDH was studied at 37 °C. Fig. 5A shows the kinetic curves of aggregation registered by the increment in the light scattering intensity. As one can see in this figure, the rate of GAPDH aggregation markedly increases with increasing the UV irradiation dose. The initial parts of the kinetic curves were analyzed using Eq. (1) which contains parameter t_0 (the duration of the lag period) and parameter K_{agg} (a measure of the initial rate of aggregation). As an example of applicability of this equation, Fig. 5 (panels B and C) demonstrates the results of fitting of Eq. (1) to the experimental data on aggregation of non-irradiated GAPDH and the enzyme irradiated by the dose of 25 J cm^{-2}. The values of parameters t_0 and K_{agg} are given in Table 2. The initial rate of aggregation (parameter K_{agg}) increases, whereas the duration of the lag period (parameter t_0) decreases with increasing the UV irradiation dose.

The measurements of the size of the particles formed in the course of aggregation give additional information on the mechanism of the aggregation process. Fig. 6A shows the dependence of the hydrodynamic radius (R_h) of the protein aggregates on time for the aggregation of non-irradiated GAPDH at 37 °C. The hydrodynamic radius of particles registered over the first 30 min corresponds to the R_h value of the native enzyme: $R_h = 4.5 \pm 0.1$ nm. In the region of 50–60 min the protein aggregates appear, and at $t > 60$ min a sharp increase in the R_h value for protein aggregates occurs. When $t = 65.5$ min, the hydrodynamic radius reaches the value of 72.5 nm. Calculations show that further increase in the R_h value follows Eq. (2) with $d_f = 1.8 \pm 0.1$. The fact that the d_f value is close to 1.8 is indicative of the fulfillment of the diffusion-controlled regime of aggregation (regime of DLCA). It should be noted that up to $t = 186$ min the population of protein particles contains, apart from large aggregates, a component corresponding to the native protein.

In the case of aggregation of UV-irradiated GAPDH we also registered the splitting of the population of protein particles into two subpopulations above a certain time value (Fig. 6B; the UV-dose was 25 J cm^{-2}). Over the first 2.5 min the average value of the hydrodynamic radius of particles was 6.5 ± 0.8 nm. The splitting of the population of protein particles became evident at $t \approx 4$ min. The initial part of the dependence of R_h on time for large protein aggregate subpopulation can be described by Eq. (3) (see inset in Fig. 6B). The applicability of Eq. (3) means that aggregation proceeds in the regime of RLCA. We set the t_0 value in Eq. (3) on the dependence of the light scattering intensity on time equal to the duration of the lag period ($t_0 = 2.7$ min). In this case the following values of parameters $R_{h,0}$ and t_{2R} were obtained: $R_{h,0} = 44 \pm 3$ nm and $t_{2R} = 4.2 \pm 0.2$ min. At $t > 16$ min the dependence of R_h on time follows Eq. (2) with $d_f = 1.8 \pm 0.1$. This transition of RLCA regime to DLCA regime at rather high values of time is due to an increase in the sticking probability of particles as the particle size increases (see [43]). It should be noted that up to $t = 100$ min the population of protein particles contains, apart from large aggregates, a subpopulation of particles of lesser size (the average value of R_h for small aggregate subpopulation is 72 ± 4 nm).

The analysis of the kinetics of aggregation of UV-irradiated GAPDH at various concentrations of the protein is of special interest. Fig. 7A shows the dependences of the light scattering intensity on time obtained for the concentrations of UV-irradiated GAPDH varied in the interval from 0.2 to 1.0 mg mL^{-1} at 37 °C. The initial parts of these dependences were analyzed using Eq. (1). The obtained values of parameters t_0 and K_{agg} are represented in Fig. 7B and C as a function of concentration of UV-irradiated GAPDH (UV-GAPDH). The duration of the lag period (parameter t_0) decreases with increasing the protein concentration (Fig. 7B). The dependence of parameter K_{agg} (a measure of the initial rate of aggregation) on the concentration of the protein (UV-GAPDH) is non-linear and follows the power law:

$$K_{agg} = \text{const} \times [\text{UV} - \text{GAPDH}]^n. \quad (5)$$

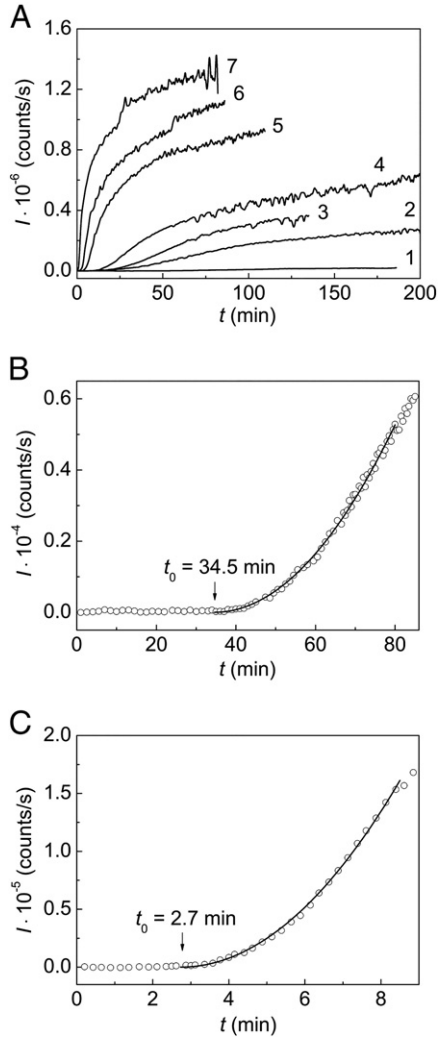

Fig. 5. Kinetics of aggregation of UV-irradiated GAPDH (0.4 mg mL^{-1}) at 37 °C. (A) Dependences of the light scattering intensity (I) on time for aggregation of GAPDH irradiated by the following doses: (1) 0, (2) 5, (3) 10, (4) 15, (5) 25, (6) 40 and (7) 60 J cm^{-2}. (B and C) Initial parts of dependences of I on time for non-irradiated GAPDH and the enzyme irradiated by the dose of 25 J cm^{-2}, respectively. Solid curves were calculated from Eq. (1).

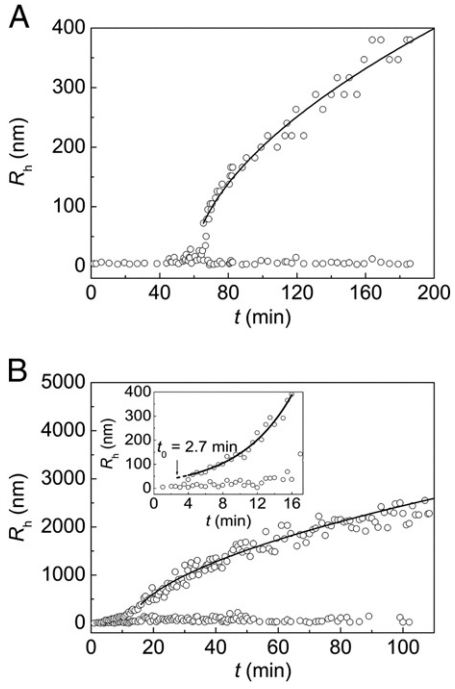

Fig. 6. Dependences of the hydrodynamic radius (R_h) of protein aggregates on time for aggregation of non-irradiated GAPDH (0.4 mg mL^{-1}) and the enzyme irradiated by dose of 25 J cm^{-2} (A and B, respectively). Solid curves in panels A and B were calculated from Eq. (2). The inset in panel B shows the initial part of the dependence of R_h on time. The solid curve was calculated from Eq. (3). Temperature of incubation was 37 °C.

Parameter n was found to be 2.1 ± 0.2. In fact, this parameter is the order of aggregation with respect to the protein. It was of special interest to compare the obtained value of n with the corresponding value for non-irradiated GAPDH. Fig. 8 shows the dependence of parameter K_{agg} on the protein concentration for aggregation of non-irradiated GAPDH at 45 °C. This temperature was selected for studying the kinetics of the aggregation process, because at 37 °C the aggregation rate for non-irradiated GAPDH was extremely low. As one can see in Fig. 8, the dependence of K_{agg} on [GAPDH] is linear up to the protein concentration of 1 mg mL^{-1}. This means that the order of aggregation with respect to the protein (n) is equal to unity. Such a case is observed when the stage of the protein molecule unfolding proceeds with substantially lower rate than the subsequent stages of aggregation of the unfolded protein molecules. The case of $n = 1$ was also observed for non-irradiated Ph*b* from rabbit skeletal muscle [41].

Table 2
Parameters of Eq. (1) for aggregation of GAPDH (0.4 mg mL^{-1}) irradiated by various doses of UV light (37 °C; 50 mM Na-phosphate buffer, pH 7.5).

UV dose, J cm^{-2}	K_{agg}, (counts/s) × min^{-2}	t_0, min
0	2.55 ± 0.04	34.5 ± 0.1
5	$(2.8 \pm 0.1) \times 10$	4.8 ± 0.1
10	$(7.6 \pm 0.2) \times 10$	8.3 ± 0.3
15	$(2.6 \pm 0.1) \times 10^2$	6.7 ± 0.2
25	$(4.8 \pm 0.2) \times 10^3$	2.7 ± 0.1
40	$(7.8 \pm 0.2) \times 10^3$	1.01 ± 0.03
60	$(1.13 \pm 0.02) \times 10^5$	0.25 ± 0.01

The aggregation test system used in the present work is based on the aggregation of protein molecules preliminarily denatured by UV radiation, and, consequently, does not contain the stage of protein unfolding. Therefore one can expect that the order of aggregation with respect to the protein will exceed unity. Actually, for the aggregation of UV-irradiated GAPDH the value of n is close to 2. Thus, test systems of this type may be used for testing the effect of various agents exclusively on the aggregation stage.

3.6. Suppression of aggregation of UV-irradiated GAPDH by α-crystallin and HP-β-CD

To demonstrate the capabilities of a test system based on the aggregation of UV-irradiated protein for testing the agents possessing chaperone-like activity, we studied the effect of α-crystallin and HP-β-CD on aggregation of UV-irradiated GAPDH at 37 °C. Fig. 9A demonstrates the suppression of the aggregation of UV-irradiated GAPDH by α-crystallin. As it is evident from this figure, the suppressing effect increases with increasing α-crystallin concentration. The measurements of the size of the protein particles in the system showed that deceleration of the increment of the light scattering intensity in the presence of α-crystallin was due to formation of protein aggregates of lesser size (Fig. 9B). It should be noted that as in the case of the aggregation of UV-irradiated GAPDH, in the absence of α-crystallin the splitting of the population of protein particles into two components takes place.

Acceleration of heat-induced aggregation of non-irradiated GAPDH under the action of HP-β-CD was caused by destabilization of the protein molecule [25,26]. Since the process of aggregation of

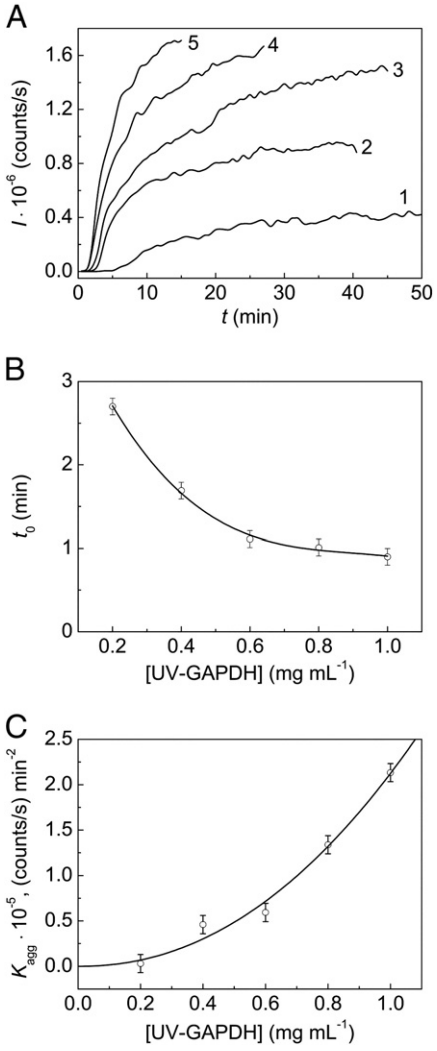

Fig. 7. Kinetics of aggregation of UV-irradiated GAPDH at various concentrations of the protein at 37 °C. (A) Dependences of the light scattering intensity (I) on time for aggregation of GAPDH irradiated by the dose of 40 J cm^{-2}. The concentrations of the enzyme were the following: (1) 0.2, (2) 0.4, (3) 0.6, (4) 0.8 and (5) 1 mg mL^{-1}. (B and C) Dependences of parameters t_0 and K_{agg} on the concentration of UV-irradiated GAPDH (UV-GAPDH). The initial parts of the dependences of I on time were used for the calculation of these parameters. Solid curve in panel C was calculated from Eq. (5) with $n = 2.1$.

UV-irradiated GAPDH does not contain a stage of protein unfolding, we may expect that HP-β-CD will exhibit protective action. Actually HP-β-CD suppresses aggregation of UV-irradiated GAPDH. The protective effect increases with increasing HP-β-CD concentration

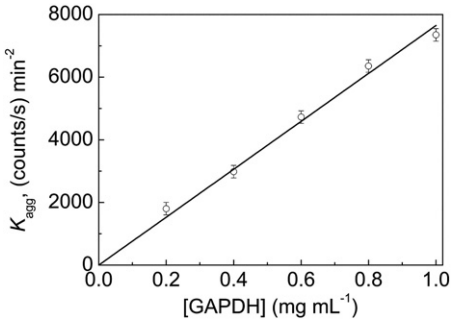

Fig. 8. Dependence of parameter K_{agg} on the protein concentration for aggregation of non-irradiated GAPDH at 45 °C.

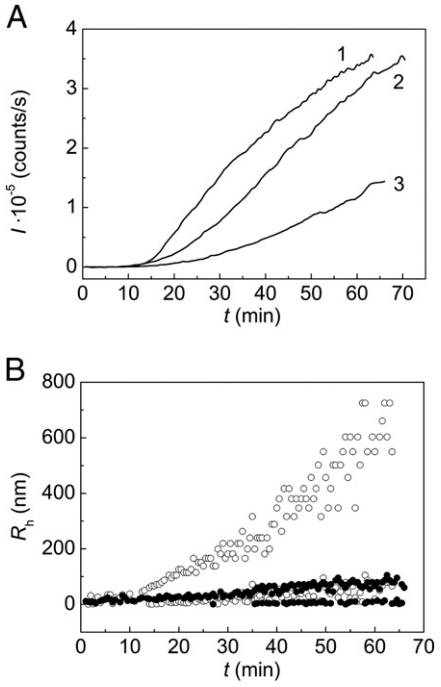

Fig. 9. Aggregation of UV-irradiated GAPDH (0.4 mg mL^{-1}; UV dose of 60 J cm^{-2}) at 37 °C in the presence of α-crystallin. (A) Dependences of the light scattering intensity on time. The concentrations of α-crystallin were the following: (1) 0, (2) 0.025 and (3) 0.05 mg mL^{-1}. (B) Dependences of the hydrodynamic radius (R_h) of protein aggregates obtained in the absence (open circles) or in the presence of 0.05 mg mL^{-1} of α-crystallin (solid circles).

(Fig. 10A). The initial parts of the dependences of the light scattering intensity on time obtained at various HP-β-CD concentrations were analyzed using Eq. (1). As one can see in Fig. 10B, the duration of the lag period (parameter t_0) linearly increases with increasing HP-β-CD concentration. Fig. 10C shows the dependence of parameter K_{agg} on HP-β-CD concentration. This dependence is S-shaped and can be analyzed using the Hill equation [51]:

$$K_{agg} = \frac{K_{agg,0}}{1 + \left([L]/[L]_{0.5}\right)^h}, \quad (6)$$

where $K_{agg,0}$ is the value of K_{agg} in the absence of HP-β-CD, L is a ligand (HP-β-CD), $[L]_{0.5}$ is the concentration of "semisaturation", i.e., the value of [L] at which $K_{agg} = K_{agg,0}/2$, and h is the Hill coefficient. The fitting of Eq. (6) to experimental data yielded the following values of parameters: $[L]_{0.5} = 11 \pm 1$ mM and $h = 1.8 \pm 0.2$ ($R^2 = 0.988$). Parameter $[L]_{0.5}$ characterizes the affinity of HP-β-CD to the protein substrate (UV-irradiated GAPDH). The lower the $[L]_{0.5}$ value, the tighter is the HP-β-CD-protein substrate complex. As in the case of α-crystallin the addition of HP-β-CD results in the formation of protein aggregates of lesser size (Fig. 11). The splitting of the population of protein particles into two components also occurs.

4. Conclusions

The fact that UV-irradiated proteins exhibit an enhanced ability to aggregate allows constructing test systems based on the aggregation of UV-irradiated proteins for detecting the influence of various agents (for example, chaperones of the protein nature, "artificial chaperones" such as cyclodextrins and others) on protein aggregation. In the present work a test system based on the aggregation of UV-irradiated GAPDH has been proposed. When studying the effect of UV light on thermostability of GAPDH, we showed that UV irradiation results in the formation of damaged protein molecules with lower

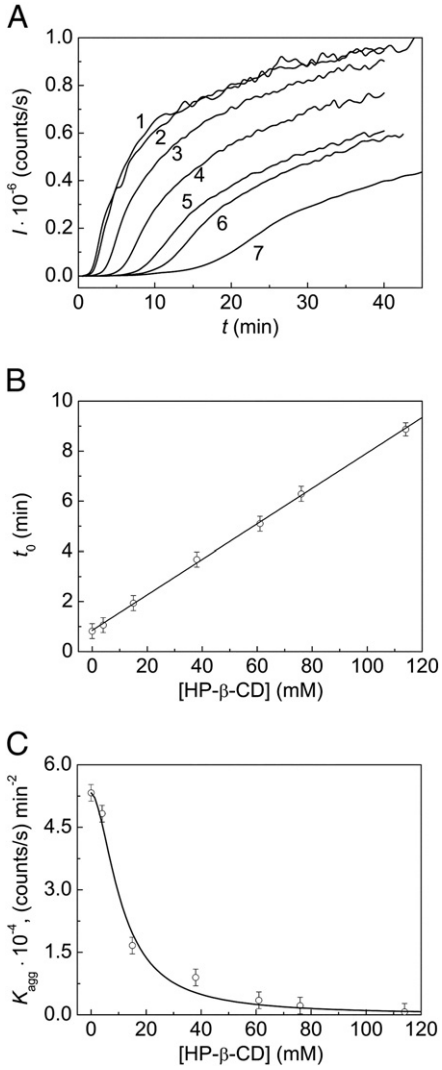

Fig. 10. Effect of HP-β-CD on aggregation of GAPDH (0.4 mg mL^{-1}) irradiated by the dose of 40 J cm^{-2}. (A) Dependences of the light scattering intensity on time. The concentrations of HP-β-CD were the following: (1) 0, (2) 4, (3) 15, (4) 38, (5) 61, (6) 76 and (7) 114 mM. Temperature of incubation was 37 °C. (B and C) Dependences of parameters t_0 and K_{agg} on the HP-β-CD concentration, respectively. Solid curve in panel C was calculated from Eq. (3).

thermostability. It should be noted that such an action of UV radiation was for the first time demonstrated for lactate dehydrogenase from rabbit skeletal muscle by Kurganov [52].

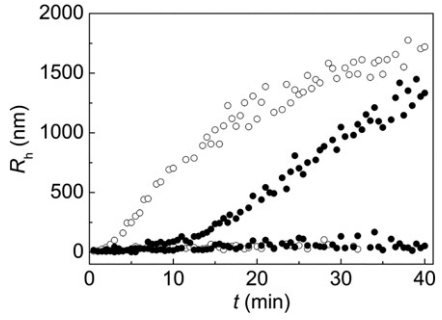

Fig. 11. Dependences of the hydrodynamic radius (R_h) of protein aggregates on time for the aggregation of GAPDH (0.4 mg mL^{-1}) irradiated by the dose of 40 J cm^{-2}. Aggregation was studied in the absence (open circles) or in the presence of 61 mM HP-β-CD (solid circles).Temperature of incubation was 37 °C.

The mechanisms of aggregation of UV-irradiated proteins are as yet imperfectly understood. In fact we can compare our data on the kinetics of aggregation of UV-irradiated GAPDH only with those for aggregation of UV-irradiated Ph*b* from rabbit skeletal muscle [50]. The main difference in the kinetic behavior of these protein substrates is the following. The time course of aggregation of UV-irradiated GAPDH during heating at 37 °C shows a lag period. The duration of the lag period (t_0) on the dependences of the light scattering intensity on time can be calculated from Eq. (1). The existence of a lag period on the kinetic curves of aggregation is typical of heat-induced aggregation of proteins, a lag phase corresponding to the initial stages of aggregation where nuclei or primary aggregates are formed from denatured protein molecules [19,24–26,30,41,44,53,54]. In the case of UV-irradiated Ph*b* the initial parts of the dependences of the light scattering intensity on time for the aggregation process studied at 37 °C can be described by the stretched exponential function [50]:

$$I = I_0 \exp\left\{(\ln 2)\left(\frac{t}{t_{2I}}\right)^n\right\}, \quad (7)$$

where I_0 is the initial value of the light scattering intensity (i.e., the light scattering intensity value at time zero), t_{2I} is the time value at which $I = 2I_0$ and n is a constant. It is significant that this equation does not contain parameter t_0. The average hydrodynamic radius of the particles registered in the solution of UV-irradiated Ph*b* was 10.4 ± 0.2 nm [50]. It was assumed that all the Ph*b* molecules denatured by ultraviolet radiation in the solution were assembled into primary aggregates. As a result, the observed aggregation is due to sticking of the primary aggregates with formation of aggregates of higher order. In contrast to UV-irradiated Ph*b*, the time course of aggregation of UV-irradiated GAPDH shows a lag period, which probably corresponds to the stage of assemblage of UV-damaged protein molecules into primary aggregates.

Analysis of the test-systems used in the literature for the study of the effects of different agents on protein aggregation shows that they do not allow registering the true influence of the agents on the aggregation stage. The observed effects include the effects of the agents on the stage of the formation of the intermediates prone to aggregation. In a test-system based on heat-induced aggregation of proteins the aggregation stage is preceded by the stage of unfolding of the protein molecule, and an agent being tested may affect both the protein unfolding stage and the stage of aggregation of unfolded protein molecules. Such a situation is observed, for example, when studying the effect of α-crystallin on thermal aggregation of GAPDH [22] or Ph*b* [55]. In a test-system where aggregation accompanying refolding of the proteins denatured by guanidine hydrochloride or urea is registered an agent being tested may affect not only the aggregation stage but also the stage of the formation of aggregation-prone intermediate from completely unfolded state.

The chief merit of test systems based on the aggregation of UV-irradiated proteins is that they enable testing the effect of various agents exclusively on the aggregation stages. The order of aggregation of UV-irradiated GAPDH with respect to the protein is close to 2, indicating that the rate-limiting stage of the aggregation process is the stage of aggregation of denatured protein molecules. The ability of α-crystallin and HP-β-CD to suppress aggregation of UV-irradiated GAPDH was demonstrated in the present work. The antiaggregation ability of HP-β-CD is of special interest because earlier we showed that HP-β-CD accelerated the aggregation of non-irradiated GAPDH due to the destabilizing action on the enzyme molecule [26]. In line with the commonly accepted opinion, one can assume that the suppression of aggregation of UV-irradiated GAPDH by HP-β-CD is due to the complexation of HP-β-CD with hydrophobic side chains of amino acids in proteins [56–62].

Acknowledgments

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