Quantification of Anti-Aggregation Activity of Chaperones: A Test-System Based on Dithiothreitol-Induced Aggregation of Bovine Serum Albumin

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Abstract

The methodology for quantification of the anti-aggregation activity of protein and chemical chaperones has been elaborated. The applicability of this methodology was demonstrated using a test-system based on dithiothreitol-induced aggregation of bovine serum albumin at 45 °C as an example. Methods for calculating the initial rate of bovine serum albumin aggregation (v_agg) have been discussed. The comparison of the dependences of v_agg on concentrations of intact and cross-linked α-crystallin allowed us to make a conclusion that a non-linear character of the dependence of v_agg on concentration of intact α-crystallin was due to the dynamic mobility of the quaternary structure of α-crystallin and polydispersity of the α-crystallin–target protein complexes. To characterize the anti-aggregation activity of the chemical chaperones (arginine, arginine ethyl ester, arginine amide and proline), the semi-saturation concentration [L]_{0.5} was used. Among the chemical chaperones studied, arginine ethyl ester and arginine amide reveal the highest anti-aggregation activity ([L]_{0.5} = 53 and 58 mM, respectively).

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Introduction

Folding of newly synthesized polypeptide chains can be accompanied by the formation of proteins prone to aggregation. Non-native proteins which implied to aggregation are also formed under stress conditions. Aggregation of non-native proteins may be prevented by small heat shock proteins (sHsps) and also by some low-molecular-weight compounds, so-called “chemical chaperones”.

sHsps, as a class of molecular chaperones, form a large family of ubiquitous proteins with molecular mass of subunit in the range 12–40 kDa, which are able to prevent protein aggregation. α-Crystallin is a representative of a family of sHsps, exhibits chaperone-like properties, including the ability to prevent the precipitation of denatured proteins [1–3]. The ability of α-crystallin to suppress heat-induced aggregation of proteins is a result of hydrophobic interactions with denatured proteins, and this ability increases when α-crystallin is heated [4,5]. sHsps, including α-crystallin, form highly dynamic assemblies of different size and composition [6,7]. Benesch and co-workers [6,8,9] suppose that the dynamic quaternary structure play an important role in sHsps chaperone function. There is some evidence that the dissociated forms of sHsps are the chaperone-active species which interact with target proteins and are subsequently sequestered into high mass complexes [10–13]. Ample evidence demonstrates the complexation of α-crystallin with nonnative proteins [1,14–18]. The formation of complexes between dissociated forms of α-crystallin and target substrates, muscle gyceraldehyde 3-phosphate dehydrogenase (GAPDH) or glycogen phosphorylase b (Phb), at elevated temperatures has been demonstrated in our studies [19–22].

The data on the importance of quaternary structure of α-crystallin for chaperone-like activity and the location of substrate-binding site(s) are contradictory. It was shown that subunit exchange was not required for chaperone function of α-crystallin. Bovine α-crystallin cross-linked with glutaraldehyde under conditions designed to minimize intermolecular reactions was able to inhibit the thermally-induced precipitation of Phb-crystallin and appeared to be more effective than the native protein under the same conditions [23]. Horwitz et al. [24] have shown that native oligomeric state of α-crystallin may not be essential for its ability to suppress non-specific aggregation, since prepared tetramers of α-crystallin had the same chaperone-like activity as the native oligomeric α-crystallin. However, according to Sharma and Otrwal [25], age-related cross-linking of α-crystallin reduces its chaperone-like activity.

Among chemical chaperones arginine (Arg) is the most effective additive in suppressing heat- and dithiothreitol (DTT)-induced


aggregation of proteins [26–31] and protein aggregation during in vitro folding [32]. It is suggested that Arg does not facilitate refolding, but can suppress aggregation of the proteins during refolding [33,34].

Solubility measurements of 20 amino acids and model peptides showed that a majority of amino acids chains, in particular aromatic amino acids, of proteins favorably interact with Arg. Such favorable interactions should be reflected on Arg binding to protein surfaces [35–39]. Tomita et al. [31] showed that heat-induced aggregation of lysozyme at around the isoelectric point occurred in a two-step process: formation of start aggregates, followed by further growth mediated by their sticking with diffusion-limited cluster-cluster aggregation. In the presence of Arg, the diffusion-limited regime changed to reaction-limited cluster-cluster aggregation. According to the data presented by Srinivas et al. [39,40], Arg is able to affect the tertiary and quaternary structure of α-crystallin and enhances the dynamics of the subunit assembly leading to enhanced chaperone-like activity.

It is important that Arg derivatives such as arginine ethylester (ArgEE) and arginine amide (ArgAd) are more effective additives for both heat-induced and refolding-induced irreversible misfolding of lysozyme than Arg [27–29,41].

Anti-aggregation activity of proline (Pro), one of the osmolytes behaving as a chemical chaperone, was demonstrated in in vitro and in vivo experiments [22,42–45]. Pro is found to prevent aggregation during protein refolding [42,43,46,47]. Experimental evidence suggests that Pro inhibits protein aggregation by binding to folding intermediate(s) and trapping the folding intermediate(s) into enzymatically inactive, “aggregation-insensitive” state(s) [48,49]. As shown by Eronina et al. [50], the suppression of aggregation at high Pro concentrations (>0.3 M) was mainly due to the protective action of Pro on the stage of unfolding of the αβmolecule.

Main problems facing the biochemists studying the anti-aggregation functions of molecular chaperones are the following: how molecular chaperones realize their anti-aggregation activity, how to compare the anti-aggregation activities of molecular chaperones of different classes and how to quantitatively characterize the mutual effects of molecular chaperones of different classes. To solve these problems, the investigator should have the strict quantitative methods of the estimation of the anti-aggregation activity of chaperones at his disposal. The goal of the present work is to elaborate the theoretical approaches to quantification of the anti-aggregation activity of chaperones and to demonstrate the applicability of these approaches using a new test-system based on DTT-induced aggregation of bovine serum albumin (BSA).

BSA is a water-soluble monomeric protein with molecular mass of 66.4 kDa [51] and isoelectric point around 4.7–5.2 [52]. Polypeptide chain of BSA consists of 583 amino acid residues [53]. The three-dimensional structure of BSA is composed of three homologous domains (I, II, III), each formed by six helices [54]. Tertiary structure is well defined: 17 disulfide bonds give some rigidity of each sub-domain but allow significant modification in the shape and size of the protein under different external conditions [51,55,56]. At neutral pH the disulfide bridges are buried in the protein molecule and not exposed to the solvent [57]. Besides, a unique free cystein (Cys-34) is located in domain I in a hydrophobic pocket of the BSA molecule [50]. BSA has two tryptophans (Trp), embedded in two different domains: Trp-134, located in proximity of the protein surface, but buried in a hydrophobic pocket of domain I, and Trp-214, located in an internal part of domain II [59].

The treatment of BSA molecules with DTT reduces S-S into SH [60]. As a result, the α-helical structure is disrupted and the β-structure is formed after unfolding, coupled with reducing disulfide bonds of BSA [61,62]. None of the disulfide bonds in BSA molecule is accessible to reducing agents in the pH range 5–7, however, between pH 7 and 10 approximately five disulfide bonds became available for reduction [57]. When the temperature increases from 35 to 55 °C, the reduction of disulfide bonds also increases [63].

Sogami et al. [64] showed that BSA was prone to intramolecular disulfide-interchange reactions which markedly broaden the population of the protein molecules. The structural fluctuations of BSA are internal without significant effect on the external shape of the protein molecules. It is supposed that fluctuations in disulfide pairing are responsible for the microheterogeneity of BSA [64].

Gobbo et al. [65] proposed a test-system based on DTT-induced aggregation of BSA for the analysis of the anti-aggregation activity of αHsp27. BSA aggregation kinetics (50 mM Na-phosphate buffer, pH 7) at 45 °C was monitored spectrophotometrically at 340 nm. This chaperone quantification test was based on the capacity of Hsp 27 to suppress DTT-induced aggregation of BSA. However, the authors did not represent the kinetic curves of aggregation in the absence and in the presence of Hsp 27 and did not discuss the quantitative methods of estimation of the anti-aggregation activity of the chaperone. Therefore it is difficult to use the work by Gobbo et al. [65] in practice.

In the present work we studied the kinetics of DTT-induced aggregation of BSA at various concentrations of the protein and DTT using dynamic light scattering (DLS). It has been demonstrated that a test-system based on the DTT-induced aggregation of BSA may be used for the quantitative estimation of the ability of different agents to suppress protein aggregation. In particular, the chaperone-like activities of intact and cross-linked α-crystallin, a representative of the family of Hsp, and of chemical chaperones Arg, ArgEE, ArgAd and Pro were quantified.

Materials and Methods

Chemicals

BSA (catalogue no. A7638, 99+ % of purity), DL-dithiothreitol (99% of purity), L-arginine monohydrochloride (Arg), L-arginine ethylester (ArgEE), L-arginine amide (ArgAd) and L-proline (Pro) (reagent grade) were purchased from Sigma-Aldrich and used without further purification.

Sample Preparation

All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA). BSA samples were prepared by dissolving solid BSA in 0.1 M phosphate buffer solutions at pH 7.0. BSA concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A_{280}^\text{cm}$ of 6.38 [66].

Isolation of α-Crystallin

α-Crystallin was isolated from freshly excised eye lenses of 2-year-old steers (Bos taurus). The eye lenses were obtained from a local slaughter-house “Pushkinskii Myasnoi Dvor”, located at Sokolovskaya St. 1, Pushkino, Moscow Region, Russia. Authors confirm that they have permission from the slaughterhouse to use these animal parts. Purification of α-crystallin, was performed according to the procedure described earlier [67,68]. α-Crystallin concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A_{280}^\text{cm}$ of 8.3 [3].
Preparation of Cross-Linked α-Crystallin

Cross-linking of α-crystallin was performed according to Augusteyn [69] with some modification. The intact protein (0.03 mM) was incubated in 40 mM phosphate buffer (pH 7.0), containing 150 mM NaCl, 1 mM EDTA and 3 mM NaN₃, with 3 mM glutaraldehyde at 20°C for 30 h. 3 mM DTT was added to block any non-reactive aldehyde groups and then the protein was dialyzed against the same buffer. The obtained samples were centrifuged at 4500 g for 30 min, using MiniSpin®. Eppendorf centrifuge and the supernatant was passed through a size-exclusion chromatography (SEC) column. The concentration of cross-linked α-crystallin was determined by micro-biuret method [70].

Size-Exclusion Chromatography

The protein samples were loaded onto the column (Toyopearl TSK-gel HW-55 fine; 2.5 cm × 90 cm) and separated into the fractions at a flow rate of 1.7 ml/min (20°C). The column was pre-calibrated with the following proteins from (Sigma-Aldrich): thyroglobulin (660 kDa), catalase (440 kDa), aldolase (158 kDa), BSA (67 kDa), α-crystallin (20 kDa). The relative error for protein mass determination was 4%.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS—PAGE)

The polypeptide composition of the protein samples was analyzed by electrophoresis in 15% PAAG in the presence of SDS and DTT [71]. Sigma-Aldrich proteins α-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa) and BSA (66 kDa) were used as standards. The gels were stained with Coomassie R-250 and scanned with an Epson Perfection 4180 scanner. The images were analyzed with ImageJ 1.41n program.

Determinations of Refractive Index, Density and Dynamic Viscosity

The values of the refractive index of Arg, ArgEE, ArgAd and Pro solutions at the different concentrations (0.1 M Na-phosphate buffer, pH 7.0) were determined in automatic microviscosimeter (Anton Paar, Austria) at 45°C. The diffusional coefficients of the solutions were determined in automatic microviscosimeter (Anton Paar, Austria) in system 1.6/1.500 mm at 45°C. The obtained values of the refractive index, density and dynamic viscosity of Arg, ArgEE, ArgAd and Pro solutions are given in Table 1. The values of refractive index and dynamic viscosity of Arg, ArgEE, ArgAd and Pro solutions were used in the DLS measurements.

Light Scattering Intensity Measurements

For light scattering measurements a commercial instrument Photocor Complex (Photocor Instruments, Inc., USA) was used. A He-Ne laser (Coherent, USA, Model 31-2002, 632.8 nm, 10 mW) was used as a light source. DynaLS software (Alango, Israel) was used for polydisperse analysis of DLS data. The diffusion coefficient D of the particles is directly related to the decay rate τc of the time-dependent correlation function for the light scattering intensity fluctuations:

\[ D = \frac{1}{2} \tau_c k^2. \]

In this equation k is the wave number of the scattered light, k = (4πn/λ)sin(θ/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 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 \( \eta \) is the dynamic viscosity of the solvent.

The kinetics of DTT-induced aggregation of BSA was studied in 0.1 M Na-phosphate buffer, pH 7.0. The buffer was placed in a cylindrical cell with the internal diameter of 6.3 mm and preincubated for 5 min at a given temperature (45°C). Cells with stopper were used to avoid evaporation. The aggregation process was initiated by the addition of an aliquot of DTT to a BSA sample to the final volume of 0.5 ml. To study the effect of α-crystallin or Arg, ArgEE, ArgAd and Pro on BSA aggregation, the

### Table 1. The values of refractive index (n), density (p) and dynamic viscosity (η) of solutions of arginine, arginine ethylester, arginine amide and proline at 45°C (0.1 M Na-phosphate buffer, pH 7.0).

| Concentration (mM) | n            | p (g/cm³) | η (mPa.s) |
|-------------------|--------------|-----------|-----------|
| Arginine          |              |           |           |
| 0                 | 1.3320 ± 0.0002 | 0.9907 ± 0.0005 | 0.4214 ± 0.0003 |
| 50                | 1.3340 ± 0.0002 | 0.9987 ± 0.0005 | 0.4339 ± 0.0003 |
| 75                | 1.3351 ± 0.0002 | 0.9985 ± 0.0005 | 0.4398 ± 0.0002 |
| 100               | 1.3361 ± 0.0002 | 1.0012 ± 0.0005 | 0.4461 ± 0.0002 |
| 150               | 1.3380 ± 0.0002 | 1.0037 ± 0.0005 | 0.4574 ± 0.0003 |
| 200               | 1.3402 ± 0.0002 | 1.0062 ± 0.0005 | 0.4613 ± 0.0002 |
| 400               | 1.3486 ± 0.0002 | 1.0162 ± 0.0005 | 0.5160 ± 0.0002 |
| Arginine ethylester |             |           |           |
| 0                 | 1.3328 ± 0.0002 | 1.0069 ± 0.0005 | 0.6372 ± 0.0003 |
| 25                | 1.3342 ± 0.0002 | 1.0078 ± 0.0005 | 0.6463 ± 0.0003 |
| 50                | 1.3365 ± 0.0002 | 1.0096 ± 0.0005 | 0.6690 ± 0.0002 |
| 75                | 1.3370 ± 0.0002 | 1.0119 ± 0.0005 | 0.6702 ± 0.0002 |
| 100               | 1.3385 ± 0.0002 | 1.0127 ± 0.0005 | 0.6826 ± 0.0003 |
| 150               | 1.3410 ± 0.0002 | 1.0158 ± 0.0005 | 0.7022 ± 0.0002 |
| Arginine amide    |              |           |           |
| 0                 | 1.3328 ± 0.0002 | 1.0053 ± 0.0005 | 0.6358 ± 0.0003 |
| 10                | 1.3325 ± 0.0002 | 1.0069 ± 0.0005 | 0.6377 ± 0.0003 |
| 30                | 1.3324 ± 0.0002 | 1.0090 ± 0.0005 | 0.6443 ± 0.0002 |
| 50                | 1.3335 ± 0.0002 | 1.0104 ± 0.0005 | 0.6501 ± 0.0002 |
| 75                | 1.3349 ± 0.0002 | 1.0130 ± 0.0005 | 0.6582 ± 0.0003 |
| 100               | 1.3359 ± 0.0002 | 1.0148 ± 0.0005 | 0.6646 ± 0.0002 |
| 150               | 1.3382 ± 0.0002 | 1.0175 ± 0.0005 | 0.6787 ± 0.0002 |
| Proline           |              |           |           |
| 0                 | 1.3327 ± 0.0002 | 1.0060 ± 0.0005 | 0.6282 ± 0.0003 |
| 150               | 1.3361 ± 0.0002 | 1.0109 ± 0.0005 | 0.6563 ± 0.0003 |
| 300               | 1.3398 ± 0.0002 | 1.0170 ± 0.0005 | 0.7010 ± 0.0002 |
| 500               | 1.3440 ± 0.0002 | 1.0236 ± 0.0005 | 0.7420 ± 0.0002 |
| 750               | 1.3483 ± 0.0002 | 1.0297 ± 0.0005 | 0.7842 ± 0.0003 |
| 1000              | 1.3525 ± 0.0002 | 1.0377 ± 0.0005 | 0.8456 ± 0.0002 |

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agents were added before the addition of DTT to a preheated solution of BSA. When studying the kinetics of aggregation of BSA, the scattering light was collected at a 90° scattering angle.

Asymmetric Flow Field Flow Fractionation (A4F) with On-Line Multi-Angle Light Scattering (MALS), Ultraviolet (UV) and Refractive Index (RI) Detectors

The Eclipse 3 separation system (Wyatt Technology Corporation, USA) based on an Agilent HPLC pump (Agilent Technologies, USA) was used for A4F experiments. BSA sample or the mixture of BSA with cross-linked α-crystallin in 0.1 M Na phosphate buffer, pH 7.0, preheated with 0.2 mM DTT for 2 h and cooled to room temperature 23°C was injected in the separation channel by Agilent autoinjection system (Agilent Technologies, USA). A 21.4 cm channel with a 350-mm channel space and ultrafiltration membrane made of regenerated cellulose with a 10-kDa molecular weight cut off (Wyatt Technology Corporation, USA) were used. The flow system was sequentially connected to UV detector (Agilent Technologies, USA), MALSS detector (DAWN HELEOS II, Wyatt Technology Corporation, USA) and RI detector (Optilab T-rEX, Wyatt Technology Corporation, USA), The elution was performed with 0.1 M phosphate buffer (pH 7.0) at a flow rate at the channel outlet of 1 ml/min, 3 ml/min cross flow. The data from the detectors were processed in ASTRA software, version 5.3.4 (Wyatt Technology Corporation, USA) to yield the final profiles. The experiment was carried out at room temperature (23°C).

Analytical Ultracentrifugation

Sedimentation velocity experiments were carried out at 45°C in a Model E analytical ultracentrifuge (Beckman), equipped with absorbance optics, a photoelectric scanner, a monochromator and an on-line computer. A four-hole An-F Ti rotor and 12 mm double sector cells were used. The rotor was preheated at 45°C in the thermostat overnight before the run. The sedimentation profiles of BSA, α-crystallin and their mixtures (0.1 M Na phosphate buffer, pH 7.0 containing 10 mM NaCl; 2 mM DTT) were recorded by measuring the absorbance at 285 nm. All cells were scanned simultaneously against the buffer containing the same additives. The time interval between scans was 3 min. The sedimentation coefficients were estimated from the differential sedimentation coefficient distribution [c(δ) versus s] or [c(δf/δ0) versus s] which were analyzed using SEDFIT program [72,73]. The c(δ) analysis was performed with regularization at a confidence level of 0.68 and a floating frictional ratio. The sedimentation coefficients were corrected to the standard conditions (a solvent with the density and viscosity of water at 20°C) using SEDFIT and SEDNTERP [74] programs.

Calculations

OriginPro 8.0 SR0 software (OriginLab Corporation, USA) and Scientist (MicroMath, Inc., USA) software were for the calculations. To characterize the degree of agreement between the experimental data and calculated values, we used the coefficient of determination R² (without considering the statistical weight of the measurement results) [75].

Theory. Quantification of the Chaperone-Like Activity

Determination of the Initial Rate of Protein Aggregation

To characterize the anti-aggregation activity of a chaperone, we should measure the initial rate of aggregation of a model target protein and compare this rate with the corresponding value measured in the absence of a chaperone. Protein aggregates possess higher light scattering capability in comparison with the non-aggregated protein molecules. Therefore the simplest way to measure the initial rate of aggregation is registration of the increment of the light scattering intensity (I) or apparent optical absorbance (A). In the early stages, the acceleration of the aggregation process takes place, suggesting that aggregation proceeds through the nucleation stage. To characterize the initial rate of aggregation, the quadratic dependence on time (t) was proposed for the description of the initial parts of the kinetic curves of aggregation [76]:

\[ I = I_0 + K_{agg} (t-t_0) \]  

(3)

or

\[ A = A_0 + K_{agg} (t-t_0) \]  

(4)

where I₀ and A₀ are the initial value of the light scattering intensity and apparent optical absorbance, respectively, at t = 0 and t₀ is the duration of lag period on the kinetic curve (t₀ is a point in time at which the light scattering intensity or apparent optical absorbance begins to increase). Parameter K_{agg} is a measure of the initial rate of aggregation. Theoretical analysis shows that the quadratic law should be valid for nucleation-dependent aggregation [76,77].

The applicability of Eq. (3) for the description of the initial parts of the kinetic curves of protein aggregation was demonstrated for thermal denaturation of Pho [50,76,78,79], GAPDH [80–82] and creatine kinase (CK) [83] from rabbit skeletal muscles and DTT-induced aggregation of α-lactalbumin [18] and insulin [84]. The practical significance of Eqs. (3) and (4) is as follows. First, the addition of a chaperone usually results in the elongation of the lag period, the quadratic dependence on time (t) begins to increase). Parameter K_{agg} is a measure of the initial rate of aggregation. Theoretical analysis shows that the quadratic law should be valid for nucleation-dependent aggregation [76,77].

Consider different modifications of Eqs. (3) and (4). First, we can extend the time interval applicable for calculation of parameters I₀ and K_{agg}, if modify these equations as follows:

\[ I = I_0 + \frac{K_{agg} \{\exp[K(t-t_0)^2] - 1\}}{K} \]  

(5)

or

\[ A = A_0 + \frac{K_{agg} \{\exp[K(t-t_0)^2] - 1\}}{K}, \]  

(6)

where K is a constant which allows for the deviation from the quadratic dependence. It is significant that at t → t₀ Eqs. (5) and (6) are transformed into Eqs. (3) and (4), respectively.

Secondly, one should bear in mind that in some cases the initial decrease in the light scattering intensity (or apparent optical absorbance) is observed on the kinetic curves of aggregation of a target protein, registered in the presence of chaperone, namely α-crystallin. Such a kinetic behavior was demonstrated, for example, when studying thermal aggregation of citrate synthase at 43°C [85] and β-amyloid peptide at 60°C [86]. There is a simple explanation for an unusual character of the kinetic curves of
aggregation. Elevated temperatures induce dissolution of α-
crystallin particles and a decrease in the light scattering intensity.
This conclusion is substantiated by the data represented in our
works [19,20,87,88]. When a decrease in the light scattering intensity
occurs in the initial part of the kinetic curves of aggregation, a reliable
determination of the initial value of the light scattering intensity ($I_0$)
of the initial value of the apparent optical absorbance ($A_0$) becomes impossible, and we can no longer apply Eq. (3) or Eq. (4). The differential forms of Eqs. (3) and (4) are useful in this case:

$$\frac{dI}{dt} = 2K_{agg}(t-t_0) \quad (t > t_0) \quad \text{(7)}$$
or

$$\frac{dI}{dt} = 2K_{agg}(t-t_0) \quad (t > t_0) \quad \text{(8)}$$

Examples of using Eqs. (5) and (7) are given in the experimental
part of the present work.

Analysis of the dependence of the initial rate of aggregation on
the initial concentration of the target protein, $[P]_0$, allows us to
determine the order of aggregation with respect to the protein and
draw inference about the rate-limiting stage of the aggregation
process. The order of aggregation with respect to the protein ($n$) is calculated in accordance with the following equation:

$$K_{agg} = \text{const} \cdot [P]_0^n. \quad \text{(9)}$$

Below we will demonstrate that the knowledge of the $n$ value is
important for characterization of the anti-aggregation activity of
chaperones of a protein nature.

In the case of thermal aggregation of Phβ (53°C; pH 6.8) [76]
and GAPDH (45°C; pH 7.5) [82] the dependence of parameter
$k_{agg}$ on the initial concentration of the target protein is linear
($n=1$). The kinetics of thermal aggregation of bovine liver
glutamate dehydrogenase (GDH) at various concentrations of
the protein was studied by Sabbaghian et al. [89] (50°C; pH 8.0).
According to our calculations, the order of aggregation with respect to the protein calculated on the basis of these kinetic data is close to unity: $n = 0.86 \pm 0.1$. The case in which $n = 1$ means that unfolding of a protein molecule proceeds with a substantially lower rate than the following stages of aggregation of the unfolded protein molecules.

When unfolding of the protein molecule is a relatively fast
process and the stages of aggregation become rate limiting,
parameter $n$ exceeds unity. For example, the analysis of the data
on thermal aggregation of β₂-crystallin from bovine lens at 60°C
(pH 6.8) [68] and thermal aggregation of yeast alcohol dehydro-
genase at 56°C (pH 7.4) [90] shows that parameter $n$ is close to 2.
An analogous situation was observed for aggregation of UV-
irradiated GAPDH (37°C; pH 7.5; $n = 2.1 \pm 0.2$) [82].

It is of interest that the equation equivalent to Eq. (3) can be
used for the description of the initial parts of the kinetic curves of aggregation in the experiments where temperature was elevated with a constant rate [91]:

$$\frac{dI}{dt} = 2K_{agg}(t-t_0) \quad (T > T_0) \quad \text{(10)}$$

where $T_0$ is the initial temperature of aggregation, i.e., the
temperature at which the light scattering intensity begins to
increase, and $k_{agg}$ is a parameter which characterizes the rate of
aggregation. Parameters $T_0$ and $k_{agg}$ can be used for quantitative
characterization of the ability of various agents to suppress protein
aggregation. The applicability of Eq. (10) was demonstrated for
aggregation of Phβ, GAPDH, CK and GDH.

According to theoretical views developed by Kurganov and co-
workers [69,91–93], the point in time $t = t_0$ or point in temperature
$T = T_0$ corresponds to the appearance of start aggregates. A start aggregate
contains hundreds of denatured protein molecules. The formation
of the start aggregates proceeds on the all-or-none principle. The intermediate states between the non-aggregated protein and start aggregates are not detected in the system.

For completeness sake additional methods of determination of the initial rate of aggregation should be discussed. When analyzing
the shape of the kinetic curves of aggregation of Phβ denatured by
UV radiation [22], we observed that Eq. (3) is not fulfilled and, to
characterize the initial rate of aggregation, we proposed to use the
time interval ($t_{2t}$) over which the initial value of the light scattering intensity is doubled. To calculate the $t_{2t}$ value, the initial part of
the dependence of the light scattering intensity on time was described by the stretched exponent:

$$I = I_0 \exp \left( \ln 2 \frac{t}{t_{2t}} \right)^m. \quad \text{(11)}$$

where $m$ is a constant. The reciprocal value of $t_{2t}$, namely $1/t_{2t}$,
may be considered as a measure of the initial rate of aggregation.
The higher the $1/t_{2t}$ value, the higher is the initial rate of
aggregation.

Characterization of Anti-Aggregation Activity of Protein Chaperones

When analyzing the dependence of the initial rate of aggregation ($v$) on the concentration of protein chaperone, one
should take into account two circumstances. First, the binding of a
chaperone to a target protein is rather firm. The dissociation
constant values for the chaperone–target protein complexes are of
the order of magnitude of several nmoles per liter (see, for example, [94]). Suppression of aggregation is usually studied under
the conditions where the initial concentrations of a chaperone and
target protein exceed sufficiently the dissociation constant for the
chaperone–target protein complex. This means that the dependence
of $v$ on [chaperone] is a titration curve which gives, in
certain cases, information on the stoichiometry of the chaperone–
target protein complex.

Second, in accordance with Eq. (9) the protein concentration
$[P]_0$ is proportional to $v^{1/n}$. This means that the decrease in the
concentration of the target protein (for example, as a result of the
complexation with a chaperone) should result in the proportional
decrease in the $v^{1/n}$ value. Thus, the coordinates $(v^{1/n},$ [chaperone–
target one]) should be used for analysis of the anti-aggregation activity of
the chaperone. The relative initial rate of aggregation $v/v_{0}$ is
determined by the ratio of the concentrations of the chaperone
and target protein, namely [chaperone]/[target protein]. Ideally,
the dependence of $(v/v_{0})^{1/n}$ on the [chaperone]/[target protein]
ratio is a straight line (Fig. 1A). The length on the abscissa axis cut
off by the straight line ($S_0$) gives the stoichiometry of the
chaperone–target protein complex. The $S_0$ value is calculated
according to the following equation:

$$S_0 = \frac{V}{V_0} = 1 - \frac{x}{S_0}, \quad \text{(12)}$$

where $x$ is the [chaperone]/[target protein] ratio. The reciprocal
value of the stoichiometry of the chaperone–target protein complex is the adsorption capacity of the chaperone with respect to the target protein: \( AC_0 = 1/S_0 \). When working with the same test-system, we can use the initial capacity of the chaperone \( AC_0 \) for the comparative analysis of the effectiveness of the anti-aggregation activity of various chaperones (for example, the protective ability of wild-type small heat shock proteins and their chemically modified form).

Consider the dependence of the initial rate of aggregation of UV-irradiated Ph\( b \) on the \( \alpha B \)-crystallin concentration obtained in [22] (37°C; pH 6.8). The \( z \) value was calculated using Eq. (11). It is significant that the target protein is Ph\( b \) completely denatured by UV-radiation. The initial part of the dependence of the \( z \) value on the \( \alpha B \)-crystallin concentration gives the following value of \( AC_0 \): \( AC_0 = 0.65 \pm 0.06 \) moles of Ph\( b \) subunit per one \( \alpha B \)-crystallin subunit. Interestingly, the deviation from linearity takes place at rather high concentrations of \( \alpha B \)-crystallin. The complicated shape of the \( z \) versus [\( \alpha B \)-crystallin] plot is probably due to the dynamic structure of \( \alpha \)-crystallin and the initial part of this dependence corresponds to the complexes of the dissociated forms of \( \alpha B \)-crystallin with the target protein. The second linear part corresponds to the formation of the \( \alpha B \)-crystallin-target protein complexes where the adsorption capacity of \( \alpha B \)-crystallin in respect to the target protein becomes decreased.

When the dependence of the initial rate of aggregation on the \([\text{chaperone}]/[\text{target protein}]\) ratio reveals a deviation from linearity, the following approach may be used for estimation of the stoichiometry of the chaperone-target protein complex. Consider, for example, the case when the initial part of the dependence of the initial rate of aggregation on \( x = [\text{chaperone}]/[\text{target protein}] \) gives way to a flatter curve at \( x > x_1 \), and this flatter part is described by the hyperbolic dependence in the interval of \( x \) values from \( x_1 \) to \( x_2 \) [see Fig. 1B]:

\[
Y = \frac{Y_0}{1 + x/x_{0.5}}, \quad (x_1 < x < x_2)
\]

(13)

where \( Y \) signifies \([z/z_0]^{1/\alpha} \), \( Y_0 \) is the \( Y \) value at \( x = 0 \), and \( x_{0.5} \) is the \( x \) value at which \( Y = Y_0/2 \). Let us choose some point between \( x_1 \) and \( x_2 \). It is seen from Fig. 1B that the slope of a tangent to the theoretical curve at the point with coordinates \([x; Y]\) is connected with the stoichiometry of the chaperone–target protein complex by the following equation:

\[
\text{slope} = \frac{dY}{dx} = \frac{Y}{(x - S)}.
\]

(14)

Hence it follows that:

\[
S = x - \frac{Y}{dY/dx}.
\]

(15)

The derivative \( dY/dx \) is calculated from Eq. (15):

\[
\frac{dY}{dx} = -\frac{Y_0}{(1 + x/x_{0.5})^2x_{0.5}}.
\]

(16)

Substitution of \( dY/dx \) in Eq. (16) produces the expression, which allows us to calculate the stoichiometry of the chaperone–target protein complex formed at a definite value of \( x \) in the interval \( x_1 < x < x_2 \):

\[
S = (x_{0.5} + 2x), \quad (x_1 < x < x_2)
\]

(17)

The adsorption capacity \( (AC) \) of the chaperone with respect to the target protein is calculated as a reciprocal value of \( S \):

\[
AC = 1/(x_{0.5} + 2x), \quad (x_1 < x < x_2)
\]

(18)

Thus, in the interval of the \( x \) values from \( x_1 \) to \( x_2 \) the value of AC decreases from \( 1/(x_{0.5} + 2x_1) \) to \( 1/(x_{0.5} + 2x_2) \). As for the initial part of the dependence of \([z/z_0]^{1/\alpha}\) on the \([\text{chaperone}]/[\text{target protein}]\) ratio (the region where \( x < x_1 \)), the adsorption capacity of the chaperone is constant and equal to \( AC_0 \).
Characterization of Anti-Aggregation Activity of Chemical Chaperones

Protective effect of chemical chaperones is revealed as a diminishing of the initial rate of aggregation \((v)\) in the presence of chemical chaperone. In the simplest case the dependence of \(v\) on the concentration of a chemical chaperone \((L)\) is hyperbolic:

\[
v = \frac{v_0}{1 + [L]/K_d},
\]

where \(v_0\) is the initial rate of aggregation in the absence of a chaperone and \(K_d\) is the dissociation constant. This equation was applied, for example, by Wilcken et al. [95] for analysis of suppression of p53 oncogenic mutant aggregation by drugs (37°C; pH 7.2). The initial rate of aggregation was calculated using Eq. (3).

When studying the suppression of aggregation of UV-irradiated protein GAPDH by chemical chaperone 2-hydroxylpropyl-β-cyclodextrin [82], we showed that the dependence of the initial rate of aggregation \(v\) expressed by parameter \(K_{0.5}\) on the concentration of chemical chaperone followed the Hill equation (see [96]):

\[
v = \frac{v_0}{1 + ([L]/K_{0.5})^h},
\]

where \([L]_{0.5}\) is the concentration of semi-saturation, i.e., the concentration of the chaperone at which \(v/v_0 = 0.5\), and \(h\) is the Hill coefficient. The value of \(h\) found to be 1.8. The values of \(h\) exceeding unity are indicative of the existence of positive cooperative interactions between chaperone-binding sites in the target protein molecule [96]. Parameter \([L]_{0.5}\) may be considered as a measure of the affinity of the chaperone to the target protein. The lower the \([L]_{0.5}\) value, the higher is affinity of the chaperone to the target protein. It is significant that the shape of the dependence of the initial rate of aggregation on the chaperone concentration should remain unchangeable at variation of the target protein concentration.

Combined Action of Chaperones

The protective activity of protein chaperones can be modulated by the low-molecular-weight chemical chaperones. For example, it was demonstrated that Arg enhanced the chaperone-like activity of α-crystallin [39,40,97]. Since each of the chaperones (protein chaperone or chemical chaperone) affects protein aggregation, strict quantitative methods should be used to characterize the combined action of chaperones. Parameter \(j\) proposed by us for analysis of combined action of inhibitors [98] may be useful for estimating the mutual inhibitory effects of chaperones:

\[
j = \frac{i_{12}}{1 - (1 - i_1)(1 - i_2)}.
\]

In this equation \(i\) is a degree of inhibition: \(i_1 = 1 - v_1/v_0\) for inhibitor 1, \(i_2 = 1 - v_2/v_0\) for inhibitor 2 and \(i_{12} = 1 - v_{12}/v_0\) for the inhibitor 1+inhibitor 2 mixture (\(v_0\) is the initial rate of aggregation in the absence of inhibitors, \(v_1\), \(v_2\) and \(v_{12}\) are the values of the initial rate of aggregation in the presence of inhibitor 1, inhibitor 2 and inhibitor 1+inhibitor 2 mixture, respectively). When the action of one inhibitor is not dependent on the presence of the other, parameter \(j\) is equal to unity. The case \(j>1\) corresponds to synergism and the case \(j<1\) corresponds to antagonism in the combined action of two inhibitors.

As mentioned above, the different parameters are used for characterization of the anti-aggregation activity of protein and chemical chaperones, namely the initial adsorption capacity \(AC_0\) and the semi-saturation concentration \([L]_{0.5}\) respectively. We can propose the following strategy for the estimation of the effects of the combined action of chaperones. Parameter \(j\) may be used for this purpose, if we study the mutual effects of chaperones of definite group, i.e., the effects of protein chaperones or the effects of chemical chaperones. In the case of protein chaperone+chemical chaperone mixtures using of parameter \(j\) becomes unreasonable. To characterize the mutual action of protein and chemical chaperones, we should study the effect of a chemical chaperone on the \(AC_0\) value for a protein chaperone or the effect of a protein chaperone on the \([L]_{0.5}\) value for a chemical chaperone. A decrease in the \(AC_0\) value in the presence of a chemical chaperone or a decrease in the \([L]_{0.5}\) value in the presence of a protein chaperone implies synergism in the combined action of protein and chemical chaperones. On the contrary, an increase in the \(AC_0\) value in the presence of a chemical chaperone or an increase in the \([L]_{0.5}\) value in the presence of a protein chaperone implies antagonism in the combined action of protein and chemical chaperones.

Results

Chromatographic and Electrophoretic Analysis of Cross-Linked α-Crystallin Preparation

Fig. 2 shows the elution profiles obtained for intact and cross-linked α-crystallin by SEC. As can be seen, intact α-crystallin is eluted at 127 min. The cross-linked protein consisting of two fractions is eluted at 107 and 128 min. The peak at 107 min is a high-molecular-weight product of inter-oligomeric cross-linking, whereas the peak at 128 min is a result of intra-molecular cross-linking. The fraction of cross-linked protein marked with gray color (Fig. 2) was collected and examined by SDS electrophoresis in 12.5% PAGE.

Fig. 3 demonstrates the SDS-PAGE patterns for the native and cross-linked α-crystallin. Even when the gel was overloaded (20±
μg protein) only traces of low-molecular-weight species can be observed in the cross-linked sample. Most of the protein (99%) was present as high-molecular-weight species, which have not entered the 5% stacking gel.

The preparations of intact and cross-linked α-crystallin were additionally characterized by DLS. The average hydrodynamic radius of intact α-crystallin particles was found to be 12.5 nm (Fig. 4A). The major peak of the particle size distribution for cross-linked α-crystallin has the similar $R_h$ value ($R_h = 16.7$ nm; Fig. 4B). Apart from this peak, there are larger particles with $R_h = 1430$ nm.

**Kinetics of DTT-Induced Aggregation of BSA**

DLS allows measuring the increment of the light scattering intensity during protein aggregation and sizing the protein aggregates. Fig. 5A shows the dependences of the light scattering intensity on time for DTT-induced aggregation of BSA registered at various concentrations of the protein (45 °C; 0.1 M Na-phosphate buffer, pH 7.0; [DTT] = 2 mM).

Fig. 5B shows the dependences of the hydrodynamic radius ($R_h$) of the protein aggregates on time obtained at various concentrations of BSA. Sizing the protein aggregates by DLS shows that the distribution of the particles by size in the course of DTT-induced aggregation of BSA remains unimodal, and the average value of $R_h$ increases monotonously with increasing the time of incubation. The value of $R_h$ for the original preparation of BSA was equal to 4.2 ± 0.1 nm.

The polydispersity index (PI) for BSA particles at 25 °C calculated in accordance of the ISO standard [99] was found to be 0.49 ± 0.01. Such a relatively high value of PI is due to the fact that the original preparation of BSA is represented by monomeric and dimeric forms (see [100,101] and our experimental data given below). The measurements of the PI value for aggregates formed upon heating of BSA (1 mg/ml) in the presence of 2 mM DTT at 45 °C were taken over 2 h. It was shown that PI value remained practically constant: PI = 0.51 ± 0.01.
The initial parts of the dependences of the light scattering intensity on time obtained at various concentrations of BSA were analyzed using Eq. (3). The calculated values of parameters $k_{\text{agg}}$ and $t_0$ are represented in Fig. 6 as a function of BSA concentration. As can be seen in Fig. 6A, the dependence of $k_{\text{agg}}$ on BSA concentration is non-linear. This dependence was treated using Eq. (9). To determine the order of aggregation with respect to the protein concentration, the plot of $\log(k_{\text{agg}})$ versus $\log([\text{BSA}])$ was constructed (inset in Fig. 6A). The slope of straight line in these coordinates gives the $n$ value: $n = 1.60 \pm 0.05$.

When measuring the duration of the lag period, we observed the decrease in the $t_0$ value from 14 ± 1 to 5.4 ± 0.2 min, as BSA concentration increased from 0.25 to 1.0 mg/ml (Fig. 6B). However, the $t_0$ value remained practically constant in the interval of BSA concentrations from 1.25 to 3.0 mg/ml (the average value of $t_0$ was found to be 9.0 ± 0.5 min).

Variation of DTT concentration shows that the initial rate of BSA aggregation is dependent on the concentration of disulfide reducing agent. The increase in DTT concentration from 1.3 to 3.3 mM results in the increase in the $k_{\text{agg}}$ value from 29.1 ± 0.5 to 135 ± 2 ([counts/s] [min]$^{-1}$) at [BSA] = 1.0 mg/ml.

Effects of Intact and Cross-Linked α-Crystallin on DTT-Induced Aggregation of BSA

As can be seen from Fig. 7A, α-crystallin suppresses DTT-induced aggregation of BSA. In this Figure the dependences ($I/I_0$) on time are represented ($I$ and $I_0$ are the current and initial values of the light scattering intensity, respectively). When the reaction mixture contains α-crystallin, the initial decrease in the light scattering intensity on the kinetic curves of aggregation is observed. For example, inset in Fig. 7A shows the initial part of the kinetic curve obtained at α-crystallin concentration of 0.5 mg/ml. This circumstance poses difficulties for using of Eq. (3) for calculation of the initial rate of aggregation, because determination of the $t_0$ value becomes impossible. Therefore, to determine parameter $k_{\text{agg}}$ characterizing the initial rate of aggregation, we apply the differential form of Eq. (3), namely, Eq. (7). In accordance with Eq. (7), the slope of the straight line for the initial positive values of $dI/dt$ gives the $2 k_{\text{agg}}$ value (Fig. 7B; [α-crystallin] = 0.05 mg/ml).

The $k_{\text{agg}}$ values were calculated at various concentrations of α-crystallin, and the plot of $(k_{\text{agg}}/k_{\text{agg},0})^{1/n}$ versus α-crystallin concentration was constructed (Fig. 8). The additional abscissa axis is shown in this Figure: $x = [\alpha$-crystallin]/[BSA], where [α-crystallin] is the molar concentration of α-crystallin calculated on subunit and [BSA] is the molar concentration of BSA. In the interval of $x$ values from 0 to $x_1 = 0.17$ the dependence of $(k_{\text{agg}}/k_{\text{agg},0})^{1/n}$ on $x$ is linear. Using Eq. (12) we estimated the stoichiometry of the initial complexes α-crystallin–target protein: $S_0 = 0.40 \pm 0.01$ α-crystallin subunits per one BSA molecule. Knowing the $S_0$ value, we can calculate the initial adsorption capacity of α-crystallin with respect to the target protein: $\alpha C_0 = 1/ S_0 = 2.50 \pm 0.06$ BSA monomers per one α-crystallin subunit. At $x > x_1$ the dependence of $(k_{\text{agg}}/k_{\text{agg},0})^{1/n}$ on $x$ becomes non-linear and follows the hyperbolic law (Eq. (13)) in the interval of the $x$ values from $x_1$ to $x_2$.
values from \( x_1 = 0.17 \) to \( x_2 = 2.6 \). Fitting of Eq. (13) to the experimental data gave the following values of parameters: \( Y_0 = 0.94 \pm 0.17 \) and \( x_0.5 = 0.093 \pm 0.029 \). In accordance with Eq. (18), the AC value (the adsorption capacity of \( \alpha \)-crystallin with respect to the target protein) decreases from 2.33 to 0.19 BSA monomers per one \( \alpha \)-crystallin subunit in the interval of \( x \) values from \( x_1 = 0.17 \) to \( x_2 = 2.6 \) (inset in Fig. 8). It should be noted that at \( x > x_2 = 2.6 \) \( \alpha \)-crystallin is incapable of completely suppressing DTT-induced BSA aggregation.

When studying the effect of cross-linked \( \alpha \)-crystallin on DTT-induced aggregation of BSA (Fig. 9A), we also used Eq. (7) for calculation of parameter \( k_{\text{agg}} \) (Fig. 9B). Fig. 10 shows the dependence of \( (k_{\text{agg}} / k_{\text{agg,0}})^{1/n} \) on the concentration of cross-linked \( \alpha \)-crystallin or \( x = [\alpha \text{-crystallin}]/[\text{BSA}] \). As can be seen from this Figure, the noted dependence is linear. Using Eq. (12) allowed us to determine the stoichiometry of the \( \alpha \)-crystallin–target protein complex (\( S_0 = 4.7 \pm 0.1 \) \( \alpha \)-crystallin subunits per one BSA molecule) and adsorption capacity of \( \alpha \)-crystallin (\( AC_0 = 0.212 \pm 0.004 \) BSA monomers per one \( \alpha \)-crystallin subunit). It is significant that \( S_0 \) and \( AC_0 \) remain constant at variation of the \([\alpha \text{-crystallin}]/[\text{BSA}]\) ratio.

Study of DTT-Induced Aggregation of BSA by Asymmetric Flow Field Flow Fractionation (A4F)

Fig. 11 shows the elution profile of BSA (1 mg/ml; 25°C) registered as a time-dependence of UV detector signal. As one can see, the sample profile runs broad range of elution times indicating the polydispersity of the size of BSA particles: three distinct peaks appear in this fractogram. According to the values of the molar mass calculated from the MALS data, the detected peaks correspond to the monomeric (\( M = 64.5 \) kDa), dimeric (\( M = 101.2 \) kDa) and trimeric forms of BSA. Peak deconvolution

Figure 8. Initial rate of DDT-induced aggregation of BSA as a function of \( \alpha \)-crystallin concentration (45°C; 2 mM DTT). The dependence of \( (k_{\text{agg}} / k_{\text{agg,0}})^{1/n} \) on \( \alpha \)-crystallin concentration (lower abscissa axis) or the ratio of the molar concentrations of \( \alpha \)-crystallin and BSA (upper abscissa axis \( x = [\alpha \text{-crystallin}]/[\text{BSA}] \); \( n = 1.6 \)). Points are the experimental data. The solid line in the interval of \( x \) values from 0 to \( x_1 = 0.17 \) was calculated from Eq. (12) at \( S_0 = 0.40 \) subunits of \( \alpha \)-crystallin per one BSA molecule. The solid line in the interval of \( x \) values from \( x_1 = 0.17 \) to \( x_2 = 2.6 \) was calculated from Eq. (13) at \( Y_0 = 0.94 \) and \( x_0.5 = 0.093 \). The inset shows the dependence of the adsorption capacity (AC) of \( \alpha \)-crystallin with respect to the target protein on \( x \).

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Figure 9. Effect of cross-linked \( \alpha \)-crystallin on DTT-induced aggregation of BSA. (A) The dependence of the light scattering intensity on time obtained at the following concentrations of cross-linked \( \alpha \)-crystallin: (1) 0, (2) 0.1 and (3) 1.0 mg/ml ([BSA] = 1.0 mg/ml; 2 mM DTT; 0.1 M phosphate buffer, pH 7.0; 45°C). \( I \) and \( I_0 \) are the current and initial values of the light scattering intensity, respectively. (B) The dependence of \( dI/dt \) on time at concentration of cross-linked \( \alpha \)-crystallin 0.05 mg/ml. Points are the experimental data. The solid curve was calculated from Eq. (7).

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Figure 10. Initial rate of DDT-induced aggregation of BSA at 45°C as a function of cross-linked \( \alpha \)-crystallin concentration. The solid line was calculated from Eq. (12) at \( S_0 = 4.7 \) subunits of \( \alpha \)-crystallin per one BSA molecule. The dotted line corresponds to the dependence of \( (k_{\text{agg}} / k_{\text{agg,0}})^{1/n} \) on concentration of intact \( \alpha \)-crystallin (\( n = 1.6 \)).

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gives the following values for the portions of monomer, dimer and trimer: 0.85, 0.14 and 0.01, respectively.

Fig. 12 demonstrates the fractograms of BSA heated at 45°C in the presence of 2 mM DTT for different intervals of time (20, 45 and 90 min). Based on the measurements of the area under fractograms, we have constructed the dependence of the portion of the non-aggregated protein (c_{non-agg}) on time (Fig. 13). Analysis of the data shows that the dependence of c_{non-agg} on time obeys the exponential law of the following type:

\[ c_{non-agg} \sim \exp^{-k_1(t-t_0)/C_1} \]  

where \( k_1 \) is the rate constant of the first order and \( t_0 \) is the duration of the lag period. Fitting of the experimental data to this equation gives the following values of parameters: \( t_0 = 6.2 \pm 0.6 \) min and \( k_1 = 0.027 \pm 0.001 \) min\(^{-1}\). Thus, at \( t > t_0 \) the decrease of the portion of the non-aggregated protein in time follows the exponential law with \( k_1 = 0.027 \) min\(^{-1}\). As expected, the \( t_0 \) value calculated from Eq. (22) (\( t_0 = 6.2 \pm 0.6 \) min) is close to the duration of the lag period determined from the kinetic curve of aggregation at [BSA] = 1 mg/ml (\( t_0 = 5.4 \pm 0.2 \) min).

The exponential decrease in the portion of the non-aggregated protein in time seemingly indicates that any monomolecular stage (conformational transition or protein unfolding) is the rate-limiting stage of the aggregation process. However, our data show that the rate constant \( k_1 \) depends on the initial protein concentration. For example, at [BSA] = 2 mg/ml the \( k_1 \) value was found to be 0.036 \( \pm 0.001 \) min\(^{-1}\) (data not presented). This means that a two-fold increase in the protein concentration results in the increase of the \( k_1 \) value by the factor of 1.32 \( \pm 0.04 \). Thus, based on the data on BSA aggregation kinetics, where the order with respect to protein was found to be 1.6, and data on AF4 we may conclude that DTT-induced aggregation of BSA can not be classified as a process with monomolecular rate-limiting stage.

Figure 11. AF4-MALS analysis of BSA (1 mg/ml) at 25°C (0.1 M phosphate buffer, pH 7.0). Molar mass versus elution time plot (points) is overlaid on the UV detector fractogram (solid line). AF4 conditions: axial (detector) flow 1 ml/min, focus flow 5 ml/min, cross flow 5 ml/min for 14 min and then linear decay to 0.1 ml/min within 20 min plus 8 min at 0 ml/min. doi:10.1371/journal.pone.0074367.g011

Study of Interaction of BSA Unfolded in the Presence of DTT with \( \alpha \)-Crystallin by Analytical Ultracentrifugation

Additional information on the interaction of BSA unfolded in the presence of DTT with \( \alpha \)-crystallin was obtained by analytical ultracentrifugation. Before analyzing the mixtures of BSA and \( \alpha \)-crystallin we studied the sedimentation behavior of intact and cross-linked \( \alpha \)-crystallin heated at 45°C for 1 h in the presence of 2 mM DTT. The general \( s^* \) distribution for heated \( \alpha \)-crystallin (Fig. 14A), besides the major peak with \( s_{20,w} = 19.4 \) S, revealed two minor peaks (\( s_{20,w} = 15 \) and 22.3 S). As in the case of intact \( \alpha \)-crystallin, the increase in the protein concentration results in the increase of the \( s^* \) value by the factor of 1.32 \( \pm 0.04 \). Thus, based on the data on BSA aggregation kinetics, where the order with respect to protein was found to be 1.6, and data on AF4 we may conclude that DTT-induced aggregation of BSA can not be classified as a process with monomolecular rate-limiting stage.

Figure 12. Fractograms of BSA (1 mg/ml) heated at 45°C in the presence of 2 mM DTT. The heating times were the following: 20 (A), 45 (B) and 90 (D) min. AF4 conditions were the same as described in legend to Fig. 11. doi:10.1371/journal.pone.0074367.g012

Figure 13. Decrease in the portion of non-aggregated protein (c_{non-agg}) in the course of DTT-induced aggregation of BSA (1 mg/ml) at 45°C. Points are the experimental data. The solid curve was calculated from Eq. (22) at \( t_0 = 6.2 \) min and \( k_1 = 0.027 \) min\(^{-1}\). doi:10.1371/journal.pone.0074367.g013
crystallin, cross-linked α-crystallin contained a set of oligomeric forms with the major species with $s_{20,w} = 22$ S (Fig. 14B). It should be noted that small oligomers with $s_{20,w} < 21$ S were lacking.

Fig. 15 shows the $c(s)$ distribution for the mixtures of BSA (1 mg/ml) and α-crystallin at various concentrations (0.05, 0.1 and 0.4 mg/ml). The mixtures were heated at 45°C for 1 h. It is noteworthy that in the case of a mixture of BSA and α-crystallin at the concentration of 0.05 mg/ml (see Fig. 15, red line) the $c(s)$ distribution did not exhibit species for unbound α-crystallin due to its small concentration. A comparison of distributions for BSA (dotted line) and mixture of BSA with α-crystallin (0.05 mg/ml; red line) suggests that the broad peak with average sedimentation coefficient 10.7 S for the mixture corresponds to the complex of α-crystallin. The red line) suggests that the broad peak with average sedimentation coefficient 10.7 S for the mixture corresponds to the complex of α-crystallin and BSA. It is important to note that the complexes with $s_{20,w}$ in the range 6.8–14.5 S may correspond to the BSA–α-crystallin complexes. At the highest concentration of α-crystallin (0.4 mg/ml) the peak with $s_{20,w} = 16.1$ S in $c(s)$ distribution may correspond to the unbound chaperone and its complex with BSA. It is important to note that the complexes with $s_{20,w}$ in the range 6.8–14.5 S were formed by dissociated species of α-crystallin and BSA (compare $c(s)$ distributions for mixtures with $c(s)$ distribution data for α-crystallin in Fig. 16A, where species with $s_{20,w}$ smaller than 15 S were lacking).

It was interesting to study the anti-aggregation ability of α-crystallin in the case of long-term exposure to 45°C. The protective effect of α-crystallin heated with BSA at 45°C for 3.5 h is demonstrated in Fig. 16. Comparison of the $s_{20,w}$ distribution for BSA with that for the mixture of BSA and α-crystallin revealed that samples with $s_{20,w}$ exceeding 50 S were lacking in the $s_{20,w}$ distribution for the mixture (Fig. 16C). Thus, the comparison of the sedimentation profiles of BSA in the absence (A) and in the presence of α-crystallin (B) and $s_{20,w}$ distributions obtained from these data is indicative of the anti-aggregation effect of α-crystallin.

We also studied the interaction of BSA (1 mg/ml) with cross-linked α-crystallin (0.05 mg/ml) at 45°C. The $c(s)$ distribution revealed two main peaks with $s_{20,w}$ equal to 5.3 and 19.2 S (Fig. 17). We supposed that the major peak with 5.3 S corresponds to BSA. It will be noted that the $c(s)$ data do not reveal species corresponding to unbound cross-linked α-crystallin. Cross-linked α-crystallin does not contribute to sedimentation profiles due to its low concentration (0.05 mg/ml). Analysis of the $c(s)$ plots in Figs. 14B, 15 (dotted line) and 17 allowed us to conclude that the peak at 19.2 S in Fig. 17 corresponded to the complex of BSA with cross-linked α-crystallin. Samples with $s_{20,w}$ in the range 6.8–14 S were lacking (Fig. 17). Thus, in the case of cross-linked α-crystallin the complexes with dissociated forms of α-crystallin were not formed.

Effect of Chemical Chaperones on DTT-Induced Aggregation of BSA

Figs. 18A–20A demonstrates suppression of DTT-induced aggregation of BSA by Arg, ArgAd and Pro. As it can be seen from Figs. 18B–20B, where the dependences of the hydrodynamic radius ($R_h$) of the protein aggregates on time are represented, the protective action of the chemical chaperones is connected with the formation of the protein aggregates of lesser size. The values of parameters $k_{agg}$ and $l_0$ calculated from Eq. (5) at various
concentrations of the chemical chaperones are given in Figs. 18C–20C. It should be noted that we do not use Eq. (3) for some kinetic curves, because the extended form of this equation (Eq. (5)) gives a better approximation. To illustrate the expedience of using Eq. (5), the curves calculated from Eqs. (3) and (5) were compared (Fig. 19, 75 mM ArgAd). As can be seen, using Eq. (5) allows us to describe the more extended part of the kinetic curve.

To analyze the dependences of $k_{\text{agg}}$ on the chemical chaperones concentration, we have used the Hill equation (Eq. (20)). Parameters $[L]_{0.5}$ and $h$ calculated from this equation are given in Table 2. The Table also contains the values of the coefficient of determination ($R^2$) characterizing the degree of agreement between the experimental data and calculated values. In the case of Pro, the Hill coefficient is equal to unity. However, for Arg, ArgEE and ArgAd the Hill coefficient exceeds unity ($h = 1.6, 1.9$ and 2.5, respectively), suggesting that there are positive cooperative interactions between the chaperone-binding sites in the target protein molecule [96]. Parameter $[L]_{0.5}$ characterizes the affinity of the chaperone to the target protein. As it can be seen from Table 2, among the chaperones studied ArgEE and ArgAd reveal the highest affinity. As for the duration of the lag-period, the increase in the $t_0$ value is observed with increasing the chaperone concentration (see insets in Fig. 18C–20C).

Fig. 19C shows the dependences of $K_{\text{agg}}/K_{\text{agg,0}}$ ratio on the ArgAd concentration obtained at BSA concentrations equal to 0.5, 1 and 2 mg/ml. The $K_{\text{agg}}/K_{\text{agg,0}}$ values corresponding to different BSA concentrations fall on the common curve. This result is consistent with the theoretical considerations.

**Combined Action of $\alpha$-Crystallin and Chemical Chaperones**

In accordance with the principles of analysis of the combined action of protein and chemical chaperones given in the Section “Theory. Quantification of the Chaperone-Like Activity” the following experiments were performed to characterize the mutual inhibitory effects of $\alpha$-crystallin and Arg. We constructed the $k_{\text{agg}}^{-1/\phi}$ on the $[\alpha$-crystallin]/[BSA] ratio plots for BSA aggregation studied in the absence and in the presence of Arg (Fig. 21).
The AC0 value for α-crystallin was estimated from the initial linear parts of the dependences of $k_{agg}^{1/n}$ on the [α-crystallin]/[BSA] ratio. The initial adsorption capacity of α-crystallin in the absence of Arg was found to be $2.48\pm0.04$ BSA monomers per one α-crystallin subunit. The same value of AC0 was obtained in the presence of 100 mM Arg (AC0 = $2.46\pm0.02$ BSA monomers per one α-crystallin subunit). Thus in the test-system under study α-crystallin and Arg act independently of one another.

When analyzing the combined action of chemical chaperones, parameter $j$ [see Eq. (21)] may be used to characterize the interaction between chemical chaperones. As an example we study the combined action of ArgEE and Pro. At [ArgEE] = 50 mM the degree of target protein aggregation inhibition $i_1 = 1 - k_{agg}/k_{agg,0}$ was found to be $0.48\pm0.09$. At [Pro] = 800 mM the degree of inhibition ($i_2$) was $0.42\pm0.11$. The degree of inhibition for the ArgEE (50 mM)+Pro (800 mM) mixture ($i_{1,2}$) was $0.73\pm0.13$. Parameter $j$ calculated from Eq. (21) was found to be $1.03\pm0.12$. $$j = \frac{k_{agg}^{1/n} [Arg]}{k_{agg,0}^{1/n}}$$
Thus, the action of one chemical chaperone is not dependent on the presence of the other.

**Discussion**

The kinetic data obtained in the present work allow us to discuss the mechanism of DTT-induced aggregation of BSA. Taking into account the data on BSA microheterogeneity caused by intramolecular disulfide interchange reactions [64], we can propose the following kinetic scheme of the aggregation process:

\[
P_{nr} \xrightarrow{R} P_r \xrightarrow{\text{DTT}} P_{red} \xrightarrow{U} \text{aggregates} \]

The first stage is the conformational transition of the initial BSA molecules with sterically hidden disulfide bonds \((P_{nr})\) into the form \(P_r\) in which disulfide bonds become accessible to the attack by DTT. Next stages are reduction of disulfide bonds \((P_{red}\) is the BSA molecule with reduced disulfide bonds\), unfolding of the protein molecule \((P_U\) is the unfolded protein\) and aggregation of the unfolded protein molecules.

When studying the kinetics of DTT-induced aggregation of \(\alpha\)-lactalbumin and insulin, we showed that the initial stage of the aggregation process was the stage of formation of the start aggregates with \(R_h\) of 80–100 nm [18,84]. No intermediate states between the non-aggregated protein and the start aggregates were detected. This means that the formation of the start aggregates proceeds on the all-or-none principle. Further growth of the protein aggregates occurs as the result of sticking of the start aggregates. The size of the start aggregates is independent of the concentration of the protein involved in aggregation. Thus, the formation of the start aggregates is analogous to the process of micelle formation. In the latter case the micelles of a definite size are formed when the critical monomer concentration is achieved. Such an analogy offers an explanation of why the formation of start aggregates proceeds according to the all-or-none principle. It should be noted that the duration of the lag period \((t_0)\) for the kinetic curves of DTT-induced aggregation of BSA is about 10 min. The lag period is followed by a period of rapid aggregation.

**Figure 20. Effect of Pro on DTT-induced aggregation of BSA \(([BSA]=1.0 \text{mg/ml}, \text{2 mM DTT})\).** (A) The dependences of the light scattering intensity on time obtained at the following concentrations of Pro: (1) 0, (2) 500 and (3) 1000 mM. Points are the experimental data. The solid curves were calculated from Eq. (5). (B) The dependences of the hydrodynamic radius \((R_h)\) of the protein aggregates on time obtained in the absence of Pro (1) and in the presence of 1000 mM Pro (2). The dependence of the \(K_{agg}/K_{agg,0}\) ratio on the concentration of Pro. Points are the experimental data. The solid curve was calculated from Eq. (20). Inset shows the dependence of the duration of the lag period \((t_0)\) on the concentration of Pro.

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Thus, the action of one chemical chaperone is not dependent on the presence of the other.

**Table 2. The values of parameters of Eq. (20) for suppression of DTT-induced aggregation of BSA by chemical chaperones \((R^2\) is the coefficient of determination).**

| Ligand | \([L]_0, \text{mM}\) | \(h\) | \(R^2\) |
|--------|-------------------|------|--------|
| Arg    | 116±6             | 1.6±0.2 | 0.9871 |
| ArgEE  | 53±2              | 1.9±0.2 | 0.9596 |
| ArgAd  | 58±2              | 2.5±0.2 | 0.9772 |
| Pro    | 800±120           | 1.0±0.1 | 0.9203 |

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The first stage is the conformational transition of the initial BSA molecules with sterically hidden disulfide bonds \((P_{nr})\) into the form \(P_r\) in which disulfide bonds become accessible to the attack by DTT. Next stages are reduction of disulfide bonds \((P_{red}\) is the BSA molecule with reduced disulfide bonds\), unfolding of the protein molecule \((P_U\) is the unfolded protein\) and aggregation of the unfolded protein molecules.

**Figure 21. Analysis of combined action of \(\alpha\)-crystallin and Arg.** The dependences of \((k_{agg})^{1/0}\) on the \([\alpha\)-crystallin]/[BSA] ratio in the absence (squares) and in the presence of 100 mM Arg (circles). Conditions: \([BSA]=1 \text{mg/ml}, [\text{DTT}]=2 \text{mM}, 0.1 \text{M Na-phosphate buffer pH 7.0, 45°C.}\)

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Thus, the action of one chemical chaperone is not dependent on the presence of the other.
DTT-induced aggregation of \( \alpha \)-lactalbumin tends to decrease with increasing protein concentration and reaches a constant value at rather high concentrations of \( \alpha \)-lactalbumin [10].

As in the case DTT-induced aggregation of \( \alpha \)-lactalbumin, aggregation of BSA in the presence of DTT is characterized by the decrease in the \( k_0 \) value with increasing protein concentration (Fig. 6B). Such a peculiarity of the aggregation kinetics indicates that DTT-induced aggregation of BSA proceeds by a mechanism of nucleation-dependent aggregation [76,102–106]. However, in contrast to \( \alpha \)-lactalbumin and insulin, nuclei formed in the course of DTT-induced aggregation of BSA are not capable of assembling into start aggregates. As can be seen in Fig. 3B, there is a monotonous increase in the average value of \( R_0 \) of the aggregates in the course of aggregation process without separation of non-aggregated and aggregated forms of BSA.

In the present work the rigorous methods for estimation of the anti-aggregation activity of protein and chemical chaperones have been elaborated. When comparing the protective action of protein chaperones, the initial adsorption capacity of the chaperone with respect to the target protein \( \Delta C_0 \) can be used as a measure of the anti-aggregation activity of chaperones. Taking into account the \( \Delta C_0 \) values for intact and cross-linked \( \alpha \)-crystallin (2.50 and 0.212 BSA monomers per one -crystallin subunit, respectively), we can say that cross-linking of \( \alpha \)-crystallin results in 11.8-fold decrease in the chaperone-like activity.

It is well known that small heat shock proteins (sHsp) tend to form large oligomers with molecular mass up to 1000 kDa. sHsp oligomers possess high mobility. There are numerous experimental data demonstrating high rate of subunit exchange between oligomers formed by sHsp [6,107,108]. The complexes between sHsp and target protein are characterized by high degree of polydispersity [109,110]. For example, when studying the interaction of Hsp18.1 with firefly luciferase denatured at 42°C by tandem mass spectrometry, Stengel et al. [8] discovered more than 300 Hsp-client protein complexes with different stoichiometry. These complexes are not static entities and can continue to incorporate additional amounts of target protein [109] [8,110]. Moreover, the Hsp subunits continue to exchange with free sHsp and sHsp–target protein complexes. By contrast, target proteins appear unable to transfer from one complex to another [110].

Since complexion of sHsp with target proteins does not result in the formation of complexes with constant stoichiometry, one may expect that the initial rate of aggregation versus the \([sHsp]/[BSA]\) ratio plot will be non-linear. It has been just such a plot which was obtained for suppression of DTT-induced aggregation of BSA by \( \alpha \)-crystallin (Fig. 8). That the dependence of the aggregation initial rate of the \([sHsp]/[BSA]\) ratio is of a non-linear character can be interpreted as a decrease in the absorption capacity on \( \alpha \)-crystallin with respect to unfolded BSA as the \([sHsp]/[BSA]\) ratio increases. It is evident that fixation of the quaternary structure of \( \alpha \)-crystallin by cross-linking should yield a linear dependence of initial rate of aggregation on the \([sHsp]/[BSA]\) ratio, because in this case monodisperse \( \alpha \)-crystallin–target protein complexes are formed. Actually, as can be seen from Fig. 10, the initial rate of aggregation on the \([sHsp]/[BSA]\) ratio plot is linear. Thus, these data support the idea that non-linear character of the dependence of initial rate of aggregation on sHsp concentration is due to dynamic mobility of quaternary structure of sHsp assemblies and polydispersity of the \( \alpha \)-crystallin–target protein complexes.

When comparing the protective action of chemical chaperones, the semi-saturation concentration \([L]_{0.5}\) can be used as a measure of the anti-aggregation activity of chaperones. The lower the \([L]_{0.5}\) value, the higher is the protective power of the chaperone. For example, taking into account the \([L]_{0.5}\) values for Arg and Pro (116 and 800 mM, respectively), we may assert that the protective action of Arg is 7 times higher than that for Pro.

Since chaperones of different classes participate in protein quality control system, we should have the corresponding quantitative methods for estimation of the effects of their combined action at our disposal. The mathematical apparatus described in the Section “Theory. Quantification of the Chaperone-Like Activity” allows us to quantitatively characterize the combined action of the agents possessing the anti-aggregation activity (for example, protein and chemical chaperones).

The data obtained in this study substantiate the use of the test system based on DTT-induced aggregation of BSA for the quantitative estimation of the protective effect of the agents possessing anti-aggregation activity. Parameter \( k_{agg} \) is used to characterize the initial rate of aggregation measured at different concentrations of the agent under study. The construction of the \( k_{agg} \) versus the agent concentration plot allows determining parameters, which characterize the protective efficiency of the agent: the adsorption capacity with respect to the target protein \( \Delta C_0 \) for protein chaperones and the semi-saturation concentration \([L]_{0.5}\) for chemical chaperones. Thus, the test system proposed in the present paper may be used for sampling the agents, which reveal a high protective efficiency and may find application in biotechnological and medical investigations [111].

### Author Contributions

Conceived and designed the experiments: VAB KAM BIK. Performed the experiments: VAB DAK NAC VFM. Analyzed the data: VAB KAM DAK NAC BIK. Contributed reagents/materials/analysis tools: NBP KOM. Wrote the paper: KAM BIK. Engaged in active discussion: VAB KAM NAC BIK.

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