A Protein Aggregation Based Test for Screening of the Agents Affecting Thermostability of Proteins

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Abstract

To search for agents affecting thermal stability of proteins, a test based on the registration of protein aggregation in the regime of heating with a constant rate was used. The initial parts of the dependences of the light scattering intensity (I) on temperature (T) were analyzed using the following empiric equation: \( I = I_{\text{lim}} \exp\left(\frac{T_{\text{agg}} - T}{B}\right)^{-1} \), where \( I_{\text{lim}} \) is the parameter characterizing the initial rate of aggregation and \( T_{\text{agg}} \) is a temperature at which the initial increase in the light scattering intensity is registered. The aggregation data are interpreted in the frame of the model assuming the formation of the start aggregates at the initial stages of the aggregation process. Parameter \( T_{\text{agg}} \) corresponds to the moment of the origination of the start aggregates. The applicability of the proposed approach was demonstrated on the examples of thermal aggregation of glycogen phosphorylase \( b \) from rabbit skeletal muscles and bovine liver glutamate dehydrogenase studied in the presence of agents of different chemical nature. The elaborated approach to the study of protein aggregation may be used for rapid identification of small molecules that interact with protein targets.

Introduction

Senisterra and coworkers [1,2] elaborated a high-throughput light-scattering-based method for screening of ligands specifically interacting with protein targets. Thermal protein denaturation is used to characterize the binding of ligands to their target protein. This method is based on the assumption that the proteins under study irreversibly denature and form aggregates during thermal denaturation. Light scattering as a measure of protein aggregation is a very sensitive technique. Protein aggregation is studied in the regime of heating with a constant rate. The dependence of the light scattering intensity on temperature has a sigmoid shape. At rather high temperatures the light scattering intensity \( I \) reaches a limiting value \( I_{\text{lim}} \). To characterize thermostability of a protein, Senisterra and coworkers used the temperature \( T_{\text{agg}} \) corresponding to the middle point of the transition, i.e., a temperature at which \( I = I_{\text{lim}}/2 \). Parameters \( I_{\text{lim}} \) and \( T_{\text{agg}} \) are determined by fitting of the experimental dependence of \( I \) on temperature with the following empiric equation, analogous to the Boltzmann equation:

\[
I = \frac{I_{\text{lim}}}{1 + \exp\left(\frac{T_{\text{agg}} - T}{B}\right)};
\]

where \( B \) is a constant.

According to the idea advanced by Senisterra and coworkers a change in the \( T_{\text{agg}} \) value in the presence of a ligand characterizes the effect of the latter on protein thermostability. To substantiate this conclusion, the authors additionally used differential scanning calorimetry (DSC), which is a source of direct information on the protein resistance to high-temperature exposure. The authors also constructed a plot demonstrating the existence of a correlation between the \( T_{\text{agg}} \) value and the position of the maximum on the DSC profiles \( T_m \).

It is evident that the accuracy of determining parameter \( T_{\text{agg}} \) is connected with the reliability of the estimation of parameter \( I_{\text{lim}} \). When trying to estimate parameter \( I_{\text{lim}} \) we should take into account that the “true” limiting level of the light scattering intensity may not be reached because of precipitation of the large-sized aggregates formed at high temperatures. Such a precipitation results in the decrease in the light scattering intensity, and the real experimental dependence of \( I \) on temperature looks like a curve passing through a maximum. The maximum value of the light scattering intensity may be lower than the \( I_{\text{lim}} \) value calculated from Eq. (1). Besides, the correlation between the increment of the light scattering intensity and the degree of protein denaturation should be controlled not only by checking the correlation between parameters \( T_{\text{agg}} \) and \( T_m \) but by stricter analysis of the relationship between turbidimetric data and calorimetric data, supplying direct information on the degree of protein denaturation.

To avoid the uncertainty in the estimation of parameter \( I_{\text{lim}} \), in the present work we proposed new parameters, which characterize the rate of aggregation. To determine these parameters, there is no
need for the full dependences of the light scattering intensity on temperature, since the proposed parameters (the initial temperature of aggregation and the parameter characterizing the rate of change in the light scattering intensity with temperature) are calculated from the initial parts of the dependences of $I$ on temperature. The use of these parameters allows us to obtain the quantitative characteristics of the effect of the agents to be tested on the rate of aggregation. Since the initial stage of thermal aggregation of the proteins is the stage of denaturation, the ligands under study involve agents affecting both the stage of denaturation and the stage of aggregation. To demonstrate the applicability of the proposed approach, thermal aggregation of glycogen phosphorylase $b$ (Ph$b$; EC 2.4.1.1) from rabbit skeletal muscles and bovine liver glutamate dehydrogenase (GDH; EC 1.4.1.3) were used as examples.

Mathematical description of the initial parts of the dependences of the light scattering intensity on temperature

As a preliminary step we discuss the kinetics of thermal aggregation of the proteins registered at a fixed temperature. To analyze the initial parts of the dependences of the light scattering intensity ($I$) on time, the following empiric equation was proposed [3]:

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

In this equation $K_{agg}$ is a constant with the dimension of (counts/ s)$\cdot$min$^{-2}$ and $t_0$ is the duration of the lag period ($t_0$ is a point in time at which the light scattering intensity begins to increase). The applicability of Eq. (2) was demonstrated for thermal aggregation of Ph$b$ [3–5], glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) [6] and creatine kinase (CK; EC 2.7.3.2) from rabbit skeletal muscles [7]. Parameter $K_{agg}$ characterizes the initial rate of aggregation, and the construction of a dependence of parameter $K_{agg}$ on the initial protein concentration ($P_0$) allows calculating the order of aggregation with respect to the protein ($n$) ($K_{agg} = const \cdot P_0^n$). The dependence of parameter $K_{agg}$ on the Ph$b$ concentration constructed on the basis of kinetic data for Ph$b$ aggregation at 53°C (pH 6.8) was linear [3], and consequently $n = 1$. This is true when the stage of unfolding of a protein molecule proceeds with a substantially lower rate than the following stages of aggregation of the unfolded protein molecules [8,9]. In other words, the rate-limiting stage of the overall process of aggregation is the monomolecular stage of protein unfolding. According to the data presented by Kurganov et al. [10] the kinetic scheme of thermal denaturation of Ph$b$ involves a stage of the conformational change of the enzyme dimeric molecule followed by dissociation of the dimer into monomers and denaturation of labile monomeric forms.

The kinetics of thermal aggregation of GDH at various concentrations of the protein was studied by Sabbaghian et al. [11] (50°C; pH 8.0). The order of aggregation with respect to the protein calculated on the basis of these kinetic data is close to unity: $n = 0.8 \pm 0.1$.

The analysis of the data on thermal aggregation of $\beta_{l}$-crystallin from bovine lens at 60°C (pH 6.8) obtained in [12] shows that parameter $n$ is close to 2 ($n = 1.99 \pm 0.07$). It is generally accepted that the initial stage of aggregation is the stage of nucleation [3,8,9,13–19]. The fact that the value of parameter $n$ for thermal aggregation of $\beta_{l}$-crystallin exceeds unity is indicative of the involvement of several molecules of denatured $\beta_{l}$-crystallin in the nucleation process.

Consider a situation when Eq. (2) may be strictly fulfilled. The theoretical analysis carried by Ferrone [16] shows that for the nucleation-dependent aggregation the accumulation of monomers incorporated in the aggregate ($\Delta$) proceeds according to the time-squared law:

$$\Delta = \frac{1}{2}JJ + ct^2,$$

where $J$ is the elongation rate of aggregates, $\theta^*$ is the elongation rate of the nucleus and $c^*$ is the concentration of the nuclei. Such an early kinetics of aggregation was demonstrated experimentally, for example, for aggregation of polyglutamine peptides [20], aggregation of a slow-folding mutant of a $\beta$-clam protein, cellular retinoic acid-binding protein (P39A CRABP I) [21], and aggregation of protein L [22].

When studying the kinetics of thermal aggregation of some proteins by dynamic light scattering (DLS) we have shown that the hydrodynamic radius ($R_h$) of the initially registered aggregates (the start aggregates) amounts to tens of nanometers [23]. This means that a start aggregate contains hundreds of denatured protein molecules. We could not detect intermediate states between the original forms of the proteins and the start aggregates. It should be noted that rather low temperatures were selected for the study of protein aggregation in this investigation (37°C for Ph$b$ and 45°C for GAPDH). The nuclei formed at the initial stages of aggregation involve several monomers. Therefore one can assume that there is no accumulation of nuclei in the system, and coalescence of the formed nuclei into larger structures, namely start aggregates, takes place. The initial increase in the light scattering intensity is connected with accumulation of the start aggregates, the size of the latter remaining unchanged (the hydrodynamic radius of the start aggregates was designated by $R_{h,0}$). It is evident that in the time interval where the $R_h$ value is constant ($R_h \approx R_{h,0}$; see [23]) the light scattering intensity is strictly proportional to the amount of the aggregated protein. Thus, in this case Eq. (2) may be used for the quantitative description of the early kinetics of the aggregation process, and parameter $K_{agg}$ is a true measure of the initial rate of aggregation. When protein aggregation is studied at elevated temperatures, the time interval corresponding to the accumulation of the start aggregates is greatly reduced, and the registered increase in the hydrodynamic radius of the protein aggregates at $T > T_0$ is due to the attachment of individual denatured protein molecules to the start aggregates or the sticking of the start aggregates. This results in the additional increment in the light scattering intensity apart from the main contribution accounted for the formation of the start aggregates. Eq. (2) should be treated as an empiric equation where parameter $K_{agg}$ is a rough characteristic of the initial aggregation rate.

When studying the dependences of the hydrodynamic radius of protein aggregates on temperature obtained in the experiments with heating of protein solutions with a constant rate, we observed that the character of these dependences is identical to that for the dependences of the hydrodynamic radius of protein aggregates on time obtained at a fixed temperature [24–26]. Therefore we propose to use the following equation analogous to Eq. (2) for analysis of the initial parts of the dependences of the light scattering intensity on temperature obtained in the regime of heating with a constant rate:

$$I = K_{agg}(T - T_0)^{2} \quad (T > T_0) \quad (4)$$

In this equation $T_0$ is the initial temperature of aggregation, i.e., the temperature at which the light scattering intensity begins to
increase, and $K_{agg}$ is a constant with the dimension of (counts/s)/C$^{-2}$. Parameter $T_0$ indicates the moment of originisation of the start aggregates, whereas parameter $K_{agg}$ characterizes the rate of aggregation. It was of interest to check the applicability of Eq. (4) for the analysis of protein aggregation in the regime of heating with a constant rate, and to clarify how parameters $T_0$ and $K_{agg}$ change with variation in the protein concentration.

**Materials and Methods**

Hepes, glucose 1-phosphate, AMP, trimethylamine N-oxide (TMAO), creatine kinase (CK) from rabbit skeletal muscles, bovine liver GDH, NAD, NADH, ADP, L-glutamate, L-arginine, and Y-crystallin were purchased from “Sigma” (USA). The concentrations of CK, GDH and Y-crystallin were determined from the absorbance at 280 nm using the extinction coefficients $A_{280}^{\text{nm}}$ of 8.8 [27], 9.7 [11] and 8.5 [28], respectively. NaCl was purchased from “Reakhim” (Russia), 2-hydroxypropyl-$\beta$-cyclodextrin (degree of substitution 3±1) (HP-$\beta$-CD) was obtained from CycloLab LTD (Hungary).

**Isolation of Phb**

Phb was isolated from rabbit skeletal muscles as described in [29]. Phb concentration was determined spectrophotometrically at 280 nm using the extinction coefficient $A_{280}^{\text{nm}}$ of 13.2 [30].

**Isolation of GAPDH**

GAPDH was isolated from rabbit skeletal muscles as described by Scopes and Stoter [31] with an additional purification step using gel-filtration on Sephadex G-100. GAPDH concentration was determined spectrophotometrically at 280 nm using the extinction coefficient $A_{280}^{\text{nm}}$ of 10.6 [32].

**Differential Scanning Calorimetry**

Thermal denaturation of Phb was investigated by DSC using the adiabatic scanning microcalorimeter DASM-4M (Institute of Biological Instruments, Russian Academy of Sciences, Pushchino, Russia) with 0.47 ml capillary platinum cells. All measurements were carried out at the rate of heating by 1°C/min using the temperature range from 20 to 85°C and constant pressure of 2.2 atm. The dependences of the heat power on temperature were calculated using the program Origin software (MicroCal, Inc., USA).

The capillary construction of calorimetric cells prevents the artifacts caused by protein precipitation, which are often observed in batch calorimetric cells as exothermic peaks. In the capillary cells large-sized aggregated particles of protein are unable to settle and stir the solution, so no extra thermal production can arise [33].

**Light Scattering Intensity Measurements**

DLS measurements were performed on a commercial instrument Photocor Complex (Photocor Instruments Inc., USA; www.photocor.com). An He-Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) was used as the light source. The temperature of sample cell was controlled by the proportional integral controller through the macro procedure of the Photocor program. Temperature scanning rate can be assigned from 10°C/min to any slower value.

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. DLS data have been accumulated and analyzed with a multifunctional real-time correlator, DynaLS software (Alango, Israel) was used for polydispersity analysis of DLS data. The diffusion coefficient $D$ of the particles is directly related to the decay rate $\tau_i$ of the time-dependent correlation function for the light-scattering intensity fluctuations: $D = 1/2\tau_i k_i^2$. In this equation $k_i$ is the wave number of the scattered light, $k_i = (4\pi n_i \lambda \sin(\theta/2))$, where $\eta$ is the refractive index of the solvent, $\lambda$ is the wavelength of the incident light in vacuum and $\theta$ is the scattering angle. The mean hydrodynamic radius of the particles, $R_h$, can then be calculated according to the Stokes-Einstein equation: $D = k_b T/6\pi \eta R_h$, where $k_b$ is Boltzmann’s constant, $T$ is the absolute temperature and $\eta$ is the shear viscosity of the solvent.

All solutions for light scattering experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead). The buffer was placed in a cylindrical cell with a diameter of 6.3 mm and incubated for 5 min at 25°C. Cells with stoppers were used for incubation at high temperature to avoid evaporation. The aggregation process was initiated by the addition of an aliquot of protein to a final volume of 0.5 ml. When studying the kinetics of aggregation of proteins, the scattering light was collected at 90° scattering angle.

Dimensions of the fast thermostat used for heating of the protein solution were the following: 15 mm (diameter) × 40 mm (height). Temperature gradients in such a thermostat are less than 0.1 degree. Notice that large temperature gradients in the sample cell would result in a developed convection of fluid. The correlation function of light, scattered on the convective flow, is quite different from the exponential correlation function in case of light scattering on diffusing particles. That is why we can reliably estimate the appearance of temperature gradients by analyzing measured correlation functions.

**Calculations**

OriginPro 8.0 SR0 software (OriginLab Corporation, USA) and Scientist software (MicroMath, Inc., USA) were used for the calculations. To characterize the degree of agreement between the experimental data and calculated values, we used the coefficient of determination $R^2$ (without considering the statistical weight of the measurement results) [34]:

$$R^2 = \frac{\sum_{i=1}^{n} (Y_{\text{obs}} - \overline{Y}_{\text{obs}})^2 - \sum_{i=1}^{n} (Y_{\text{obs}} - Y_{\text{calc}})^2}{\sum_{i=1}^{n} (Y_{\text{obs}} - \overline{Y}_{\text{obs}})^2},$$

where $\overline{Y}_{\text{obs}} = \frac{1}{n}\sum_{i=1}^{n} Y_i$ is the average of the experimental data $(Y_{\text{obs}})$, $Y_{\text{calc}}$ is the theoretically calculated value of the function $Y$ and $n$ is the number of measurements.

**Results and Discussion**

**Turbidimetric Registration of Phb Aggregation**

Thermal aggregation of Phb was studied in the regime wherein temperature was elevated with a constant rate (1°C/min). Fig. 1A
shows the dependences of the light scattering intensity on temperature for aggregation of Phb registered in the regime of heating with a constant rate (1°C/min). Conditions: 0.08 M Hepes-buffer, pH 6.8, containing 0.1 M NaCl. (A) The original experimental dependences. The Phb concentrations were as follows: (1) 0.95 and (2) 1.9 mg/ml. (B) The results of fitting the experimental curves to Eq. (1). Points are the experimental data. The solid curves are calculated from Eq. (1). The dotted horizontal lines correspond to the limiting values of the light scattering intensity ($I_{lim}$). doi:10.1371/journal.pone.0022154.g001

Denaturation of Phb Registered by DSC and Comparison of the Turbidimetric and Calorimetric Data

When studying thermal aggregation of Phb, we should take into account that the initial stage of the general process of aggregation is the stage of protein denaturation. To characterize Phb denaturation, we used DSC. Fig. 2 shows DSC profiles obtained at the Phb concentrations of 0.95 and 1.9 mg/ml (curves 1 and 2, respectively). The heating rate was 1°C/min. The increase in the light scattering intensity at rather high temperatures is due to precipitation of the large-sized aggregates. Fig. 1B demonstrates the results of fitting Eq. (1) to the experimental data. The following values of parameters were established: $I_{lim} = (0.84 \pm 0.01) \times 10^6$ counts/s, $T_{agg} = 56.1 \pm 0.1$, $B = 1.57 \pm 0.02$ ($R^2 = 0.999$) at the Phb concentration of 0.95 mg/ml and $I_{lim} = (1.05 \pm 0.01) \times 10^6$ counts/s, $T_{agg} = 55.5 \pm 0.1$, $B = 1.34 \pm 0.02$ ($R^2 = 0.999$) at the Phb concentration of 1.9 mg/ml.

Denaturation of Phb (0.08 M Hepes-buffer, pH 6.8, containing 0.1 M NaCl). The dependences of the excess heat capacity ($C_p^e$) on temperature, obtained at the following concentrations of Phb: (1) 0.95 and (2) 1.9 mg/ml. $C_p^e$ was calculated per dimer of Phb with the molecular mass of 194.8 kDa. The heating rate was 1°C/min. doi:10.1371/journal.pone.0022154.g002

Figure 2. Thermal denaturation of Phb (0.08 M Hepes-buffer, pH 6.8, containing 0.1 M NaCl). The dependences of the excess heat capacity ($C_p^e$) on temperature, obtained at the following concentrations of Phb: (1) 0.95 and (2) 1.9 mg/ml. $C_p^e$ was calculated per dimer of Phb with the molecular mass of 194.8 kDa. The heating rate was 1°C/min. doi:10.1371/journal.pone.0022154.g002

Figure 3. The relationships between the increment of the light scattering intensity accompanying Phb aggregation and the portion of the denatured Phb ($\gamma_{den}$) calculated from the DSC data. Phb concentrations were as follows: (1) 0.95 and (2) 1.9 mg/ml. The inset shows the initial parts of the curves. Points are the experimental data. The solid curves are calculated from Eq. (6). doi:10.1371/journal.pone.0022154.g003
Phb aggregation and the $\gamma_{\text{den}}$ value at two concentrations of Phb. It can be seen that the increase in the light scattering intensity ($I$) is not a linear function of $\gamma_{\text{den}}$. Thus, in the case of thermal aggregation of Phb the increment of the light scattering intensity cannot be considered as a direct measure of the degree of protein denaturation. It should be noted that in some cases a linear proportionality between the increase in the light scattering intensity and the degree of protein denaturation is really fulfilled. This, for example, was demonstrated by us for thermal aggregation of GAPDH [35].

The analysis of the relationship between the increment of the light scattering intensity ($I$) and the portion of the denatured Phb presented in Fig. 3 showed that at $\gamma_{\text{den}}<0.5$ the $I$ value was a power function of $\gamma_{\text{den}}$:

$$I = K \gamma_{\text{den}}^N.$$  \hspace{1cm} (6)

The following values of parameters $K$ and $N$ were found: $K=(4.0 \pm 0.1) \times 10^6$ counts/s and $N=1.32 \pm 0.02$ ($R^2 = 0.999$) at the Phb concentration of 0.95 mg/ml and $K=(6.6 \pm 0.1) \times 10^6$ counts/s and $N=1.34 \pm 0.02$ ($R^2 = 0.999$) at the Phb concentration of 1.9 mg/ml.

When comparing calorimetric and turbidimetric data obtained in the regime of heating with a constant rate, we should take into account the following features of the temperature dependences of excess heat capacity and the light scattering intensity. There is no threshold temperature on the DSC profile. The dependence of excess heat capacity on temperature asymptotically approaches zero level with decreasing temperature. The lower the temperature, the lower is the amount of the denatured protein accumulated to the moment when the given temperature has been achieved. On the contrary, an initial increase in the light scattering intensity is registered at definite temperature designated as $T_0$. According to the mechanism of thermal aggregation of proteins proposed in our works [12,23,35–37], the initial increase in the light scattering intensity corresponds to the moment of the origination of the start aggregates. The formation of the start aggregates proceeds on the all-or-none principle. The intermediate states between native enzyme forms (or denatured protein molecules) and start aggregates have not been detected.

### Analysis of the Initial Parts of the Dependences of the Light Scattering Intensity on Temperature

In the present work the focus is concentrated on the initial parts of the dependences of the light scattering intensity on temperature. Fig. 4A shows the influence of the protein concentration on the initial parts of the kinetic curves of aggregation of Phb heated with a constant rate of 1 °C/min. The Phb concentration was varied in the interval from 0.1 to 1.5 mg/ml. The experimental curves are satisfactorily described by the Eq. (4). The $R^2$ values lie in the range from 0.995 at [Phb] = 0.1 mg/ml to 0.990 at [Phb] = 1.5 mg/ml. The calculated values of parameters $T_0$ and $K_{\text{agg}}$ are represented in Fig. 4B and C as a function of the Phb concentration. Parameter $T_0$, characterizing the temperature at which the light scattering intensity begins to increase, decreases monotonously with increasing the concentration of Phb (Fig. 4B).

When interpreting the dependence of parameter $K_{\text{agg}}$, characterizing the rate of aggregation on the Phb concentration, we should be aware that this dependence can not be used for estimation of the order of aggregation with respect to the protein because the $K_{\text{agg}}$ values on the $K_{\text{agg}}$ versus the Phb concentration plot correspond, in fact, to different temperatures. It is of interest that at the Phb concentration higher than 0.5 mg/ml parameter $K_{\text{agg}}$ is a constant value, which is independent of the protein concentration (Fig. 4C). When the Phb concentration is lower than 0.5 mg/ml, decreasing the protein concentration results in the decrease in the $K_{\text{agg}}$ value.

It was of interest to investigate whether the character of the dependences of parameters $T_0$ and $K_{\text{agg}}$ on the protein concentration observed for Phb remains the same for other proteins. Fig. 5 shows the analysis of the initial parts of the

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**Figure 4. Analysis of the kinetics of Phb aggregation registered in the regime of heating at the rate of 1 °C/min.** (A) The initial parts of the dependences of the light scattering intensity on temperature obtained at the following concentrations of Phb: (1) 0.1, (2) 0.25, (3) 0.5, (4) 0.75, (5) 1.0 and (6) 1.5 mg/ml. Points are the experimental data. The solid curves were calculated from Eq. (4). (B and C) The dependences of parameters $T_0$ and $K_{\text{agg}}$ calculated from Eq. (4) on the Phb concentration, respectively.

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parameters of the equation were calculated from Eq. (4). The parameters were obtained at the following concentrations of GAPDH: (1) 0.1, (2) 0.75 and (3) 1.5 mg/ml. Points are the experimental data. The dependences of the light scattering intensity on temperature obtained for aggregation of GAPDH at various concentrations of the protein. The \( T_0 \) values for approximation of these dependences by Eq. (4) lie in the range from 43.4 to 47.5°C (Fig. 5A). The value of parameter \( T_0 \) remains practically constant with variation of the GAPDH concentration (Fig. 5B). The average value of \( T_0 \) is equal to 45.2°C. As it can be seen from Fig. 5C the parameter \( K_{agg} \) increases monotonously with increasing the protein concentration.

The dependences of the light scattering intensity on temperature for aggregation of human interleukin-1\( \beta \) presented by Chrunyk et al. [38]. These dependences were obtained at various protein concentrations. The heating rate was 0.5°C/min. The following values of parameters of Eq. (4) were found: \( T_0 = 50.0 \pm 0.1, 48.4 \pm 0.1 \) and 47.5 ± 0.1°C, \( K_{agg} = 517 \pm 15, 1130 \pm 50 \) and 2200 ± 100°C^2 for the interleukin-1\( \beta \) concentration equal to 1, 2 and 4 mg/ml, respectively. The \( R^2 \) values for approximation of the experimental curves by Eq. (4) were equal to 0.965, 0.913 and 0.846 at the interleukin-1\( \beta \) concentration of 1, 2 and 4 mg/ml, respectively. Thus, the enhancement of the interleukin-1\( \beta \) concentration results in the diminishing of the \( T_0 \) value and the increase in the \( K_{agg} \) value. It is worthy of mention that to characterize the temperature aggregation Chrunyk et al. [39] proposed to use a temperature corresponding to the length cut off on the temperature axis by the straight line, which is a continuation of the linear part of the dependence of the light scattering intensity on temperature reachable at the inflexion point.

In the work of Raibekas [40] the dependences of the light scattering intensity on temperature for aggregation of interleukin-1 receptor antagonist (IL-1ra) are represented in the coordinates \( I/ I_{lim} = T \). The heating rate was 1°C/min. The dependences \( I/ I_{lim} \) on temperature were obtained at various concentrations of IL-1ra and characterized by parameter \( T_{agg} \) corresponding to the level \( I/ I_{lim} = 0.5 \). In the interval concentrations of IL-1ra from 1 to 10 mg/ml parameter \( T_{agg} \) decreases by 5.3°C, namely from 56.4 to 51.1°C (\( \Delta T_{agg} = 5.3°C \)). We have analyzed the dependences of \( I/ I_{lim} \) on temperature using an equation, which is a modification of Eq. (4):
\[ I/I_{\text{lim}} = K_{\text{agg}}(T - T_0)^2. \quad (T > T_0) \]  

In this equation \( K_{\text{agg}} \) is a constant. The change in the \( T_0 \) value characterizes the displacement of the dependence of \( I/I_{\text{lim}} \) on temperature along the abscissa axis, whereas parameter \( K_{\text{agg}} \) characterizes the steepness of the initial part of the dependence of \( I/I_{\text{lim}} \) on \( T \). The following values of parameters of Eq. (7) were found: \( T_0 = 53.7 \pm 0.1^\circ C \) and \( K_{\text{agg}} = 0.080 \pm 0.002 \mathrm{~(C)^{-2}} \) \((R^2 = 0.994)\) at \([\text{IL-1ra}] = 1 \mathrm{mg/ml}\) and \( T_0 = 47.3 \pm 0.1^\circ C \) and \( K_{\text{agg}} = 0.044 \pm 0.001 \mathrm{~(C)^{-2}} \) \((R^2 = 0.997)\) at \([\text{IL-1ra}] = 10 \mathrm{mg/ml}\).

If one assumes that the \( I_{\text{lim}} \) value is strictly proportional to the
protein concentration, the calculations show that $K_{\text{agg}}$ should increase by a factor of 5.5 when the IL-1ra concentration increases from 1 to 10 mg/ml. The displacement of the dependence of $I/\Delta I_{\text{lim}}$ on temperature in this concentration interval was characterized by the change in parameter $T_h$ and $\Delta T_0$ was equal to 6.4 °C. This value is less than $\Delta T_{\text{agg}}$ (5.3 °C). The discrepancy between the values of $\Delta T_0$ and $\Delta T_{\text{agg}}$ is explained by the lesser steepness of the initial part of the dependence of $I/\Delta I_{\text{lim}}$ on temperature and, consequently, the lesser slope of this dependence in the inflexion point at $[\text{IL-1ra}] = 10 \text{ mg/ml}$.

Thus, the quantitative analysis of the initial parts of the dependences of the light scattering intensity on temperature using Eq. (4) allows us to estimate the threshold temperature $T_h$ and parameter $K_{\text{agg}}$, which may be considered as a measure of the rate of aggregation. Parameter $T_h$ has a clear physical sense. When the measurement of the light scattering intensity is used for registration of protein aggregation, the initial increment of the light scattering intensity corresponds to the instant of the formation of the start aggregates containing hundreds of denatured protein molecules [25,26]. The value of parameter $T_h$ is a function of the protein concentration and, as a rule, decreases with increasing the protein concentration. As it could be expected, the value of parameter $K_{\text{agg}}$ characterizing the aggregation rate tends to grow as the protein concentration increases. In some cases the leveling off for the $K_{\text{agg}}$ value is observed at rather high concentrations of the protein, as with Ph6 and GAPDH. Such unexpected decrease in the growth of the aggregation rate with increasing protein concentration may have a simple explanation. When the protein concentration increases, the diminishing of the initial aggregation temperature ($T_0$) occurs, and, consequently, the initial rate of aggregation is measured in the region of lower temperatures.

Two parameters we proposed to characterize the temperature dependency of the light scattering intensity ($T_h$ and $K_{\text{agg}}$) provide more detailed information on the character of protein aggregation than parameter $T_{\text{agg}}$. Really, the value of parameter $T_{\text{agg}}$ is determined by two factors, namely the threshold temperature $T_h$ and the steepness of the initial part of the dependence of the light scattering intensity on temperature expressed by parameter $K_{\text{agg}}$ (or parameter $K_{\text{agg}}^{1/2}$).

Apart from the protein concentration there is another factor affecting the shape on the light scattering intensity versus temperature plot, namely the heating rate. One could expect that an increase in the heating rate would result in the displacement of the dependence of the light scattering intensity on temperature towards higher temperatures. Actually, such a picture was observed for aggregation of α-amylase from Bacillus amyloliquefaciens (BAA). Duy and Fitter [41] studied aggregation of BAA at various values of the heating rate ($\phi$). The protein concentration was 50 µg/ml. The experimental data were represented by the coordinates ($I/\Delta I_{\text{lim}}, T$). An analysis of the dependences of $I/\Delta I_{\text{lim}}$ on temperature based on Eq. (7) gives the following values of parameters: $T_h = 74.0 \pm 0.1 \degree C$ and $K_{\text{agg}} = 0.0052 \pm 0.0002 (\degree C)^{-2}$ ($R^2 = 0.964$) at $\phi = 0.1 \degree C/min$ and $T_h = 79.8 \pm 0.1 \degree C$ and $K_{\text{agg}} = 0.0070 \pm 0.0005 (\degree C)^{-2}$ ($R^2 = 0.949$) at $\phi = 1.0 \degree C/min$. Thus the enhancement of the heating rate from 0.1 to 1.0 °C/min results in the displacement of the dependence of the light scattering intensity on temperature along the abscissa axis by 5.8 °C towards higher temperatures. The change in the steepness of the initial part of the dependence of the light scattering intensity on temperature is not marked. Parameter $K_{\text{agg}}$ increases by a factor of 1.35, when the heating rate is changed from 0.1 to 1.0 °C/min. It should be noted that the change in the value of parameter $T_{\text{agg}}$ corresponding to the level $I/\Delta I_{\text{lim}} = 0.5$ in this interval of the heating rates was equal to 3.3 °C. Consequently, $\Delta T_{\text{agg}}$ is less than $\Delta T_0$. Apart from the light scattering measurements the authors studied thermal unfolding of BAA using intrinsic fluorescence spectroscopy. Unfolding transitions were analyzed in terms of wavelength shifts of the emission peak and by calculating the peak ratios of intensities as measured at 330 nm and 350 nm (excitation wavelengths of 280 and 295 nm were applied). It was shown that for both heating rates (0.1 and 1.0 °C/min) aggregation that was characterized by the normalized change in the light scattering intensity appeared rather concurrent with respect to the unfolding transition as obtained from tryptophan fluorescence.

Estimation of the Size of Protein Aggregates

Consider what additional information on the aggregation process studied in the regime of heating with a constant rate may be obtained from the measurements of the size of the protein aggregates. Fig. 8A shows the initial parts of the dependences of the hydrodynamic radius ($R_h$) of the protein aggregates on temperature obtained for GAPDH aggregation at various concentrations of the protein. These dependences are linear, and the following equation was used for their analysis [24,25]:

$$R_h = R_{h,0} \left[ 1 + \frac{1}{\Delta T_{2R}} (T - T_0) \right]. \quad (T > T_0) \quad (8)$$

In this equation $R_{h,0}$ is the hydrodynamic radius of the start aggregates, $T_0$ is the temperature at which the start aggregates appear and $\Delta T_{2R}$ is the temperature interval over which the hydrodynamic radius of the protein aggregates increases from $R_{h,0}$ to 2$R_{h,0}$. The reciprocal value of parameter $\Delta T_{2R}$ is a measure of the aggregation rate. The higher the $1/\Delta T_{2R}$ value, the higher is the aggregation rate. Taking $T_0$ in this equation to be identical to parameter $T_h$ in Eq. (4), we can calculate other parameters, namely $R_{h,0}$ and $\Delta T_{2R}$. As it can be seen from Fig. 8B, the marked decrease in the $R_{h,0}$ value occurs with increasing GAPDH concentration (from 64±3 nm at [GAPDH] = 0.1 mg/ml to 14±3 nm at [GAPDH] = 1.5 mg/ml). Such a decrease in the $R_{h,0}$ value is accompanied by the increase in the rate of aggregation expressed by the reciprocal value of parameter $\Delta T_{2R}$ (Fig. 8C).

Fig. 9A shows the initial parts of the dependences of the hydrodynamic radius of protein aggregates on temperature obtained for CK aggregation at various concentrations of the protein. These dependences are exponential, and the following equation was used for their analysis [24–26]:

$$R_h = R_{h,0} \exp \left[ \frac{\ln 2}{\Delta T_{3R}} (T - T_0) \right]. \quad (T > T_0) \quad (9)$$

In this equation $\Delta T_{3R}$ is the temperature interval over which the hydrodynamic radius is doubled. When the concentration of the protein increases, a slight diminishing of the $R_{h,0}$ value takes place (from 44±2 nm at [CK] = 0.1 mg/ml to 34±2 nm at [CK] = 1.5 mg/ml) with simultaneous increase in the $1/\Delta T_{3R}$ (Fig. 9B and C).

As is with CK, the initial parts of the dependences of the hydrodynamic radius of protein aggregates on temperature obtained for GDH aggregation at various concentrations of the protein are exponential (Fig. 10A). The values of parameters $R_{h,0}$ and $1/\Delta T_{3R}$ as a function of the GDH concentration are represented in Fig. 10B and C. The increase in the GDH concentration results in the diminishing of the $R_{h,0}$ value with simultaneous increase in the $1/\Delta T_{3R}$ value.
The following explanation can be proposed for the decrease in parameter $T_0$ with increasing the protein concentration (Fig. 4–6). The increase in the protein concentration favors nucleation and further formation of start aggregates. Therefore one may expect that the temperature corresponding to the moment of origination of the start aggregates will decrease with increasing the protein concentration. This picture resembles the well-known phenomenon of the decrease in the duration of the lag period on the kinetic curves of aggregation registered at a fixed temperature when the protein concentration is increased.

Figure 8. Estimation of the size of the protein aggregates formed in the course of GAPDH aggregation registered in the regime of heating at the rate of $1\, ^\circ C$/min (10 mM Na-phosphate buffer, pH 7.5, containing 0.1 M NaCl). (A) The initial parts of the dependences of the hydrodynamic radius ($R_h$) of the protein aggregates on temperature obtained at the following GAPDH concentrations: (1) 0.1, (2) 0.25, (3) 0.5 and (4) 1.5 mg/ml. (B and C) The dependences of parameter $R_{h,0}$ and the reciprocal value of parameter $\Delta T_{2R}$ calculated from Eq. (8) on the GAPDH concentration, respectively. doi:10.1371/journal.pone.0022154.g008

Figure 9. Estimation of the size of the protein aggregates formed in the course of CK aggregation registered in the regime of heating at the rate of $1\, ^\circ C$/min (30 mM Hepes–NaOH buffer, pH 8.0, containing 0.1 M NaCl). (A) The initial parts of the dependences of the hydrodynamic radius ($R_h$) of the protein aggregates on temperature obtained at the following CK concentrations: (1) 0.1, (2) 0.75 and (3) 1.5 mg/ml. (B and C) The dependences of parameter $R_{h,0}$ and the reciprocal value of parameter $\Delta T_{2R}$ calculated from Eq. (9) on the GAPDH concentration, respectively. doi:10.1371/journal.pone.0022154.g009
other words, the sticking probability for the colliding particles for this regime of aggregation is equal to unity. We interpret the exponential character of the dependence of the hydrodynamic radius of the protein aggregates on temperature observed for CK and GDH as an indication for the fulfillment of the regime of reaction-limited cluster-cluster aggregation wherein the sticking probability for the colliding particles is lower than unity [24–26].

Consider the differences between the model of protein aggregation used in the present work and models proposed by other investigators. A general model of protein aggregation was recently elaborated by Li and Roberts [42]. This model takes into account the conformational transition of monomers between folded and unfolded states, nucleation of the smallest aggregate species, growth of aggregates via chain polymerization and aggregate growth due to aggregate-aggregate association. When studying the kinetics of thermal aggregation of proteins by dynamic light scattering, we observed that at the moment of time when the initial increment in the light scattering intensity is observed, protein aggregates containing hundreds of denatured protein molecules are registered. These primary aggregates were called the start aggregates. We could not detect intermediate states between the non-aggregated form of the protein and the start aggregates. Thus we infer that the formation of the start aggregates proceeds on the all-or-none principle. This pattern was demonstrated for thermal aggregation of β-crystallin from bovine lens [12,37], GAPDH from rabbit skeletal muscle [6,23,33,37,43], yeast alcohol dehydrogenase I [26,37], tobacco mosaic virus coat protein [37,44], Phb from rabbit skeletal muscle [5,23,29,37,43,45], aspartate aminotransferase from pig heart mitochondria [46] and creatine kinase from rabbit skeletal muscle [7]. On the basis of these experimental data a new model of protein aggregation was proposed [12,23,36,37]. According to this model nuclei are assembled rather quickly in the start aggregates and further growth of aggregates is due to sticking of the start aggregates and aggregates of higher order. It was demonstrated that the size of the start aggregates is independent of the concentration of the protein involved in aggregation. This fact allowed us to draw an analogy between the formation of the start aggregates and micelle formation. In the latter case the micelles of a definite size are formed when the critical micelle concentration is achieved. Such an analogy offers an explanation of why the formation of start aggregates proceeds according to the all-or-none principle.

The idea on the formation of primary aggregates in the course of protein aggregation was also used by Durand and coworkers. When studying thermal aggregation of β-lactoglobulin at pH 7.0 by size exclusion chromatography, Durand et al. [47–51] showed that there was a clear separation between a narrow peak that corresponded to residual native protein and a broad peak that corresponded to the aggregates. This observation implied that the aggregates of the minimum size contained many monomers and that no or very few stable oligomers were formed. The authors supposed that the first stage of thermal aggregation of β-lactoglobulin was the step of the formation of “primary aggregates”.

Shiraki and coworkers [52] studied heat-induced aggregation of lysozyme at around the isoelectric point. Dynamic light scattering and transmission electron microscopy revealed that aggregation occurred in a two-step process: formation of start aggregates, followed by further growth mediated by their sticking with diffusion-limited cluster-cluster aggregation.

Recent investigations of dithiothreitol-induced aggregation of alpha-lactalbumin and insulin using dynamic light scattering convincingly demonstrate that the aggregation process
proceeds through the stage of formation of the start aggregates [53,54].

It is notable that according to present views the assembly of amyloid fibrils proceeds through the stage of the formation of protofibrils. Association of protofibrils results in the formation of fibrils and then clusters of fibrils and bundles [35,36]. It is evident that protofibrils are equivalent to the start aggregates in the case of aggregation producing amorphous aggregates (this analogy was discussed in Golub et al. [23]).

Screening of the Ligands Affecting Denaturation or Aggregation of Phb and GDH on the Basis of the Data of the Aggregation Kinetics

Consider the use of this new approach to characterization of the aggregation rate for the quantitative estimation of the ability of various agents to suppress protein aggregation. Our investigations have shown that Phb is a convenient model for testing the compounds affecting protein denaturation and aggregation [4,5,25,29,45,57–60]. Fig. 11 shows the effect of some agents on Phb aggregation. The suppression of aggregation in the presence of 1 mM AMP is accompanied by the increase in the $T_0$ value from 47.2°C (curve 1; the control) to 51.4°C (curve 2; 1 mM AMP) and the decrease in parameter $K_{agg}$ from $(1.83 \pm 0.02) \times 10^5$ (curve 1) to $(1.20 \pm 0.02) \times 10^5$ (counts/s)/(°C)$^{-2}$ (curve 2). Based on the data on kinetics of thermal inactivation of Phb and DSC data [58,60], we can conclude that the anti-aggregation ability of allosteric activator AMP is due to the stabilization of the native Phb molecule. The acceleration of Phb aggregation in the presence of HP-$\beta$-CD (curve 3 in Fig. 11) is accompanied by the decrease in the $T_0$ value to 46.1°C and the increase in parameter $K_{agg}$ to $(3.27 \pm 0.03) \times 10^5$ (counts/s)/(°C)$^{-2}$. This action of HP-$\beta$-CD is caused by the destabilization of the Phb molecule as a result of binding of HP-$\beta$-CD to the native form of the enzyme and early intermediates of Phb unfolding as suggested by the data on the kinetics of thermal inactivation and DSC data [45]. It is known that osmolytes stabilize the protein structure [61–63]. Therefore, when testing the effect of osmolytes on protein aggregation, one can expect that osmolytes will reveal the protective action. Fig. 11 demonstrates that a markedly expressed retardation of thermal aggregation of Phb is observed in the presence of 1 M TMAO. The $T_0$ value increases to 55.1°C and the $K_{agg}$ value decreases to $(1.54 \pm 0.02) \times 10^5$ (counts/s)/(°C)$^{-2}$ (curve 4). At last, it is of interest to discuss the effect of the agents of protein nature responsible for suppression of protein aggregation in the cell, namely small heat shock proteins. Meremyanin et al. [25,29] showed that $\alpha$-crystallin, one of the representatives of small heat shock protein family, effectively hindered thermal aggregation of Phb. Curve 5 in Fig. 11 presents an initial part of the dependence of the light scattering intensity on temperature for Phb aggregation measured in the presence of $\alpha$-crystallin at the concentration of 1 mg/ml.

The protective action of $\alpha$-crystallin is reflected in the increase in the $T_0$ value (to 52.6°C) and the marked decrease in the $K_{agg}$ value (to $(0.27 \pm 0.01) \times 10^5$ (counts/s)/(°C)$^{-2}$). The $R^2$ values for approximation of the curves 1–5 shown in Fig. 11 by Eq. (4) were found to be 0.981, 0.954, 0.907, 0.906 and 0.972, respectively. The values of parameters of $T_0$ and $K_{agg}$ obtained during testing of the effect of various agents on Phb aggregation are given in Table 1.

Fig. 12 shows the effect of specific ligands (NADH, ADP, L-glutamate and L-leucine) on aggregation of GDH studied in the regime of heating with a constant rate. The values of parameters $T_0$ and $K_{agg}$ are given in Table 2. The increase in parameter $K_{agg}$ in the presence of 0.2 mM NADH means that NADH displays the destabilizing action on the enzyme. Judging from the $K_{agg}$ values, the enhancement of GDH stability is observed in the presence of allosteric activators, ADP and L-leucine, and in the presence of substrate, L-glutamate. ADP (0.5 mM) eliminates the destabilizing effect of NADH (0.2 mM). NAD has no effect on GDH aggregation (see Table 2).

The effect of ADP on aggregation of GDH was studied in greater detail. Fig. 13A shows the initial parts of the dependences of the light scattering intensity on temperature obtained for GDH aggregation at various concentrations of ADP. The values of parameters $T_0$ and $K_{agg}$ as a function of the ADP concentration are represented in Fig. 13B and C. A sharp increase in parameter $T_0$ is observed in the presence of 0.05 mM ADP, namely from 55.2°C at [ADP] = 0 to 38.7°C at [ADP] = 0.05 mM (Fig. 13B). The changes in parameter $T_0$ in the interval of ADP concentration from 0.05 to 0.5 mM are insignificant. The average value of $T_0$ in this interval of ADP concentrations is equal to 39.1±0.2°C. A decrease in parameter $T_0$ is observed at the ADP concentrations higher than 0.5 mM. As it follows from Fig. 13C, the increase in ADP concentration is accompanied by the diminishing of parameter $K_{agg}$. The dependence of $K_{agg}$ on ADP concentration in the interval 0.05–0.5 mM was analyzed using the following equation:

$$
\text{Table 1. Parameters of aggregation of Phb (0.5 mg/ml) in the presence of different ligands (0.08 M Hepes-buffer, pH 6.8, containing 0.1 M NaCl).}
$$

| Ligand      | $T_0$ (°C) | $K_{agg}$ (counts/s)/(°C)$^{-2}$ |
|-------------|------------|----------------------------------|
| -           | 47.2±0.1   | $(1.83 \pm 0.02) \times 10^5$    |
| AMP (1 mM)  | 51.4±0.1   | $(1.20 \pm 0.02) \times 10^5$    |
| HP-$\beta$-CD (19 mM) | 46.1±0.1 | $(3.27 \pm 0.03) \times 10^5$    |
| TMAO (1 M)  | 55.1±0.1   | $(1.54 \pm 0.02) \times 10^5$    |
| $\alpha$-crystallin (1 mg/ml) | 52.6±0.1 | $(0.27 \pm 0.01) \times 10^5$    |

![Figure 11. Effect of different agents on Phb aggregation. The initial parts of the dependences of the light scattering intensity on temperature for aggregation of Phb (0.5 mg/ml) in the presence of the following agents: (1) control, (2) 1 mM AMP, (3) 0.19 M HP-$\beta$-CD, (4) 1 M TMAO and (5) $\alpha$-crystallin at the concentration of 1 mg/ml. Points are the experimental data. The solid curves were calculated from Eq. (4). doi:10.1371/journal.pone.0022154.g011](image-url)
where $K_{agg,0}$ is the value of $K_{agg}$ at $[ADP] = 0$, and $K_{diss}$ is the microscopic dissociation constant for the ADP-GDH complex. The $K_{diss}$ value was found to be $0.52 \pm 0.07$ mM. It is reasonable to suppose that this value of $K_{diss}$ corresponds to the temperature of $39.1^\circ C$.

Conclusion

This article has described a new approach to testing the agents affecting the stability of proteins. The approach is based on the quantitative analysis of the initial increment in the light scattering intensity caused by aggregation of the denatured protein molecules in the experiments where aggregation is studied in the regime of the elevation of temperature with a constant rate. The proposed approach allows estimating the temperature at which the initial increase in the light scattering intensity is registered ($T_0$) and establishing parameter $K_{agg}$, which characterizes the rate of aggregation. Temperature $T_0$ is a moment of the origination of the start aggregates. Such aggregation systems may be used for testing the compounds affecting protein stability as a result of direct binding to the native protein molecule (for example, substrates and modifiers of the enzymes); the compounds stabilizing the protein structure owing to the effects of excluded volume (for example, osmolytes); and the compounds possessing chaperone-like activity (for example, small heat shock proteins). If we want to select agents affecting exclusively the stage of denaturation, we should demonstrate that these agents have no effect on the stage of aggregation. In turn, this would require

| Ligand          | $T_0$ (°C) | $K_{agg}$ [(counts/s) °C$^{-2}$] |
|-----------------|-----------|---------------------------------|
|                 | 34.3±0.1  | (8.7±0.1)×10$^2$                |
| NADH (0.2 mM)   | 35.0±0.1  | (11.6±0.2)×10$^2$               |
| NAD (0.2 mM)    | 34.5±0.1  | (8.4±0.1)×10$^2$                |
| ADP (0.5 mM)    | 39.3±0.1  | (4.7±0.1)×10$^2$                |
| NADH (0.2 mM)+  | 36.4±0.1  | (4.7±0.1)×10$^2$                |
| ADP (0.5 mM)    | 36.4±0.1  | (4.7±0.1)×10$^2$                |
| L-Leucine (50 mM)| 36.0±0.1  | (2.8±0.1)×10$^2$                |
| L-Glutamate (50 mM)| 32.8±0.1 | (4.9±0.1)×10$^2$                |

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additional aggregation systems where a preliminary denatured protein undergoes thermal aggregation. A protein denatured by ultraviolet radiation may be used for this purpose [64]. Test-systems based on aggregation of UV-irradiated proteins allow registering the direct action of the agents under study on the stages of aggregation.

When discussing the practical utility of the method of study of protein-ligand interactions proposed in the present work, we should like to note, first of all, that this method may be used for characterization of the proteins themselves. The results of investigation of protein stability in buffer solutions of different composition may be useful for selection of optimal buffer for purification procedure and storage of the proteins under study. The protein mutant forms obtained by genetic engineering may also be characterized by the method under discussion. Furthermore, screening against cofactors, substrate analogs, effectors and metals may be used to establish the conditions that favor protein stability. These stabilizing agents may be used during protein crystallization to improve the crystallization rate. Finally, an important line of application of the screening procedure based on the measurements of the light scattering intensity increase, which accompanies protein aggregation, is the search for compounds of potential medicinal significance.

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Author Contributions

Conceived and designed the experiments: TE BK. Performed the experiments: TE OM BK SK RA. Analyzed the data: TE BK. Contributed reagents/materials/analysis tools: TE BK. Wrote the paper: TE BK. Engaged in active discussion: TE KM BK.

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