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Protein aggregates as depots for the release of biologically active compounds

Natalya V. Artemova, Alexei S. Kasakov, Zoya M. Bumagina, Elena M. Lyutova, Bella Ya. Gurvits*

A.N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky prospect, 33, 119071 Moscow, Russia

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ABSTRACT

Protein misfolding and aggregation is one of the most serious problems in cell biology, molecular medicine, and biotechnology. Misfolded proteins interact with each other or with other proteins in non-productive or damaging ways. However, a new paradigm arises that protein aggregation may be exploited by nature to perform specific functions in different biological contexts. From this consideration, acceleration of stress-induced protein aggregation triggered by any factor resulting in the formation of soluble aggregates may have paradoxical positive consequences. Here, we suggest that amorphous aggregates can act as a source for the release of biologically active proteins after removal of stress conditions. To address this concept, we investigated the kinetics of thermal aggregation in vitro of yeast alcohol dehydrogenase (ADH) as a model substrate in the presence of two amphiphilic peptides: Arg-Phe or Ala-Phe-Lys. Using dynamic light scattering (DLS) and turbidimetry, we have demonstrated that under mild stress conditions the concentration-dependent acceleration of ADH aggregation by these peptides results in formation of large but soluble complexes of proteins prone to refolding.

In response to environmental stress (heat shock, oxidizing conditions, toxic compounds) labile proteins lose their native conformations and seek alternatively stable aggregated states. Molecular chaperones can protect the non-native proteins by binding hydrophobic unraveled or misfolded surfaces thereby preventing them from interaction with each other or with other proteins in non-productive or damaging ways [1–6].

The efficiency of chaperone-mediated processes in vitro, however, depends on the nature of the protein substrate, protein concentration, aggregate types, size, and solubility, varying environmental conditions, such as pH, ionic strength, temperature, salt type and concentration, co-solutes and preservatives. Change in these conditions can drastically change the capacity of chaperones or chaperone-like compounds for preventing aggregation of protein substrates [7,8].

Under certain conditions, so-called anti-chaperone activity, unexpected chaperone capacity for accelerating protein aggregation, is observed. It is noteworthy that protein disulfide isomerase [9–12] and trigger-factor (peptidyl–prolyl *cis–trans* isomerase) [13], which catalyze folding, heat shock protein Hsp47 [14], chaperone-like proteins and various peptides [12,15–18] accelerate protein aggregation. These results raise the question of diversity and complexity of the molecular mechanisms involved in adaptation of an organism to different kinds of stress, especially to heat shock.

The enhanced aggregation of a protein substrate is often observed in the presence of another protein/peptide able to affect its physicochemical properties, such as net charge, hydrophobicity, or tendency to beta structure formation resulting in accumulation of soluble aggregates that can act as a source for the release of biologically active proteins after removal of stress conditions. In this aspect, acceleration of protein aggregation triggered by any factor resulting in the formation of large complexes by non-covalently cross-linking the aggregation-prone substrate(s) and/or smaller amorphous aggregates may have seemingly paradoxical positive consequences.

It is interesting that many non-pathogenic proteins and peptides (which are not amyloid in nature) can form highly variable aggregates. Some aggregates display an amorphous organization, whereas others form highly ordered amyloid-like fibrils [7,19,20], suggesting that protein aggregation may be a common structural property of polypeptide chains and a much more widespread event in the living world than previously suspected. Moreover, the intra- or extracellular protein and/or peptide aggregates may comprise natural biomolecular nanostructures able to perform specific physiological functions in cells [21–23].

Therefore, the investigation of the complex molecular machineries aimed at hindering or promoting the appearance of protein aggregates is very important. Since our interest was to study the latter events, we addressed the molecular mechanisms of accelerated protein aggregation at the early onset of heat stress around physiological conditions when the temperature does not exceed 42 °C. We have investigated the kinetics of thermal aggregation in vitro of yeast alcohol dehydrogenase (ADH) as a model substrate in the presence of two charged aromatic peptides: arginyl phenylalanine (RF) and alanyl phenylalanyl lysine (AFK). The peptides

* Corresponding author. Fax: +7 495 9542732.
E-mail address: bella@inbi.ras.ru (B.Y. Gurvits).

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containing both hydrophobic and positively charged amino acid residues were used in this study as a model to explore the potential of small amphiphilic compounds to associate with an unfolded substrate and enhance protein solubilization from aggregates. The reason behind this choice is that among many low molecular weight compounds tested, arginine is one of the most widely used additives effective in suppressing aggregation, assisting refolding of aggregated proteins, and enhancing the solubility of aggregation-prone molecules [24–28]. However, current understanding of the molecular mechanisms by which short arginine/lysine-containing amphiphilic peptides exert their effects on the protein aggregation process under mild stress conditions is limited.

The concentration-dependent acceleration of ADH heat-induced aggregation upon addition of either AFK or RF to the incubation medium has been observed under appropriate conditions. Confirmed by the amount of soluble protein and enzymatic activity measurements, it is demonstrated that the peptides render ADH amorphous aggregates more accessible to disaggregation.

Materials and methods

Materials. Tris, NAD, the RF dipeptide, and molecular weight markers were obtained from Sigma. Yeast alcohol dehydrogenase (ADH) was from MP Biomedicals (Germany). The AFK tripeptide synthesis was performed in Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences. All other chemicals used were of analytical grade. Water was subjected to deionization using Easy Pure II, RF ultrapure water system (Barnstead, USA).

DLS studies. For dynamic laser light scattering measurements a commercial instrument Photocor Complex was used (Photocor Instruments Inc., USA; www.photocor.com). This instrument allows measuring dynamic light scattering at various scattering angles. A He–Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) has been used as a light source. DLS data have been accumulated and analyzed with multifunctional real-time correlator Photocor-PC. DynaLS software (Alango, Israel) was used for polydisperse analysis of DLS data. Origin 7.0 software (OriginLab Corporation, USA) was used for the calculations.

The kinetics of thermal aggregation of ADH (0.3 mg/ml) was studied by DLS in 25 mM Tris–HCl buffer, pH 7.0. The buffer was preincubated in the absence or presence of aliquots of the di- or tripeptide in a cylindrical cell with the internal diameter of 6.3 mm at 42 °C for 10 min. In each experiment, the total volume in the cell was 0.5 ml. The aggregation process was initiated by the addition of an aliquot of ADH to the buffer. The scattering light was collected at 90° scattering angle and the accumulation time of the autocorrelation function was 30 s.

Turbidimetry. The aggregation of ADH under the same conditions was determined by measuring the apparent absorbance at 360 nm caused by increased turbidity. The aggregation was followed in a Beckman DU 650 spectrophotometer. In each experiment, the total volume in the 1-cm cuvettes was 1.5 ml. The buffer was preincubated in the absence or presence of the peptides in the cell holder at 42 °C. The absorbance in each cell was recorded automatically every 2 min for 30–120 min. The values of ΔA_{360} were normalized to the initial moment of time ($t = 0$).

At the end of the incubation process, the samples were cooled to room temperature and precipitation of protein aggregates was performed by centrifugation at 10,000*g* for 45 min. The supernatant and the precipitate, the latter being dissolved in 25 mM Tris–HCl buffer, pH 7.0, and centrifuged under the same conditions, were subjected either to electrophoresis under denaturing and non-denaturing conditions or determination of enzymatic activity.

Electrophoresis. SDS–PAGE was carried out according to Laemmli method [29] in a 12% polyacrylamide gel. The spots were visualized by silver staining. Native PAGE in a 10% polyacrylamide gel was used for determination of molecular weight and oligomeric state of the protein substrate.

Enzyme assay. The activity of ADH (EC 1.1.1.1) was measured in 50 mM potassium phosphate buffer, pH 8.0, containing 2 mM NAD and 1 mM ethanol in a final volume of 1 ml as described previously [30]. The buffer was preincubated at 25 °C in the absence of the enzyme in the cell of a Beckman DU 650 spectrophotometer. The reaction was started by the addition to the temperature-equilibrated buffer of an aliquot of the supernatant or dissolved ADH precipitate formed in the process of aggregation at 42 °C in the absence or presence of the peptides, and the increase in the apparent absorbance at 340 nm was measured. Absorbance variations were always linear within 3 min; each activity assay was performed in duplicate. The values were normalized to the initial moment of time ($t = 0$). The enzymatic activity was expressed as absorbance units per second. The results were compared with the activities of diluted native enzyme at known concentrations using a standard curve. The percentage reactivation yield was calculated with respect to the activity of the native enzyme.

Protein concentration was determined according to the method of Bradford [31].

Results and discussion

Influence of AFK and RF peptides on kinetics of ADH aggregation registered by DLS

To characterize the effect of AFK peptide on the kinetics of thermal aggregation of ADH (0.3 mg/ml) at 42 °C in 25 mM Tris–HCl buffer, pH 7.0, we have analyzed the dependences of the light scattering intensity on time in the absence and presence of the tripeptide (Fig. 1).

The light scattering intensity of ADH aggregates (Fig. 1 A, curve 1). Addition of AFK at a concentration of 1 mM resulted in changes in the light scattering intensity towards higher values (curve 2). In the presence of 2 mM AFK, the kinetic curve of the light scattering intensity increment abruptly changes at the end of the initial 3-min time point (Fig. 1 A, curve 3) when the intensity reaches the level that remains constant at further heating. At a concentration of 4.5 mM the aggregation was almost completely suppressed (curve 4). For comparison, it has been widely reported that arginine is an effective protector only at high concentrations (0.1–2.0 M).

The distribution of aggregates by their size with time was shown in Fig. 1B. In the absence of AFK, the ADH aggregate formation demonstrates complicated dynamics of the dependences of the hydrodynamic radii (R_h) values on time. The bimodal distribution of particles by their size is observed: in addition to the aggregates of low R_h values (Fig. 1B, curve 1'), the aggregates of larger size appear (curve 1). Two populations of particles have been also revealed in the presence of AFK, however, at the higher levels of average aggregate sizes (curves 2 and 2', or 3 and 3').

In the presence of 2 mM AFK, the rate constant of the kinetic curve of the dependence of the R_h values on time changes for the lower level at the end of the initial 7-min time point (Fig. 1 B, inset), in accordance with the kinetic curve of the light scattering intensity (Fig. 1 A, curve 3). The complicated kinetics of ADH aggregation in the presence of AFK at a concentration of 2 mM is suggested to reflect a transition state between stimulation (Fig. 1 A, curve 2) and inhibition (curve 4) of the aggregation process. AFK at a concentration of 4.5 mM, deters formation of larger particles that ultimately leads to a lower average aggregate size (data not shown).

It should be noted that in our experiments, the appearance of bimodal distribution of ADH particles measured by DLS (Fig. 1 B) could be attributed to the oligomeric structure of the enzyme preparation. Two types of aggregates may be initiated by different