Effects of Arginine on Kinetics of Protein Aggregation Studied by Dynamic Laser Light Scattering and Tubidimetry Techniques

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Prevention of undesirable protein aggregation is an extremely important strategy in protein science, medicine, and biotechnology. Arginine is one of the most widely used low molecular weight solution additives effective in suppressing aggregation, assisting refolding of aggregated proteins, and enhancing the solubility of aggregation-prone unfolded molecules in vitro. However, the mechanism of suppression of protein aggregation by arginine is not well understood. To address the mechanism, two model systems have been investigated: protection of alcohol dehydrogenase (ADH) and insulin from heat- and dithiothreitol-induced aggregation, respectively, in the presence of arginine. Using dynamic light scattering (DLS) technique, we have demonstrated the concentration-dependent suppression of light scattering intensity of both ADH and insulin aggregates upon addition of arginine to the incubation medium, a significant effect being revealed in the physiological concentration range of arginine (1−10 mM). DLS studies showed that arginine shifted the populations of nanoparticles with higher hydrodynamic radii to the lower ones, suggesting that the preventive effect of arginine on the protein aggregation process arises because it suppresses intermolecular interactions among aggregation-prone molecules. The results of turbidity measurements were also shown to be consistent with these findings.

Introduction

Undesirable aggregation of unfolded proteins occurs in living biological systems on many occasions, including co-translational misfolding of the synthesized polypeptide chains, mutations, or stress (heat, oxidizing conditions, and toxic compounds) that result in the malfunctioning of cells and organs. The challenging problem of aggregation is serious not only in cell biology and medicine but also in biotechnological, pharmaceutical, and food industry applications involving proteins. The development of genetic engineering techniques for overexpression of proteins has increased attention to the practical aspects of protein folding. Both basic protein science and biotechnology require that the overexpressed protein attain the correctly folded conformation.

Molecular chaperones and co-chaperones perform diverse cellular functions. They are involved in the folding of nascent proteins, prevention of protein aggregation, refolding of denatured proteins, and assisting the targeting of proteins for degradation by the proteasome and lysosomes. Chaperones can protect the nonnative proteins by binding hydrophobic unraveled or misfolded surfaces, thereby preventing them from interaction with each other or with other proteins in nonproductive or damaging ways. Moreover, protein aggregates can be solubilized and their nonnative conformers can be correctly refolded by chaperones (*1*−*6*).

Though molecular chaperones possess one common feature (they have hydrophobic domains exposed at the surface, enabling them to recognize and to bind unfolded proteins), the structural features of a protein that make it a chaperone are far from clear. Hence, the keen interest in chaperones does not seem to relax, and the list of new chaperone-like proteins discovered is increasing quite rapidly.

However, protein engineering (*7*) and the chaperone and chaperone-like protein application in biotechnology (*8*) are costly and require additional time-consuming and laborious steps for refolding and purification of many proteins to the bioactive form. Rarely high yields of soluble proteins are produced, only a minute fraction of which are correctly refolded proteins (*9*).

A practical and relatively simple approach (compared with using chaperone systems) to solving the aggregation problem is the utilization of low molecular weight "artificial chaperones". The strategy that was inspired by the known mechanisms of action of naturally occurring chaperones have been developed to prevent aggregation in vitro and promote efficient refolding of denatured proteins via the addition of chemical compounds, including denaturants (typically guanidine, urea, and detergents), cyclodextrins, poly(ethylene glycol) (*10*−*12*), and surfactants (*13*).

However, the effects of natural molecular and artificial chaperones do not completely solve the aggregation problem. The use of denaturants may lead to destabilization of proteins, and their removal at the final steps of the purification procedure for the high recovery of bioactive proteins becomes essential. The efficiency of chaperone-mediated processes 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; cosolutes; and preservatives. Moreover, because proteins are diverse in structure−functional peculiarities, additives that work well for a particular protein may not function universally (*14*, *15*).

In search for better additives naturally occurring nondenaturing reagents, such as osmolytes (*16*, *17*), diamines (*18*),

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polyamines (*19*), amino acids, and their derivatives (*20*−*22*) have been used.

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 unfolded molecules (*20*, *23*−*27*).

Although arginine has been extensively studied and frequently used for protein solubilization from insoluble pellets, as well as for purification and stabilization of proteins during storage, the molecular mechanisms by which arginine exerts its effects on aggregation in most cases are far from clear and have remained to be elucidated. The available data for development of appropriate qualitative and quantitative models of arginine action are insufficient, and hence, more extensive study of the kinetics of protein aggregation in the presence of arginine is needed to get a comprehensive understanding of the arginine mode of action.

To address the molecular mechanisms of the protective effects of arginine, two model protein systems, aggregation of thermally denatured yeast alcohol dehydrogenase (ADH) and dithiothreitol-induced aggregation of human recombinant insulin without and with arginine at physiological concentrations, were investigated using dynamic light scattering (DLS) and turbidimetry measurements.

Findings concerning molecular mechanisms of suppression of protein aggregation by naturally occurring low molecular weight agents could provide clues for designing effective additives for optimization protein engineering processes, as well as for drug development to prevent protein misfolding diseases.

Materials and Methods

Materials. Tris, dithiothreitol (DTT), human recombinant insulin, L-arginine, and molecular weight markers were obtained from Sigma. Yeast alcohol dehydrogenase (ADH) was from MP Biomedicals (Germany). 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 both dynamic and static light scattering at various scattering angles with a stepper-motor-controlled turntable. A He−Ne laser (Coherent, USA, Model 31-2082; 632.8 nm, 10 mW) has been used as a light source. The temperature of the sample cell was controlled by the proportional integral derivative (PID) temperature controller to within ±0.1 °C. The quasi-cross-correlation photon counting system with two photomultiplier tubes (PMTs) was used to increase the accuracy of particle sizing in the range of 0.5−10 nm. DLS data have been accumulated and analyzed with a multifunctional real-time correlator Photocor-PC that has both logarithmic multiple-tau and linear time-scale modes. DynaLS software (Alango, Israel) was used for polydisperse analysis of DLS data. Origin 7.0 software (OriginLab Corp., USA) was used for the calculations.

To analyze the dependence of both the light scattering intensity and the hydrodynamic radius (R_h) of particles on time, we registered the aggregation of proteins at fixed temperatures. To improve the accuracy of measurements of small particles, a quasi-cross-correlation photon counting system with two PMTs is used.

The kinetics of thermal aggregation of ADH was studied by DLS in 25 mM Tris-HCl buffer, pH 7.0. The buffer was preincubated in a cylindrical cell with the internal diameter of 6.3 mm at 48 °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. To study the effect of arginine on ADH aggregation, aliquots of both components were added into the cell simultaneously. The scattering light was collected at 90° scattering angle, and the accumulation time of the autocorrelation function was 30 s.

In a separate set of experiments, the kinetics of insulin aggregation was determined under the same conditions in 100 mM sodium−phosphate buffer, pH 7.0, at room temperature (25 °C). Insulin was dissolved in 20 mM NaOH and then in sodium−phosphate buffer to a concentration of 1 mg/mL. An aliquot of this solution was added to the buffer in the cell. The aggregation was initiated by the addition of DTT to the final concentration of 20 mM. To study the effect of arginine on insulin aggregation, aliquots of arginine solutions were added into the cell simultaneously with insulin.

Turbidimetry. The aggregation of ADH upon thermal denaturation at elevated temperatures in 25 mM Tris-HCl buffer, pH 7.0, in the absence or presence of different concentrations of arginine was determined by measuring the apparent absorbance at 360 nm caused by increased turbidity. The aggregation was followed in a Beckman DU 650 spectrophotometer equipped with a thermostated six-cell-holder accessory. The temperature of the samples in the cells was controlled using a circulating water bath. 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 arginine in the cell holder at 48 °C (the average time for the mixture to reach the predetermined temperature of the cell holder was ∼5 min), and the aggregation was started by the addition of a small volume of the substrate protein to the temperature-equilibrated buffer. The apparent absorbance was measured as a function of time. 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 ($t = 0$).

The effects of arginine on insulin aggregation induced by 20 mM DTT were determined in 100 mM sodium−phosphate buffer, pH 7.0, at room temperature (25 °C) using the same test system.

For calculation of the molar ratio of model protein:arginine, the molecular masses of ADH monomer and insulin B-chain were taken as 37 and 3.4 kDa, respectively.

Electrophoresis. SDS-PAGE was carried out according to the Laemmli method (*28*) in a 12% polyacrylamide gel at 360 V, 35 mA. 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. Molecular weight markers (kDa), bovine serum albumin (201, 134, and 67) and chymotrypsinogen (*24*), were used as standards.

Protein concentration was determined according to the method of Bradford (*29*).

Results and Discussion

Influence of Arginine on Kinetics of Heat-Induced Aggregation of ADH Registered by DLS. Thermal aggregation of ADH was studied by DLS at 48 °C in 25 mM Tris-HCl buffer, pH 7.0, at various concentrations of the substrate. DLS technique allows measuring the light scattering intensity and the size of particles in the course of protein aggregation.

To characterize the effect of arginine on the kinetics of thermal aggregation of ADH, we have analyzed the dependence

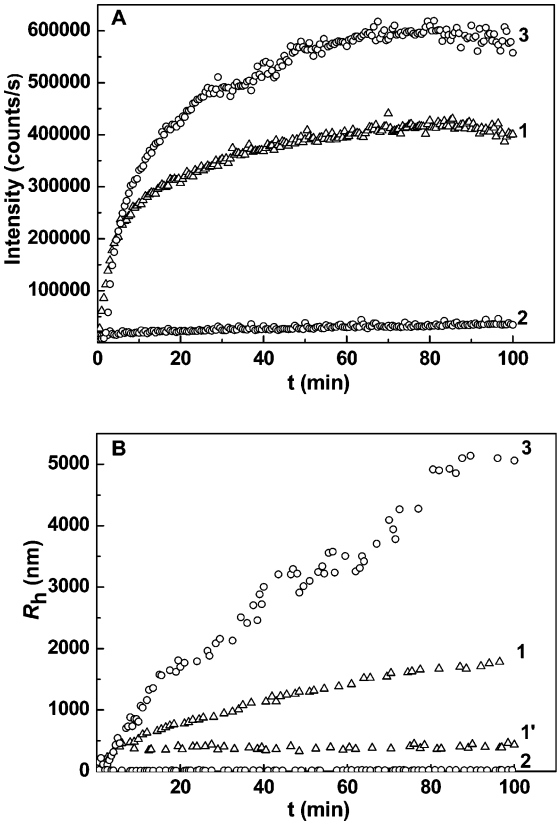

Figure 1. Kinetics of thermal aggregation of ADH (0.2 mg/mL) in 25 mM Tris-HCl buffer, pH 7.0, at 48 °C. (A) Dependence of the light scattering intensity on time in the absence (1) and presence of 2 mM arginine (2) or 100 mM NaCl (3). (B) Dependence of the hydrodynamic radius value (R_h) of aggregates on time in the absence (1) and presence of 2 mM arginine (2) or 100 mM NaCl (3). At the initial period of incubation, the aggregate population of ADH (control) is splitting into two components (curves 1 and 1′).

of the light scattering intensity on time in the absence and presence of arginine (Figure 1A).

The light scattering intensity of ADH aggregates increases with time and reaches the limiting value after heat treatment for 100 min (Figure 1A, curve 1). Addition of arginine resulted in concentration-dependent changes in the light scattering intensity toward lower values (data not shown). At a concentration of arginine of 2 mM the intensity reaches the lowest level (Figure 1A, curve 2) suggesting that in this case arginine almost completely suppresses heat-induced aggregation of ADH.

The distribution of aggregates by their size with time was also demonstrated in the course of ADH aggregation in the presence of arginine (Figure 1B). In this experiment, only low-sized particles have been revealed. The R_h values at the level of about 10−20 nm remain almost constant during heating of ADH for at least 100 min (curve 2).

In the absence of arginine, the ADH aggregate formation demonstrates complicated dynamics of the R_h value changes in time. At ADH concentration of 0.2 mg/mL, bimodal distribution of particles by their size is observed: in addition to the aggregates of low R_h values (Figure 1B, curve 1′), the aggregates of larger size appear (Figure 1B, curve 1). The results show that arginine deters formation of larger particles that ultimately leads to a lower average aggregate size.

The experiments were conducted to study the effect of arginine on the kinetics of ADH aggregation versus a salt control (NaCl), to draw a comparison between two different cation types. Although NaCl ions have been also considered to prevent protein aggregation, in our experiments, NaCl exhibited different

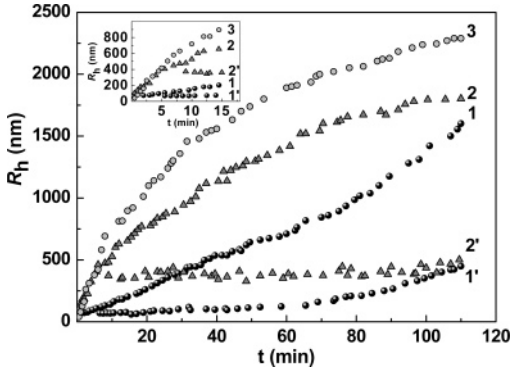

Figure 2. Kinetics of thermal aggregation of ADH at 48 °C in 25 mM Tris-HCl buffer, pH 7.0, at different concentrations of the substrate. Dependence of the hydrodynamic radius value (R_h) of aggregates on time obtained at ADH concentrations of 0.15 (1), 0.2 (2), and 0.25 mg/mL (3). At the initial period of incubation, the aggregate population is splitting into two components (curves 1 and 1′ at an ADH concentration of 0.15 mg/mL; curves 2 and 2′ at an ADH concentration of 0.2 mg/mL). The inset shows the distribution of R_h at the initial period of aggregation.

kinetic behavior in comparison with arginine. At a concentration of 100 mM, NaCl dramatically activated the aggregation process. A pronounced increase in both the light scattering intensity (Figure 1A, curve 3) and the R_h values of high-sized ADH aggregates has been observed (Figure 1B, curve 3).

To address the complicated dynamics of the R_h value changes in time in the control samples, we studied kinetics of thermal aggregation of ADH taken alone at 48 °C in 25 mM Tris-HCl buffer, pH 7.0, at different concentrations of the substrate. Figure 2 shows the dependence of hydrodynamic radii (R_h) values on time in the process of aggregation of ADH in the concentration range from 0.15 to 0.25 mg/mL.

At lower concentration of ADH (0.15 mg/mL), bimodal distribution of particles by their size is observed: in addition to the aggregates of low R_h values (Figure 2, curve 1′) the aggregates of larger size appear (Figure 2, curve 1). However, at the initial period of aggregation (up to 5 min), monomodal distribution of aggregates is registered. At higher ADH concentration (0.2 mg/mL), the size distribution function of the protein aggregates becomes bimodal at the higher point in time (9 min). The R_h values of nanoparticles (curves 1′ and 2′) were in the range of about 100 and 350 nm that remain practically constant during heating of ADH at concentrations of 0.15 and 0.2 mg/mL for about 70 and 100 min, respectively, but the R_h of particles of larger size dramatically increases with time (curves 1 and 2). At ADH concentrations exceeding 0.25 mg/mL, formation of only large aggregates occurred, and the monomodal distribution of the aggregates by their size with time was observed during heating for more than 110 min (curve 3). The R_h values increase much faster than those for the lower ADH concentrations.

The monomodal distribution was also shown for the ADH aggregation in the presence of 2 mM arginine or 100 mM NaCl (Figure 1B).

It should be noted that, in our experiments, the appearance of bimodal distribution of ADH particles measured by DLS (Figure 1 and 2) could be attributed to the oligomeric structure of the commercial preparation of the enzyme. According to the non-denaturing PAGE analysis, dimers and tetramers have been revealed in the ADH preparation, whereas SDS PAGE demonstrated homogeneous monomer of about 40 kDa (Figure 3).

Two types of ADH aggregates may be initiated by different conformational changes in the oligomers under stress conditions.

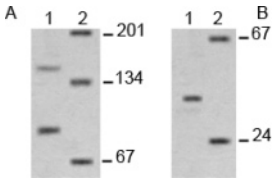

Figure 3. (A) Native PAGE of ADH in a 10% polyacrylamide gel: (lane 1) commercial preparation of ADH; (lane 2) molecular weight markers, bovine serum albumine, monomer, dimer, and trimer (67, 134, and 201 kDa, respectively). (B) (lane 1) SDS-PAGE of ADH commercial preparation in a 12% polyacrylamide gel; (lane 2) molecular weight markers, bovine serum albumine, monomer (67 kDa) and chymotrypsinogen (24 kDa). The spots were visualized by silver staining.

Discrete species formed from noncovalent association of partially folded intermediates were populated at the initial stages of the aggregation reaction. This approach of isolating in the process of aggregate multimers formed as a result of different conformation transitions of oligomers was applicable to many other proteins (*30*−*33*). A new aspect of the arginine protective effect on protein aggregation may be based on different interactions of arginine with substrate oligomers being in different conformational states.

Kinetics of Dithiothreitol-Induced Aggregation of Insulin Registered by DLS. Similar results were observed in a separate set of experiments using another model system: suppression of dithiothreitol-induced aggregation of insulin in the presence of arginine. The effect of arginine at a concentration of 8.5 mM on the kinetics of aggregation of insulin (0.2 mg/mL) in 100 mM sodium−phosphate buffer, pH 7.0, at room temperature has been demonstrated by DLS analysis of the dependence of both the light scattering intensity of insulin aggregates and the hydrodynamic radii on time (Figure 4).

The light scattering intensity of insulin aggregates was completely suppressed by arginine during incubation for about 60 min. Further incubation resulted in a negligible increment of the intensity level (Figure 4A, curve 2). The analysis of the hydrodynamic radii values of insulin particles (Figure 4B) shows the monomodal distribution of aggregates by their size with time in the absence (curve 1) and presence (curve 2) of arginine, the low-sized particles being revealed only at the end of the 60 min period of incubation. With longer aggregation times, an increase in the hydrodynamic radii values was registered.

Kinetics of Thermal Aggregation of ADH Measured by Turbidimetry. The results of turbidity measurements were also shown to be consistent with the findings obtained by DLS. The suppression of thermal aggregation of ADH was observed in the presence of arginine in a concentration-dependent manner. The examples of typical curves for ADH aggregation are presented in Figure 5A.

The effect of arginine on the aggregation of ADH (0.2 mg/mL) at 48 °C was noticeable even at a concentration as low as 0.05 mM (curve 2), the almost complete prevention of ADH aggregation being registered at arginine concentration of 2 mM (curve 5). In the latter case, a significant increase in the lag-period of aggregation was observed. The data were found to be highly reproducible in a series of experiments carried out with ADH.

Kinetics of Dithiothreitol-Induced Aggregation of Insulin Registered by Turbidimetry. Similar suppression of insulin aggregation in the presence of arginine in a concentration-dependent manner was observed (Figure 5B), a significant effect being expressed in the presence of 4 mM arginine.

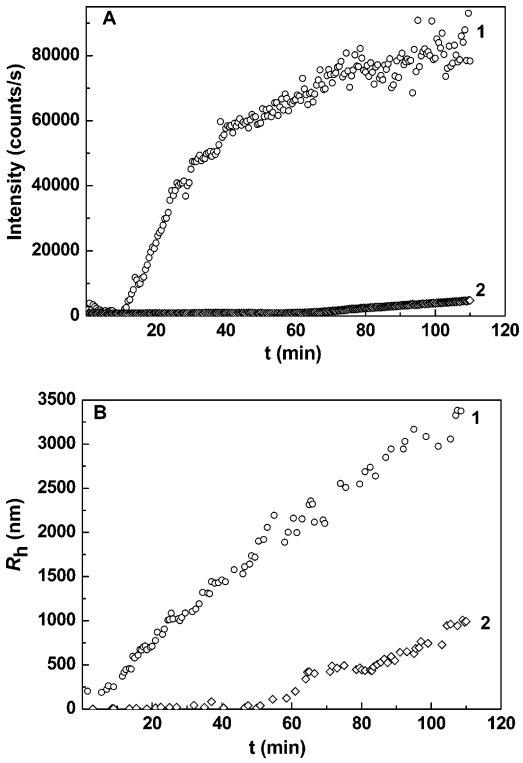

Figure 4. Effect of arginine at a concentration of 8.5 mM on the kinetics of dithiothreitol-induced aggregation of insulin (0.2 mg/mL) in 100 mM sodium−phosphate buffer, pH 7.0: (A) Dependence of the light scattering intensity of insulin aggregates on time in the absence (1) and presence of arginine (2); (B) dependence of the hydrodynamic radius value (R_h) of insulin aggregates on time in the absence (1) and presence of arginine (2).

The aggregation was almost completely prevented by arginine at a concentration of 8.5 mM. This was accompanied by the increase in the lag-period duration (curves 4 and 5).

Taken together, the results provide additional evidence for the protective effect of arginine on protein aggregation.

The results obtained are reminiscent of the finding reported earlier concerning the role of arginine in the process of protein refolding in two model systems: the association of insulin with a monoclonal antibody to insulin and the association of folding intermediates and aggregates of carbonic anhydrase (aggregation during refolding) (*27*). The authors observed that arginine hydrochloride in solution slowed protein−protein association and accelerated dissociation and that the reversibility of small multimer formation implies that early association reactions are at least partially equilibrium-controlled. It was suggested that since arginine hydrochloride shifts equilibrium toward the smaller oligomers, it should promote formation of the native protein during refolding. This was probed experimentally by measuring the multimer distribution by DLS and size-exclusion HPLC.

In addition, arginine has been shown to increase the refolding yield of several proteins from unfolded states, such as lysozyme (*34*), γ-interferon (*35*), carbonic anhydrase II (*36*), antibodies (*37*), and immunotoxin (*38*).

It has been widely reported that arginine is an effective protector only at high concentrations (0.1−2.0 M), suggesting that the interactions of arginine with proteins are weak. Little is known about the arginine action in the micro- and millimolar concentration range regarding protein folding and aggregation. In this paper, it has been demonstrated that the protective effect of arginine is expressed at paradoxically low concentrations

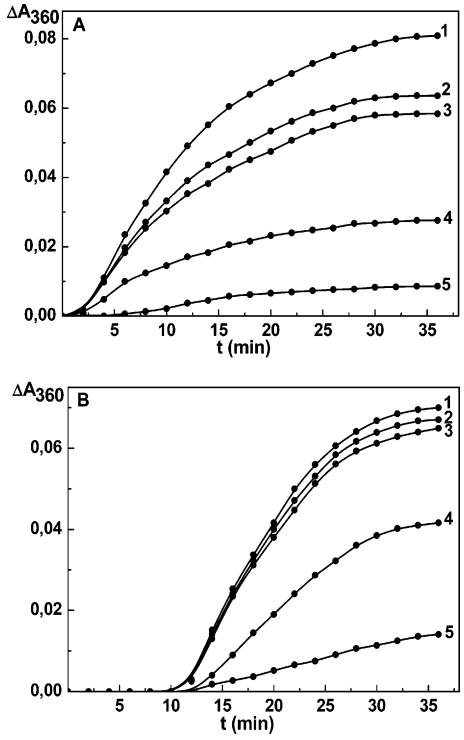

Figure 5. (A) Aggregation of ADH (0.2 mg/mL) determined by measuring the apparent absorbance at 360 nm (ΔA_{360}) caused by increased turbidity in 25 mM Tris-HCl buffer, pH 7.0, at 48 °C in the absence (1) or presence of arginine at concentrations of 0.05 (2), 0.3 (3), 1 (4), and 2 (5) mM. (B) Aggregation of insulin (0.2 mg/mL) determined by measuring the apparent absorbance at 360 nm (ΔA_{360}) caused by increased turbidity upon addition of 20 mM DTT to 100 mM sodium−phosphate buffer, pH 7.0, in the absence (1) or presence of arginine at concentrations of 0.5 (2), 2 (3), 4 (4), and 8.5 (5) mM.

(0.1−10 mM), the lowest ones reported in connection with protein aggregation.

Arginine has a unique side chain, the guanidinium group, which has been shown to bind certain functional groups. Because both ADH and insulin B-chain possess aromatic amino acids, we suggest that the observed effect could be attributed to the extensive binding of arginine to either substrate as a result of the interaction between the guanidinium group of arginine and aromatic side chains, which are buried inside the native proteins, but which would be exposed at the misfolded surfaces. Moreover, electrostatic interactions could be also involved, taking into consideration that ADH is an acidic protein (pI 5.4). The importance of the guanidinium group has also been suggested to be a factor in increasing protein solubility, as is true for guanidinium hydrochloride (*39*).

Such kinds of interaction were suggested to be responsible for protein aggregation or solubility of aggregates (*25*, *26*, *38*).

The capacity of arginine to interact directly with proteins has been demonstrated, e.g., binding with bovine serum albumin (*40*) and ribonuclease A (*41*).

Arginine is more frequently used as a solution additive to prevent proteins from aggregation in the process of refolding. Current understanding of the mechanisms by which arginine exerts its effects in a direct process of protein aggregation under stress conditions is limited. Although there is insufficient evidence for drawing firm conclusions, the results of DLS and turbidity measurements of the kinetics of the model protein aggregation presented in this paper may yield additional information concerning the mechanism of the effects of arginine on proteins.

Conclusions

To address the molecular mechanisms by which arginine exerts its effects on protein aggregation, two model systems, aggregation of thermally denatured alcohol dehydrogenase (ADH) and of dithiothreitol-induced aggregation of insulin with and without arginine, have been investigated using dynamic light scattering (DLS) and turbidimetry.

We have demonstrated the concentration-dependent suppression of both ADH and insulin aggregation upon addition of arginine to the incubation medium, a significant effect being revealed in the physiological concentration range of arginine (1−10 mM). With ADH as a substrate, aggregate formation demonstrated complicated dynamics of the hydrodynamic radii (R_h) value changes with time: bimodal distribution of nanoparticles by their size is observed. In the presence of arginine, only one type of low-sized particles has been revealed. Arginine was shown to shift the populations of nanoparticles with higher R_h values to the lower ones, suggesting that the preventive effect of arginine on protein aggregation arises because it suppresses intermolecular interactions among aggregation-prone molecules.

Thus, the results presented in this paper may give additional information for elucidating the mechanism which can explain how arginine suppresses aggregation.

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