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ORIGINAL ARTICLE

L-Arginine induces protein aggregation and transformation of supramolecular structures of the aggregates

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Abstract Protein misfolding, self-assembly, and aggregation are an essential problem in cell biology, biotechnology, and biomedicine. The protein aggregates are very different morphologically varying from soluble amorphous aggregates to highly ordered amyloid-like fibrils. The objective of this study was to elucidate the role of the amino acid L-arginine (Arg), a widely used suppressor of protein aggregation, in the regulation of transformations of soluble aggregation-prone proteins into supramolecular structures of higher order. However, a striking potential of Arg to govern the initial events in the process of protein aggregation has been revealed under environment conditions where the protein aggregation in its absence was not observed. Using dynamic light scattering we have demonstrated that Arg (10–100 mM) dramatically accelerated the dithiothreitol-induced aggregation of acidic model proteins. The inhibitory effect on the protein aggregation was revealed at higher concentrations of Arg. Using atomic force microscopy it was shown that aggregation of α-lactalbumin from bovine milk induced upon addition of Arg reached a state of formation of supramolecular structures of non-fibrillar species profoundly differing from those of the individual protein in type, size, and shape. The interaction of another positively charged amino acid L-lysine with α-lactalbumin also resulted in profound acceleration of the aggregation process and transformation of supramolecular structures of the aggregates.

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Abbreviations

DLS	Dynamic light scattering
Arg	L-Arginine
Lys	L-Lysine
AFM	Atomic force microscopy
TEM	Transmission electron microscopy
DTT	Dithiothreitol
ADH	Alcohol dehydrogenase
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
R_h	Hydrodynamic radius
CD	Circular dichroism

Introduction

It is becoming increasingly clear that non-pathogenic proteins and peptides are able to form supramolecular assemblies of different morphology, from amorphous aggregates to amyloid-like fibrils. Though these structures are comparable with aggregates of polypeptides involved in pathogenesis of a great number of diseases known as "conformational diseases" (Stefani 2004; Artemova et al. 2008; Chiti and Dobson 2009; Maury 2009), in striking contrast to the disease-causing aggregates, there are supramolecular structures having beneficial biological activities.

In the light of new insights into the aggregate formation as a common structural feature of polypeptide chains and a physiologically relevant phenomenon, understanding molecular machineries involved in structural transformations of aggregates is of great importance for regulation of

properties and activity of supramolecular assemblies, which may influence many physiological or pathological events (Stefani and Dobson 2003; Chiti and Dobson 2006; Artemova et al. 2012).

These observations have awakened fresh interest in low-molecular weight biogenic agents to be considered as regulators of transformations of soluble aggregation-prone proteins into structures of higher order. In search for naturally occurring reagents, amino acids and their derivatives can meet the requirements for reliable research tools to study mechanisms of protein aggregation (Taneja and Ahmad 1994; Shiraki et al. 2005). Among many amino acids tested, L-arginine (Arg) is one of the most widely used agents effective in suppressing aggregation, assisting refolding of aggregated proteins, enhancing the solubility of aggregation-prone unfolded molecules and stabilization of proteins during storage (Arakawa and Tsumoto 2003; Tsumoto et al. 2004; Baynes et al. 2005; Lyutova et al. 2007; Li et al. 2010; Tomita et al. 2011; Kawasaki and Kamijo 2012).

It is assumed that these effects are based on the unique structure and physicochemical properties of Arg that possesses the most basic side chain with similarity to the denaturing agent guanidine and on its ability to bind to the polypeptide backbone, as well as to side chains of negatively charged and aromatic amino acids (Ishibashi et al. 2005; Shah et al. 2012). Although Arg has been extensively studied, the available data for development of appropriate molecular mechanisms by which Arg exerts its effects on aggregation in most cases have remained to be elucidated. Hence, more extensive study of the kinetics of protein aggregation in the presence of Arg is required to obtain a comprehensive understanding of the Arg mode of action.

In search for new evidence to the fascinating hypothesis that the amino acid has the potency to govern the initial events in the process of protein aggregation, we decided to look more closely at the structural transformation of the aggregates upon addition of Arg to the solution of a model protein. Our aim was to study in vitro how Arg can guide protein between different aggregation pathways under a variety of conditions, especially those that destabilize the state of a protein and promote aggregate formation.

The reason behind this choice is that Arg is a good model to explore the potential of low-molecular weight compounds having the electrostatic and hydrophobic properties to associate with aggregation-prone proteins and influence their folding state and morphology of the aggregates. We assume that appearance of a complementary amino acid in the environment of oppositely charged protein domains can induce structural transformations in a protein molecule.

Using dynamic light scattering (DLS) and atomic force microscopy (AFM), we present the results of investigation of a model test system, dithiothreitol (DTT)-induced aggregation of bovine milk α-lactalbumin, in the absence or presence of Arg. The effects of Arg have also been investigated using alternative protein test systems for identification of molecular recognition modules that promote amino acid–protein interactions. In a separate set of experiment, guanidine or L-lysine (Lys) were used instead of Arg to elucidate the role of the guanidinium group in the effects of Arg on protein aggregation. Transmission electron microscopy (TEM) was used to reveal morphological features of supramolecular structures of α-lactalbumin formed in the process of the Lys-induced aggregation.

Findings concerning molecular mechanisms of action of naturally occurring low-molecular weight agents on protein aggregation could provide clues for drug development to prevent protein "conformational diseases" in medical and pharmaceutical applications, as well as for designing effective additives for optimization of protein engineering processes and elaboration of novel biomaterials with properties analogous to natural protein assemblies.

Materials and methods

α-Lactalbumin from bovine milk, hen egg white lysozyme, alcohol dehydrogenase (ADH) from yeast *Saccharomyces cerevisiae*, Arg-HCl, Lys-HCl, guanidine-HCl, DTT and ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were obtained from Sigma. Mica (V-1 GRADE) for AFM was obtained from SPI Supplies, USA. All other chemicals used were of analytical grade. All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA).

Dynamic laser light scattering

For DLS measurements, a commercial instrument Photocor Complex was used (Photocor Instruments, USA; http://www.photocor.com) as described in our previous works where DLS was used for the study of the kinetics of DTT-induced aggregation of proteins (Lyutova et al. 2007; Bumagina et al. 2010; Artemova et al. 2011).

This system allows measuring both the light scattering intensity and the hydrodynamic radius (R_h) of particles in a wide range from 1 nm to 5 μm. An He–Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) has been used as a light source. To analyze the dependence of both the light scattering intensity and the R_h values of particles on time, we registered the aggregation of proteins at fixed temperatures. A quasi-cross-correlation photon counting system with two photomultiplier tubes was used, which allows to improve accuracy of measurement of small particles.

DynaLS software (Alango) was used for polydisperse analysis of DLS data. The scattering light was collected at the angle of 90° and the accumulation time of the autocorrelation function was 30 s.

Study of the kinetics of protein aggregation

The aggregation of α-lactalbumin, lysozyme, or ADH at the concentrations in the range of 0.1–2 mg/mL was followed in 25 mM sodium-phosphate buffer, pH 7.0, containing 120–150 mM NaCl, in the absence or presence of the amino acids (Arg, Lys), or guanidine at the concentrations in the range of 10–500 mM. Reduction of the proteins was initiated by adding DTT to the sample to a final concentration of 20 mM. The experiments were performed at 37 °C. Aliquots of Arg solutions were added into the cell either at the beginning of the incubation process, simultaneously with DTT, or at various stages of incubation of a reduced protein taken alone with DTT. In each experiment, the total volume in a cylindrical quartz cell with the internal diameter of 6.3 mm was 0.5 mL. To follow the aggregation of α-lactalbumin in its calcium-free state, 1 mM EGTA was added to the incubation medium. The representative kinetics of protein aggregation is demonstrated as reproducible results of 5–10 experiments.

Atomic force microscopy

Each sample of 5 μL of the original solution was placed on the surface of freshly cleaved mica, exposed for 4 min at room temperature, washed with double distilled water, and, after removal of excess water with filter paper, dried. Specimens were scanned using a commercial Scanning Probe atomic force Microscope Smart SPM AIST-NT (Advanced Integrated Scanning Tools for Nanotechnology, Zelenograd, Russia) in the tapping mode with fpN01HR cantilever (Nanotuning, Russia) having a tip radius curvature of about 1 nm, which is small, compared to the structures measured. Several images were measured for each sample. The obtained images were analyzed using the Gwiddion software (Czech Metrology Institute, Czech Republic).

Transmission electron microscopy

Samples containing the protein aggregates of α-lactalbumin were applied to a formvar- and carbon-coated 300-mesh copper grids and stained with 2 % phosphotungstic acid, pH 7.2. The transmission electron micrographs were visualized at nominal magnifications of ×30,000–50,000 using a JEOL JEM-100CX microscope (Japan) operating at 80 kV. The images were collected using a negative photographic film Kodak EL Camera and a flatbed scanner Umax Astra 6700 with a resolution of 600 dpi.

Circular dichroism (CD) measurements

CD spectra were recorded on the Chirascan CD spectrometer (Applied Photophysics Ltd., UK). Changes in the secondary structure of α-lactalbumin in the absence or presence of 10 mM Arg were monitored in the far-UV region (190–260 nm) in 25 mM sodium-phosphate buffer, pH 7.0, containing 120 mM NaCl, 1 mM EGTA, and 20 mM DTT at 37 °C in a temperature-controlled Quartz Suprasil cell of 0.1 mm path length (Helma Analytics). Spectra were recorded with a 0.5-nm resolution setting a 1.2 nm bandwidth and response time of 3 s. The concentration of the protein was determined by measurement of the absorbance at 280 nm. The α-lactalbumin concentration was 2 mg/mL. Each spectrum was an average of at least three scans with the baseline scan subtracted. The data are presented as total molar ellipticity [θ].

Calculations

Origin 7.0 (OriginLab Corporation, USA) software was used for the calculations.

Results

Acceleration of α-lactalbumin aggregation by Arg

A mammalian milk protein, α-lactalbumin, is a small acidic globular protein (theoretical p*I* is 4.8) with a molecular weight of 14.2 kDa containing four disulfide bonds and no free thiol groups (Christina et al. 2000). It demonstrates significantly different structural stability in its calcium-bound and calcium-free apo form. Under stress conditions, the Ca^{2+}-depleted form of α-lactalbumin attains a classical molten globule state (Kuwajima 1996).

To characterize the effects of Arg on the kinetics of aggregation of α-lactalbumin studied by DLS technique, we examined the dependences on time of the light scattering intensity and the hydrodynamic radius (R_h) of the aggregates formed in the process of incubation of the reduced α-lactalbumin at the concentration of 1 mg/mL. The level of aggregation of α-lactalbumin alone appears to be insignificant under incubation for at least 60 min (Fig. 1a, curve 3). Upon addition to the incubation mixture of Arg at the final concentration of 100 mM, the aggregation process was dramatically accelerated.

A representative example of this phenomenon demonstrates that upon addition of Arg to the solution of α-lactalbumin after its incubation in the presence of DTT for

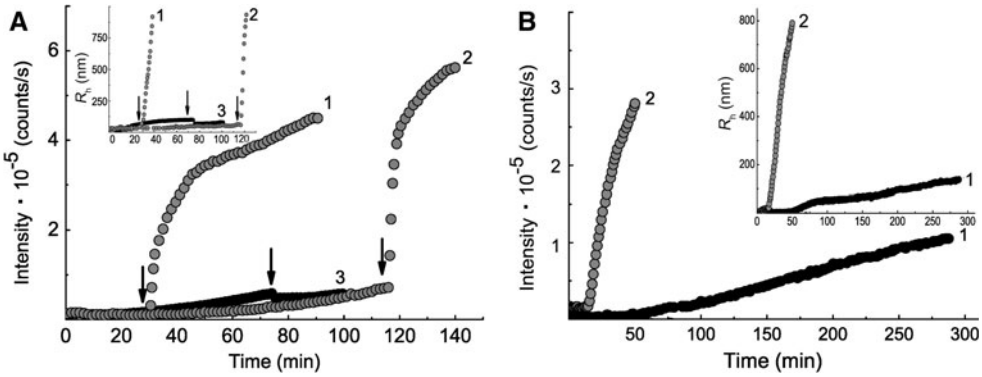

Fig. 1 The effect of L-Arg on the kinetics of aggregation of α-lactalbumin assessed by the dynamic light scattering technique. Dependences of the light scattering intensity (**a**) and the hydrodynamic radius (R_h) (*inset*) on time of the particles formed in the solution of α-lactalbumin (1 mg/mL) in the process of its incubation in the absence (curves 3) or presence (curves 1 and 2) of Arg. The incubation was carried out at 37 °C in 25 mM sodium-phosphate buffer, pH 7.0, containing 1 mM EGTA, 120 mM NaCl and 20 mM DTT. Arg at the final concentration of 100 mM was added to the samples after incubation of α-lactalbumin in the presence of DTT for 25 or 115 min (indicated by the arrows, curves 1 and 2, respectively). To the control sample, buffer was added instead of Arg after incubation of α-lactalbumin in the presence of DTT (indicated by the arrows, curves 3). (**b**) Dependences of the light scattering intensity and the hydrodynamic radius (R_h) (see inset) on time of the particles formed in the solution of α-lactalbumin (1 mg/mL) in the process of its incubation in the absence (curves 1) or presence (curves 2) of 100 mM Arg added to the sample at the beginning of the incubation process, simultaneously with DTT

about 25 min, the light scattering intensity of α-lactalbumin aggregates considerably increased (Fig. 1a, curve 1). The distribution of aggregates by their size with time varied in accordance with the kinetic curves of the light scattering intensity (Fig. 1a, inset, curve 1). Particles with R_h of >100 nm are immediately registered in the system. At further incubation, the population of nanoparticles of the aggregates was rapidly shifted to the level of higher hydrodynamic radii.

We have also demonstrated that addition of Arg (100 mM) to the solution of α-lactalbumin at a later stage of the incubation process induces the analogous changes in the kinetics of aggregation of the protein substrate. In this case, Arg was added to the solution of α-lactalbumin incubated in the presence of DTT for 115 min. The increase in the light scattering intensity (Fig. 1a, curve 2) and in the R_h values of the aggregates (Fig. 1a, inset, curve 2) on time is similar to those observed upon addition of Arg at the initial stage of the incubation process.

Analogous kinetics of aggregation was observed after the addition of Arg to the solution of α-lactalbumin at the very beginning of the incubation process, simultaneously with DTT (Fig. 1b). In this case, the Arg-induced dramatic increase in the light scattering intensity (curve 2) and in the R_h values of α-lactalbumin particles (inset, curve 2) was observed after a lag period of about 12 min, rather short in comparison with the control (curves 1). The initial rate of the aggregation process induced by Arg is significantly greater than that of the control. Thus, a striking potential of Arg, a widely used suppressor of protein aggregation, to induce paradoxical enhancement in aggregation of the model protein is demonstrated.

Morphology of the α-lactalbumin aggregates formed in the presence of Arg revealed by AFM

The samples containing α-lactalbumin and Arg were withdrawn from the incubation mixtures at the initial period of aggregation, when the light scattering intensity and the R_h values of the aggregates of α-lactalbumin considerably increased, namely after incubation of the protein in the presence of 100 mM Arg and DTT for 35 min (Fig. 1b, curves 2). Each sample of 5 μL of the original solution was placed on the surface of freshly cleaved mica, dried and subjected to AFM analysis. The AFM images of structures generated in the process of aggregation of α-lactalbumin in the presence of 100 mM Arg are demonstrated in Fig. 2.

Analysis of the AFM images of structures generated at the initial stage of the process of aggregation of α-lactalbumin (1 mg/mL) in the presence of Arg revealed formation of dispersed asymmetric granular units of 3–5 nm in apparent diameter, some of them being associated into rarely occurring short chains of 10–40 nm in length (Fig. 2a). Figure 2b demonstrates that along with small-sized species, unbranched chains of 50–100 nm in length composed predominantly of granular units of 10 nm in apparent diameter are formed. These chains packed at high density vary widely in length, suggesting that during prolonged incubation, the short species may bind together by end-to-end association, resembling pieces of necklace consisting of irregular-shaped beads termed "baroque pearl chains" (Fig. 2c). It should be mentioned, that the process of aggregation of α-lactalbumin taken alone developed very slowly and the structures generated in the control samples withdrawn from the incubation mixture at the

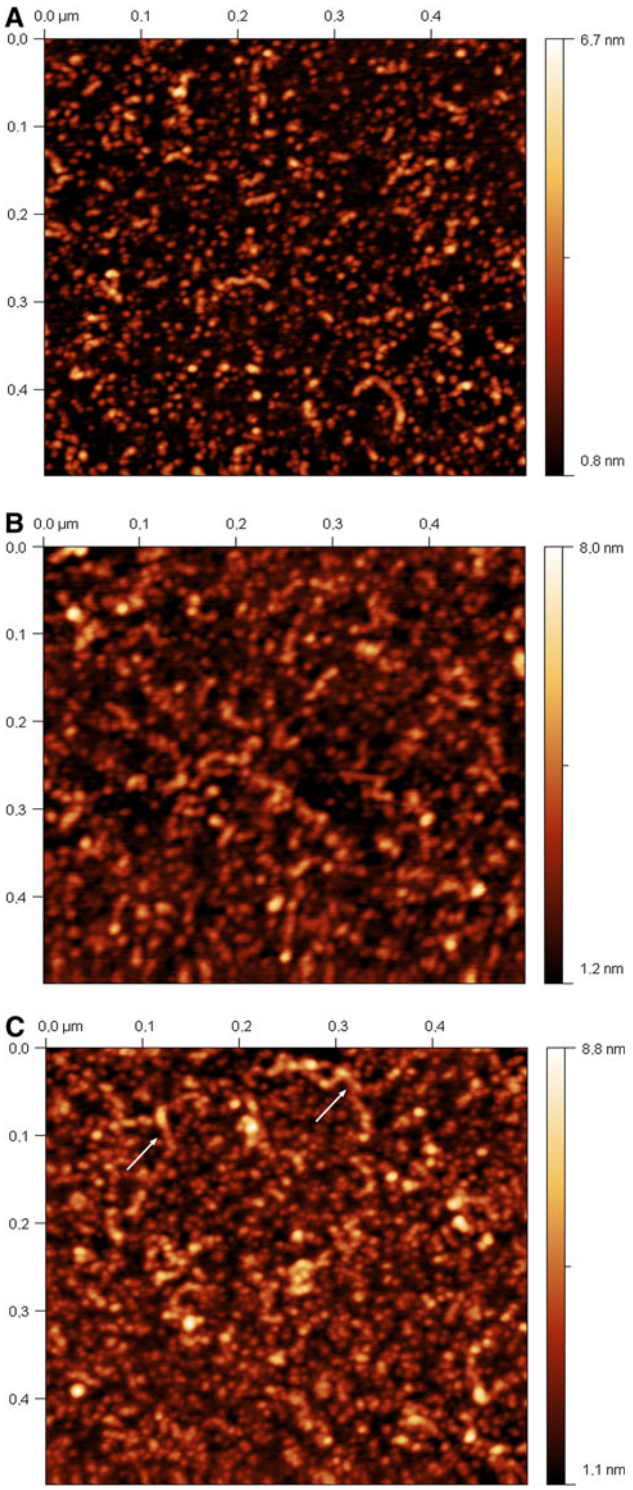

Fig. 2 The representative AFM images of structures generated in the process of aggregation of α-lactalbumin (1 mg/mL) in the presence of 100 mM Arg. Formation of dispersed asymmetric granular units of 3–5 nm in apparent diameter, some of them being associated into rarely occurring short chains of 10–40 nm in length (**a**), unbranched chains of 50–100 nm in length composed predominantly of granular units of 10 nm in apparent diameter (**b**), the short species bound together by end-to-end association to form the longer chains resembling "pieces of necklace", consisting of irregular-shaped beads (shown by *arrows* in **c**)

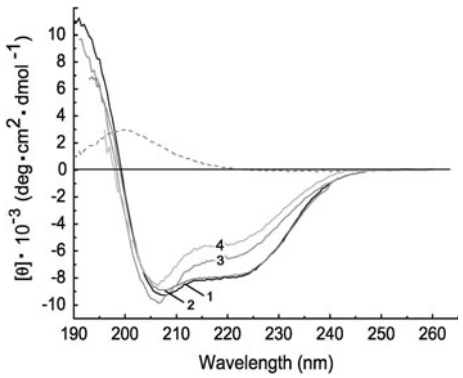

Fig. 3 Effect of Arg on the secondary structure of α-lactalbumin monitored by far-UV CD (190–260 nm). The CD spectrum of α-lactalbumin at a concentration of 2 mg/mL taken alone (*1*); α-lactalbumin incubated with 10 mM Arg (*2*); α-lactalbumin incubated with 20 mM DTT in the absence (*3*) or presence (*4*) of Arg. The spectra 2 and 4 correspond to the difference spectra obtained by subtracting the individual spectrum of Arg (*dotted line*) from that of a mixture of α-lactalbumin and Arg measured in the absence or presence of DTT, respectively. Experiments were performed at 37 °C in 25 mM sodium-phosphate buffer, pH 7.0, containing 1 mM EGTA and 120 mM NaCl

initial stage of the aggregation process appeared in the form of amorphous aggregates (data not shown).

The AFM images show that in the presence of Arg at low concentrations morphologically distinct supramolecular structures are formed, profoundly differing by shape and size from those generated in the process of aggregation of α-lactalbumin in its absence.

The secondary structure of α-lactalbumin in the presence of Arg monitored by CD

An important question to be addressed is whether the observed effects are generated by initial transformation of the protein secondary structure upon addition of Arg. Using CD spectroscopy in the far-UV region (190–260 nm) as a commonly applied technique for definition of changes in the secondary structure of proteins, we studied the conformational states of α-lactalbumin in the absence or presence of Arg.

The CD spectrum of native-like α-lactalbumin exhibited a strong negative ellipticity in the region 208–222 nm, characteristic of a protein having α-helix structure (Fig. 3, curve 1). The spectrum of α-lactalbumin incubated in the presence of Arg appears to be similar to that of native-like α-lactalbumin suggesting that addition of Arg did not change the secondary structure of the protein (curve 2). Incubation of α-lactalbumin with 20 mM DTT resulted in a decrease in the negative ellipticity at 222 nm indicating a partial loss of the α-helix structure at the initial stage of the DTT-induced protein denaturation (curve 3). In the presence of DTT and 10 mM Arg, detectable changes in the

CD spectrum of the α-lactalbumin under the same conditions were observed, with substantial amount of the secondary structure being retained (curve 4). This spectrum represents an intermediate state in the unfolding transition between the dominant α-helix and fully unfolded conformation of the protein molecule.

In the absence of DTT, Arg may interact with the acidic residues on the surface of the native-like protein and predispose its structure to transformation into a molten globule-like state, as a result of changes in overall net charge and hydrophobicity of the protein molecule. In this case, the CD spectrum (curve 2) appears to be identical to that of the folded protein (curve 1), the result being very close to data previously reported by other authors who showed that CD spectra of bovine α-lactalbumin in the molten globule and native states are similar (Permyakov and Berliner 2000; Vassilenko and Uversky 2002). However, the changes observed in the CD spectrum of α-lactalbumin measured in the presence of DTT and Arg (curve 4) suggest that Arg may accelerate the aggregation process as a result of its preferential binding to unfolded or partially folded intermediates of the DTT-reduced protein.

The concentration-dependent effect of Arg on the aggregation of α-lactalbumin, lysozyme and alcohol dehydrogenase

To modulate the effects of Arg playing the roles of accelerator or suppressor of protein aggregation, we designed a series of experimental conditions by using a number of different proteins, varying protein and Arg concentrations, as well as NaCl content in the sodium-phosphate buffer composition.

The analyses of the kinetics of aggregation of α-lactalbumin have shown that addition of Arg accelerated the aggregation process in a concentration-dependent manner (Fig. 4a–d). The representative kinetic curves of the aggregation of α-lactalbumin (2 mg/mL) demonstrate a potential of Arg at a concentration as low as 10 mM to induce aggregation of the model protein under experimental conditions where the protein aggregation in its absence was not observed. At this concentration, an increase in both the aggregation rate and the hydrodynamic radius values and a decrease in the lag period were observed (Fig. 4a, b, curves 2). Addition of Arg at the concentration of 100 mM results in a dramatic acceleration of the aggregation process (curves 3).

At higher salt concentrations (150 mM NaCl), the light scattering intensity and the values of the hydrodynamic radius (R_h) of the particles formed in the solution of α-lactalbumin either in the absence or presence of Arg substantially enhanced (Fig. 4c, d), probably because the addition of NaCl masks the repulsive electrostatic interaction between the charged groups. However, the concentration-dependent acceleration of the aggregation process by Arg in the low concentration range of 10–100 mM could still be detected (Fig. 4c, d, curves 2 and 3). At the concentrations exceeding 200 mM, Arg inhibited aggregation in a concentration-dependent manner (curves 4 and 5). The aggregation process was fully suppressed in the presence of 1 M Arg (data not shown).

It is interesting to note that at the intermediate concentration of 200 mM, Arg combines the roles of accelerator and suppressor of protein aggregation. The amino acid decreased the lag period of aggregation, on the one hand, and decreased the light scattering intensity and the values of the hydrodynamic radius (R_h) of the particles, on the other hand (curves 4).

For comparison, in a separate set of experiment, we have used a small highly basic globular protein (pI 11), hen egg white lysozyme, as a model protein instead of α-lactalbumin. Although these two oppositely charged globular proteins containing 129 and 123 amino acids, respectively, present homologous three-dimensional structures, including four disulfide bonds (McKenzie and White 1991; Iyer and Qasba 1999), no acceleration of lysozyme aggregation could be demonstrated in the presence of low concentrations of Arg under the same experimental conditions. In contrast to α-lactalbumin, Arg at the concentrations of 10 and 100 mM caused suppression of lysozyme aggregation (Fig. 4e, f, curves 2 and 3, respectively), suggesting a critical role for negatively charged residues of the protein substrate structure in the Arg accelerating effect on the aggregation development.

This suggestion was supported by the dependences on time of the light scattering intensity and the hydrodynamic radius (R_h) of the particles formed in the process of incubation of ADH in the absence or presence of Arg at the concentrations in the range of 100–500 mM. The yeast ADH molecule of about 36.7 kDa is a homooligomer composed of four subunits with a high acidic residue content (theoretical pI 5.4). The dual effects of Arg on the kinetics of aggregation of ADH were observed: the addition of Arg at lower concentrations (100 mM) caused a substantial increase in the aggregation rate and a decrease in the lag period, whereas Arg at higher concentrations inhibited the aggregation process, the results being quite similar to those observed in the case of α-lactalbumin (see Fig. 4c, d).

Effects of guanidine hydrochloride on the α-lactalbumin aggregation

Taking into consideration the unique structure of Arg that possesses the side chain with similarity to the denaturing agent guanidine, we examined the effects of guanidine hydrochloride on the kinetics of α-lactalbumin aggregation.

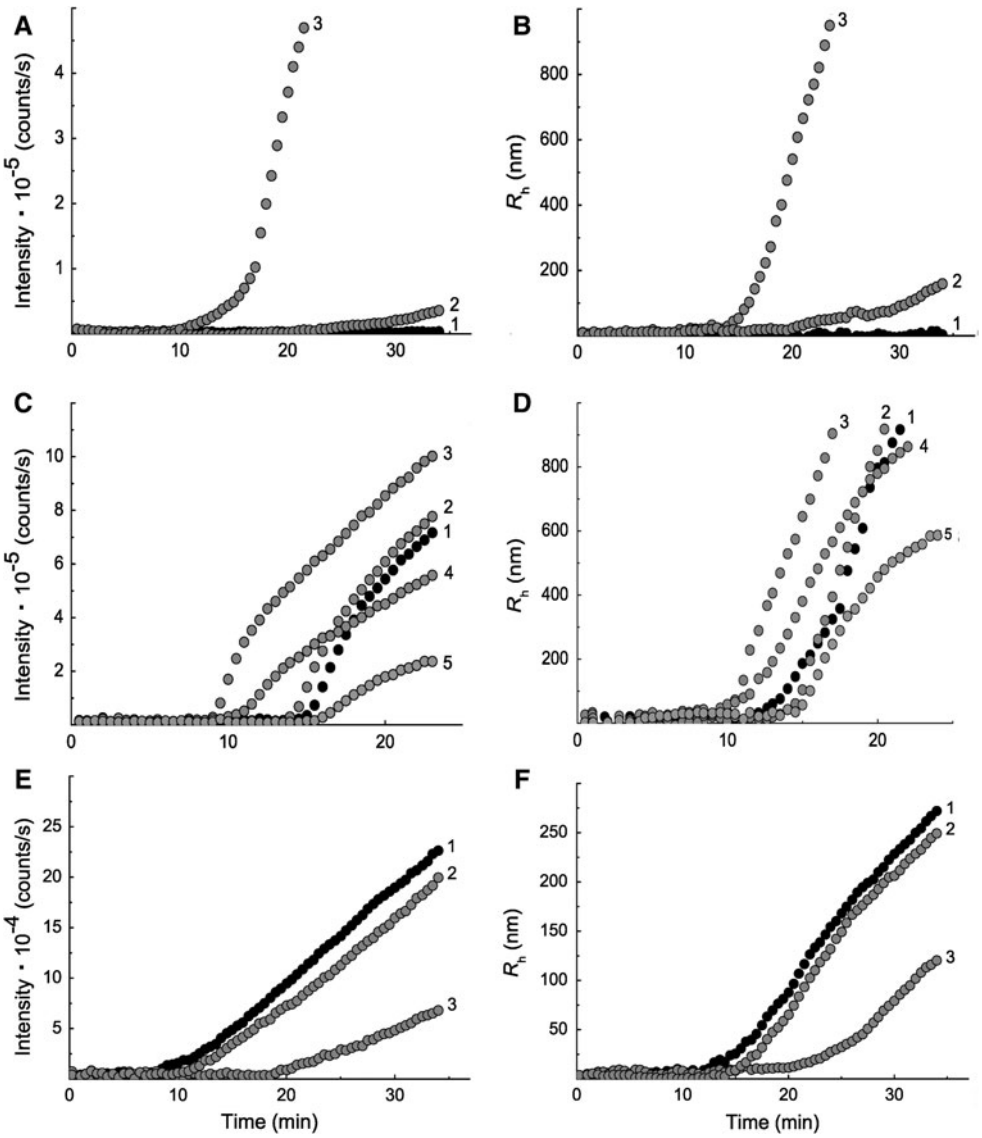

Fig. 4 The concentration-dependent effect of L-Arg on the kinetics of aggregation of α-lactalbumin and lysozyme. Dependences of the light scattering intensity (**a**, **c**) and the hydrodynamic radius (R_h) (**b**, **d**) on time of the particles formed in the solution of α-lactalbumin (2 mg/mL) in the process of its incubation in the absence (*curves 1*) or presence of Arg at the concentrations of 10 and 100 mM (**a** and **b**, *curves 2* and *3*, respectively); **c** and **d** α-lactalbumin was incubated in the absence (*curves 1*) or presence of Arg at the concentrations of 10, 100, 200 and 500 mM (*curves 2*, *3*, *4* and *5*, respectively). Arg was added to the samples at the beginning of the incubation process, simultaneously with DTT. The incubation was carried out at 37 °C in 25 mM sodium-phosphate buffer, pH 7.0, containing 1 mM EGTA, 20 mM DTT and 120 mM NaCl (**a**, **b**) or 150 mM NaCl (**c**, **d**). Dependences of the light scattering intensity (**e**) and the hydrodynamic radius (R_h) (**f**) on time of the particles formed in the solution of lysozyme (0.2 mg/mL) in the absence (*curves 1*) and presence of Arg at the concentrations of 10 and 100 mM (*curves 2 and 3*, respectively). The incubation was carried out at 37 °C in 25 mM sodium-phosphate buffer, pH 7.0, containing 20 mM DTT and 150 mM NaCl

The results show that guanidine at low concentrations accelerates, whereas at higher concentration inhibits the DTT-induced aggregation process.

A representative example of this phenomenon is demonstrated in Fig. 5a, b. The analysis of the light scattering intensity and the R_h values of the aggregates show that the addition of guanidine at the concentration of 100 mM results in a substantial increase in the aggregation rate and a decrease in the lag period (curves 2). However, the aggregation process was fully suppressed in the presence of 500 mM guanidine (curves 3). Thus, guanidine has a dual role in modulating protein aggregation, the result being similar to that observed in the case of Arg.

Effects of Lys on protein aggregation

To test whether the property of Arg to promote protein aggregation is related to the guanidinium moiety, we

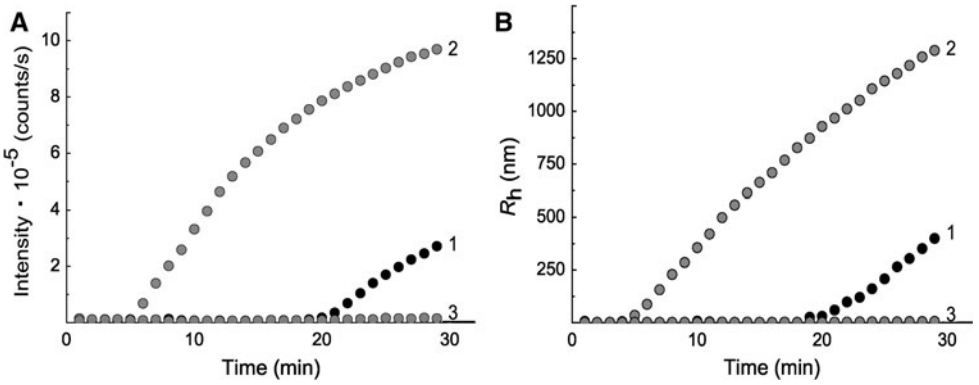

Fig. 5 Dependences of the light scattering intensity (**a**) and the hydrodynamic radius (R_h) (**b**) on time of the particles formed in the solution of α-lactalbumin (1 mg/mL) in the process of its incubation in the absence (*curves 1*) or presence of guanidine hydrochloride at the concentrations of 100 and 500 mM (*curves 2* and *3*, respectively). Guanidine was added to the samples at the beginning of the incubation process, simultaneously with DTT. The incubation was carried out at 37 °C in 25 mM sodium-phosphate buffer, pH 7.0, containing 1 mM EGTA, 20 mM DTT and 150 mM NaCl

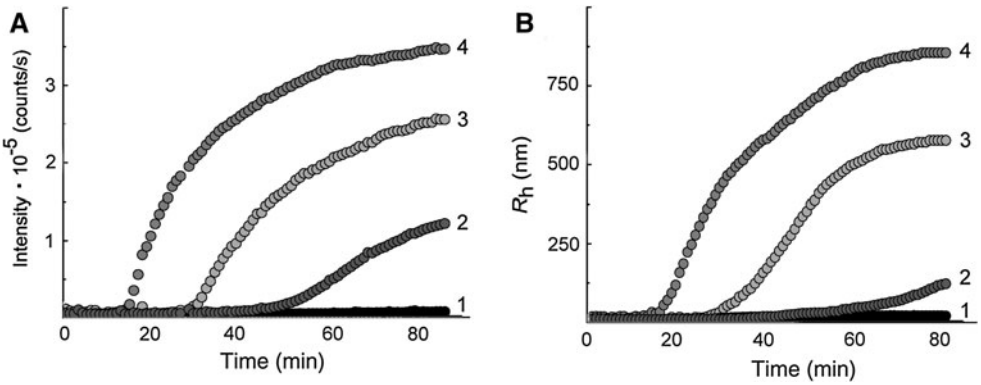

Fig. 6 The concentration-dependent effect of L-Lys on the kinetics of aggregation of α-lactalbumin. Dependences of the light scattering intensity (**a**) and the hydrodynamic radius (R_h) (**b**) on time of the particles formed in the solution of α-lactalbumin (1 mg/mL) in the process of its incubation in the absence (*curves 1*) or presence of Lys at the concentrations of 50, 100 and 300 mM (*curves 2, 3* and *4*, respectively). Lys was added to the samples at the beginning of the incubation process, simultaneously with DTT. The incubation was carried out at 37 °C in 25 mM sodium-phosphate buffer, pH 7.0, containing 1 mM EGTA, 20 mM DTT and 120 mM NaCl

studied the kinetics of aggregation of α-lactalbumin in the absence or presence of another positively charged amino acid, Lys, instead of Arg. Under experimental conditions used in this study, the aggregation of α-lactalbumin alone was not observed under incubation for at least 90 min (Fig. 6a, b, curves 1). A representative example of the kinetics of aggregation demonstrates that upon addition to the solution of α-lactalbumin of Lys at the concentrations in the range of 50–300 mM, the light scattering intensity of the aggregates gradually increased and the lag period of aggregation decreased in a concentration-dependent manner (Fig. 6a, curves 2–4). The distribution of aggregates by their size with time varied in accordance with the kinetic curves of the light scattering intensity (Fig. 6b, curves 2–4). At further incubation, the population of particles of the aggregates was rapidly shifted to the level of higher hydrodynamic radii.

The effects of Lys on the aggregation of ADH were found to be similar to those of α-lactalbumin (data not shown). It is interesting to note that in contrast to the action of Arg, no suppression of the aggregation of either α-lactalbumin or ADH was observed, even at concentrations of Lys as high as 1–2 M. The concentration-dependent enhancement of the aggregation of α-lactalbumin or ADH by Lys was demonstrated at all tested concentrations of the proteins, suggesting that the guanidinium group's interactions with amino acid residues of the model protein may not necessarily be behind the ability of Arg to play the role of an accelerator of protein aggregation.

Morphology of the α-lactalbumin aggregates formed in the presence of Lys revealed by TEM

Formation of aggregates of α-lactalbumin (1 mg/mL) in the presence of 100 mM Lys was assessed in protein samples withdrawn from the incubation mixture containing all components used for DLS measurements, as described above. The samples were taken at the points in time, at

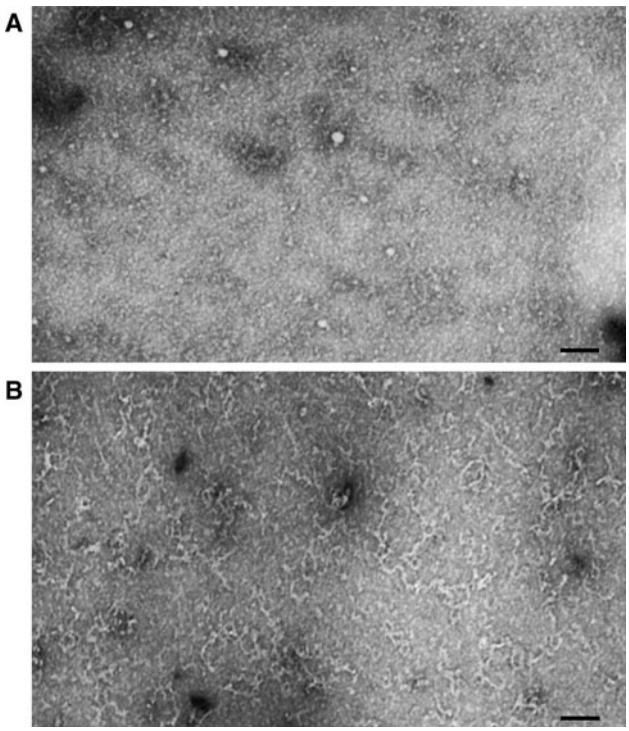

Fig. 7 Transmission electron micrographs of aggregates generated in the process of incubation of α-lactalbumin (1 mg/mL) in the presence of Lys (100 mM). Formation of amorphous granular units of 3–5 nm in apparent diameter, most of them being associated into short chains of 10–40 nm in length or rarely occurring compact circular-shaped aggregates of 10–20 nm (**a**); the longer chains of 100–500 nm in length composed predominantly of granular units of 3–5 nm (**b**). The *scale bars* represent 100 nm

which the R_h values reach the maximum level, namely after incubation for 80 min (see Fig. 6b, curve 3), and the structures of aggregates were visualized using TEM.

Analysis of the electron micrographs revealed formation of amorphous granular units of 3–5 nm in apparent diameter, most of them being associated into short chains of 10–40 nm in length or rarely occurring compact circular-shaped aggregates of 10–20 nm (Fig. 7a). Figure 7b demonstrates that along with small-sized species, the longer chains of 100–500 nm in length composed predominantly of units of 3–5 nm in apparent diameter are formed.

Although further experiments using AFM and TEM imaging of protein samples are required to explore the generality of these observations, the results obtained under experimental conditions of the present study demonstrate the structural similarity of the α-lactalbumin aggregates generated in the presence of Arg or Lys.

Discussion

Our observations add new evidence to the fascinating hypothesis that an amino acid has the potency to govern the initial event in the process of aggregation and formation of supramolecular structures of a destabilized model protein. The most interesting result is the demonstration that aggregation of α-lactalbumin induced upon addition of Arg rapidly reaches a state of formation of aggregate structures profoundly differing from those of the individual protein substrate. In the presence of 100 mM Arg, the AFM images revealed the predominant formation of structures resembling "baroque pearl chains".

It should be noted that the "baroque pearl chains" of protofibrills of pathogenic Alzheimer's β-amyloid peptide Ab(1–42), occurring at the early stages of aggregation, similar to those revealed in the present study with the individual non-pathogenic protein substrate α-lactalbumin, have been previously demonstrated using AFM technique (Kowalewski and Holtzman 1999). In particular, the length of linear aggregates was about 100 nm, the apparent width was typically between 10 and 15 nm, and the height rarely exceeded 5 nm. It was suggested that the aggregates were formed by association of smaller, globular, or ellipsoidal species. Moreover, based on AFM imaging in situ under aqueous solution on hydrophilic mica, the authors also demonstrated the formation of pseudomicellar aggregates, which at higher Ab(1–42) concentrations had the tendency to form assemblies, reminiscent of protofibrils.

It seems more appropriate to term such assemblies "transient suprastructures", instead of "protofibrils", as in many cases, they may have no tendency for gradual transformation into mature fibrils. The biological functions of a great variety of the transient and miscellaneous oligomeric states of "intermediate" non-fibrillar species have remained relatively unexplored so far and inspired a special interest. Arg and other agents affecting assembly or disassembly of "transient suprastructures" should be valuable in the design of suppressors of the fibrillization process.

A number of studies have been described in the literature on the effects of small molecules of biological origin including nucleotides, polyamines, polyanions, phosphates, polyvalent metal ions, carbohydrates, osmolytes, etc., on the protein aggregation processes (Uversky et al. 2001; Kudou et al. 2003; Bouma et al. 2003; Natalello et al. 2009). However, the efficiency of the additives depends on the nature and structure–functional peculiarities of the protein substrate; protein concentration; aggregate types, size, and solubility; varying environmental conditions, such as pH, ionic strength, temperature, salt type and concentration.

The mechanisms underlying the Arg-induced transformation of protein aggregates from an amorphous state to a highly ordered supramolecular state remain unknown. Our results partially support the previously reported data concerning the potency of Arg to bring about significant changes in the quaternary structure of α-crystallin leading

to an increase in the exposure of its hydrophobic surfaces and chaperone-like activity, but to a decrease in molecular mass of the oligomeric structure and polydispersity of the protein (Srinivas et al. 2003).

Though Arg has been widely used as a suppressor of protein aggregation and an enhancer of refolding yield and solubility of proteins, in the present paper we demonstrate the suppression or acceleration of the aggregation of model proteins at high or low concentrations of Arg, respectively. The mechanisms underlying the dual effect of Arg involve, on the one hand, the electrostatic interactions with negatively charged protein domains that result in initiation or acceleration of the aggregation process, probably because the addition of Arg masks the repulsive electrostatic interaction between the protein charged groups and changes the total hydrophobicity of Arg–protein complexes via the aliphatic structure of Arg. Moreover, the guanidinium group, although charged, is poorly hydrated in aqueous solutions and thus partially exhibits a hydrophobic behavior (Mason et al. 2004).

On the other hand, the suppression of protein aggregation might be attributed to the deceleration of protein–protein association reactions as a result of the interactions between the guanidinium group of Arg with the aromatic residues of a protein molecule, which are buried inside the native protein or involved in intramolecular interactions, but which would be localized at the surface of the molecule under stress conditions (Baynes et al. 2005). The fact that guanidine hydrochloride but not Lys suppresses the protein aggregation, supports these observations. It was also reported previously that the aromatic residues of proteins have a significantly stronger interaction with the guanidinium group than with the aliphatic segment of Arg and that the terminal ends of Arg have the least association with the aromatic residues (Shah et al. 2012).

There are a few other studies concerning the ability of Arg to accelerate aggregation or disturb the structure of some proteins that seems to contradict the generally accepted belief that Arg is a universal suppressor of aggregation. It has recently been demonstrated that Arg can enhance or suppress heat-induced aggregation of concentrated protein solutions depending on the structure and concentration of the model protein substrate (Shah et al. 2011). Arg was also found to exert protein-denaturing effects on aminoacylase similar to those of guanidine hydrochloride, which might help to understand the mechanism by which Arg suppresses incorrect refolding (Xie et al. 2004). Moreover, Arg was reported recently to exert its destabilizing effects on the conformational stability of solvent-exposed regions in an IgG1 monoclonal antibody subjected to elevated temperatures (Thakkar et al. 2012), promote the heat-induced unfolding and aggregation of human growth hormone and interferon-α2b (Cirkovas et al. 2011), or interfere with thioflavin T fluorescence assay for detection of amyloid fibril formation by bovine serum albumin (Liu et al. 2010).

In conclusion, the data presented here suggest that the Arg-induced transformation of the protein structure may be of interest for generating the aggregation process and fabricating novel supramolecular materials, under environment conditions where the aggregation of the protein substrate in the absence of the amino acid was not observed. Such a strategy could be of great value in medical, pharmaceutical and biotechnological application (Morozova-Roche et al. 2007; Arakawa et al. 2007).

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Conflict of interest The authors declare that they have no conflict of interest.

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