Biophysical Chemistry 146 (2010) 108–117

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Mechanism of suppression of dithiothreitol-induced aggregation of bovine α-lactalbumin by α-crystallin

Zoya M. Bumagina [a], Bella Ya. Gurvits [a,*], Natalya V. Artemova [a], Konstantin O. Muranov [b], Igor K. Yudin [c], Boris I. Kurganov [a]

[a] A.N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky prospect 33, Moscow 119071, Russia
[b] Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, Kosygina 4, Moscow 119991, Russia
[c] Oil and Gas Research Institute, Russian Academy of Sciences, Gubkina 3, Moscow 117971, Russia

ARTICLE INFO

Article history:
Received 14 September 2009
Received in revised form 10 November 2009
Accepted 11 November 2009
Available online 14 November 2009

Keywords:
α-Crystallin
α-Lactalbumin
Aggregation
Molecular chaperone
Dynamic light-scattering

ABSTRACT

The kinetics of dithiothreitol (DTT)-induced aggregation of α-lactalbumin from bovine milk has been studied using dynamic light-scattering technique. Analysis of the distribution of the particles formed in the solution of α-lactalbumin after the addition of DTT by size showed that the initial stage of the aggregation process was the stage of formation of the start aggregates with the hydrodynamic radius (R_h) of 80–100 nm. Further growth of the protein aggregates proceeds as a result of sticking of the start aggregates. Suppression of α-lactalbumin aggregation by α-crystallin is mainly due to the increase in the duration of the lag period on the kinetic curves of aggregation. It is assumed that the initially formed complexes of unfolded α-lactalbumin with α-crystallin were transformed to the primary clusters prone to aggregation as a result of the redistribution of the denatured protein molecules on the surface of the α-crystallin particles.

1. Introduction

When studying the kinetics of thermal aggregation of proteins using dynamic light-scattering (DLS), it was demonstrated that the initial stage of the aggregation process was formation of the start aggregates [1,2]. Each start aggregate contains hundreds of denatured protein molecules. DLS measurements provide a simultaneous registration of the native forms of protein substrates and protein aggregates. The intermediate states between the native enzyme forms and start aggregates were not detected. The hydrodynamic radius of the start aggregates was tens of nanometers. The start aggregate formation was revealed in the process of thermal aggregation of the following model substrates: β_L-crystallin from bovine lens [1], glyceraldehyde-3-phosphate dehydrogenase and glycogen phosphorylase *b* from rabbit skeletal muscle [2–6], aspartate aminotransferase from pig heart mitochondria [7] and tobacco mosaic virus coat protein [8].

α-Crystallin, a member of small heat shock protein family, functions as a molecular chaperone by interacting with unfolded or misfolded proteins to prevent their aggregation [9]. It has a broad molecular mass distribution of 300 to 1000 kDa and forms large oligomer assemblies that display great heterogeneity [10]. In the presence of α-crystallin deceleration of thermal aggregation was observed accompanied by a decrease in the hydrodynamic radius of the start aggregates [1,7]. It should be noted that the chaperone-like activity of α-crystalline is enhanced at temperatures above 30 °C [11,12]. Therefore, to avoid complication at interpreting the results of thermal aggregation experiments, another way to denature model proteins should be used.

It is of special interest to elucidate whether the mechanism of aggregation involving the stage of formation of the start aggregates is fulfilled in the case of dithiothreitol (DTT)-induced aggregation of model substrates containing disulfide bonds, such as α-lactalbumin, insulin, and lysozyme. It should be noted that α-crystallin itself is not susceptible to DTT since it does not contain disulfide bonds.

A mammalian milk protein, α-lactalbumin, is a small (14.2 kDa) calcium-binding protein containing four disulfide bridges. It demonstrates a 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 [13,14]. α-Lactalbumin partially denatured by reducing its disulfide bonds by DTT is widely used as a simple model substrate in folding studies [15–18]. A detailed study has been performed using DLS measurements to gain insight into the kinetics of DTT-induced aggregation of α-lactalbumin and to record size distribution of particles in the absence and presence of α-crystallin [19–21]. However, the mechanisms involved in aggregation of α-lactalbumin under stress conditions and in the interaction of α-crystallin with a denatured substrate thereby preventing its uncontrolled aggregation are far from being clear.

In the present work the kinetics of DTT-induced aggregation of α-lactalbumin was studied at various concentrations of the protein

Abbreviations: DLCA, diffusion-limited cluster–cluster aggregation; DLS, dynamic light scattering; DTT, dithiothreitol; RLCA, reaction-limited cluster–cluster aggregation.

* Corresponding author. Tel.: +7 495 954 3066; fax: +7 495 954 2732.
E-mail address: bella@inbi.ras.ru (B.Y. Gurvits).

0301-4622/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2009.11.002

using DLS. Analysis of the distribution of the particles formed in the solution of α-lactalbumin after the addition of DTT by size allowed us to conclude that the aggregation proceeds through the stage of formation of the start aggregates with the hydrodynamic radius (R_h) of 80–100 nm. Character of the dependence of the hydrodynamic radius of the particles formed in the presence of α-crystallin indicated that immediately after the addition of DTT the complexes of unfolded α-lactalbumin with α-crystallin were formed in the system. It is assumed that the duration of the lag period on the kinetic curves of aggregation obtained in the presence of α-crystallin is determined by the transformation of the α-crystallin–unfolded α-lactalbumin complexes into the primary clusters (with $R_h \approx 20$ nm) prone to aggregation as a result of the redistribution of the unfolded α-lactalbumin molecules on the surface of the α-crystallin particles.

2. Materials and methods

2.1. Materials

Type I α-lactalbumin from bovine milk, DTT and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Sigma. All other chemicals were of reagent grade. All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA).

2.2. Isolation and purification of α-crystallin

Purification of α-crystallin from freshly excised lenses of 2-year-old steers was performed according to the procedure described earlier [22]. The α-crystallin concentration was determined from the absorbance at 280 nm using the extinction coefficient $A^{1\%}_{280}$ of 8.5 [23].

2.3. DTT-induced aggregation of α-lactalbumin

Aggregation of α-lactalbumin (0.2–1.6 mg mL^{-1}) in the absence or presence of varying concentrations of α-crystallin was studied in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA. Reduction of α-lactalbumin was initiated by adding DTT to 0.5 mL of the sample to a final concentration of 20 mM. The experiments were performed at 37 °C.

2.4. Dynamic light-scattering studies

DLS measurements were performed by a commercial instrument Photocor Complex (Photocor Instruments Inc., USA; www.photocor.com) as described in our previous works where DLS was used for the study of the kinetics of thermal aggregation of proteins [1,4,5,24]. 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 derivative (PID) temperature controller to within ±0.1 °C. The 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 multifunctional real-time correlator Photocor-FC. DynaLS software (Alango, Israel) was used for polydispersity analysis of the DLS data. The diffusion coefficient D of the particles is directly related to the decay rate τ_c of the time-dependent correlation function for the light-scattering intensity fluctuations: $D = 1/2\tau_c k^2$. In this equation k is the wavenumber of the scattered light, $k = (4\pi n/\lambda)\sin(\theta/2)$, where n is the refractive index of the solvent, λ is the wavelength of the incident light in vacuum and θ is the scattering angle. The mean hydrodynamic radius of the particles (R_h) can then be calculated according to 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 η is the shear viscosity of the solvent.

To analyze the time-course of the increase in the light-scattering intensity (I) accompanying aggregation of α-lactalbumin, we used the approaches elaborated by us previously [25]. To calculate the duration of the lag period (t_2) on the dependences of the I value on time, we used the empiric equation:

$$I = [K_{LS}(t - t_2)]^2, \quad (1)$$

where K_{LS} is a constant with the dimension (counts/s)$^{1/2}$ min^{-1} (index LS means "Light Scattering"). It should be noted that in the present work the duration of the lag period was designated as t_2, in order to preserve the notation used by us earlier [2].

When studying the kinetics of thermal aggregation of proteins, we observed that the typical dependences of the hydrodynamic radius (R_h) of the protein aggregates on time were as follows. The initial parts of the dependences of R_h on time were linear. The following equation may be used for the description of these parts of the dependences of R_h on time [1]:

$$R_h = R_{h,0}\left[1 + \frac{t - t_3}{t_{2R}}\right], \quad (2)$$

where $R_{h,0}$ is the hydrodynamic radius of the protein aggregates at $t = t_3$, i.e., at the moment of time at which the hydrodynamic radius of the protein aggregates begins to increase (the notation proposed in [2] is used), and t_{2R} is the time interval over which the hydrodynamic radius increases from $R_{h,0}$ to $2R_{h,0}$. The reciprocal value of parameter t_{2R} characterizes the rate of aggregation. The higher the $1/t_{2R}$ value, the higher is the rate of aggregation.

Analysis of the full dependences of R_h of the protein aggregates for thermal aggregation of Ph*b* on time [1,3,5–8] showed that above the definite point of time designated as t^* the dependences of R_h on time followed the power law:

$$R_h = R_h^*[1 + K_1(t - t^*)]^{1/d_f}, \quad (3)$$

where R_h^* is the R_h value at $t = t^*$, K_1 is a constant and d_f is the fractal dimension of the aggregates. The d_f value was found to be close to 1.8. This value of d_f is typical of the regime of diffusion-limited cluster–cluster aggregation (DLCA), wherein each collision of the interacting particles results in their sticking [26–28].

In the presence of α-crystallin a decrease in the rate of aggregation of the protein substrates occurs, and the dependence of the hydrodynamic radius of the protein aggregates on time becomes exponential [1,5,7]:

$$R_h = R_{h,0}\left\{\exp\left[\frac{\ln 2}{t_{2R}}(t - t_3)\right]\right\}, \quad (4)$$

where t_3 is the moment of time at which the R_h value begins to increase and t_{2R} is the time interval over which the R_h value is doubled. Such a character of the dependence of R_h on time indicates that the aggregation process proceeds in the regime of reaction-limited cluster–cluster aggregation (RLCA), wherein the sticking probability for the colliding particles is lower than unity [28].

2.5. Fluorescence spectroscopy

The tryptophan fluorescence measurements were performed using a fluorescence spectrophotometer Cary eclipse (USA) with a temperature-controlled cuvette holder. Samples were excited at 297 nm and fluorescence emission spectra were recorded from 300 to 400 nm. The excitation and emission bandwidths were set at 5 nm. Before measurement, protein samples (α-crystallin and/or α-lactalbumin) were preincubated for 30 min at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mM EGTA, in the absence or presence of 20 mM DTT. An aliquot of the incubation mixture was then added to 500 μL of the buffer to a final protein concentration of 0.1 mg mL^{-1}. The fluorescence measurements were performed using a quartz cuvette with a 1 cm path length. Each spectrum was the