Process Biochemistry 48 (2013) 250–259

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

An analysis of the factors that affect the dissociation of inclusion bodies and the refolding of endostatin under high pressure

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ARTICLE INFO

Article history:
Received 26 August 2012
Received in revised form
26 November 2012
Accepted 22 December 2012
Available online 3 January 2013

Keywords:
High hydrostatic pressure
Refolding
Endostatin
Inclusion bodies
Aggregates

ABSTRACT

An optimization of the refolding of endostatin (ES), by a study of the conditions that can affect (i) dissociation of inclusion bodies (IBs) and (ii) renaturation under high hydrostatic pressure (HHP), is described. IBs produced by bacteria cultivated at 25 °C were shown to be more soluble than those produced at 37 °C and their dissociation by application of 2.4 kbar at 20 °C was shown to be further enhanced at −9 °C. A red shift in intrinsic fluorescence spectra and an increase in binding of the hydrophobic fluorescent probe bis-ANS show subtle changes in conformation of ES in the presence of 1.5 M GdnHCl at 2.4 kbar, while at 0.4 kbar the native conformational state is favored. The 25% refolding yield obtained via compression of IBs produced at 37 °C by application of 2.4 kbar, was increased to 78% when conditions based on the insights acquired were utilized: dissociation at 2.4 kbar and −9 °C of the IBs produced at 25 °C, followed by refolding at 0.4 kbar and 20 °C. Besides providing insights into the conformational transitions of ES structure under HHP, this work proposes innovative conditions that are likely to have wide applicability to the HHP-induced refolding of proteins in general.

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1. Introduction

Many of the proteins with a biomedical relevance are found at low concentrations in their native sources. *Escherichia coli* is a microorganism that is extremely useful for producing proteins of commercial interest at large scale and for structural and functional studies. However, the production of recombinant polypeptides in *E. coli* frequently results in an incomplete folding process that usually leads to the accumulation of insoluble aggregates, known as inclusion bodies (IBs), in the cytoplasm or in the periplasmic space of the host cells. Therefore, refolding is often problematic and results in low yields of soluble and biologically active proteins.

The aggregation of heterologous proteins is caused by a high local concentration of nascent polypeptides on the ribosome [1], which are not protected from aggregation due to insufficient number of chaperones present during the overexpression of the recombinant protein or even to an absence of chaperones [2]. It has been shown that IBs contained ordered structural segments of cross-β structures with specific amino acid sequences, which were surrounded by folded domains or disordered segments. These β-strands do not necessary have a β-sheet conformation in the corresponding soluble folded form of the protein. The number of cross-β segments is variable, and their sizes typically range between 7 and 10 amino acid residues in length [3]. The proteins in the IBs were considered to be completely inactive. However, the current knowledge of IBs has evolved, and today, it is recognized that proteins within the IBs present a native-like structure [4]. An analysis by infrared spectroscopy (FTIR) showed that despite an increase in the intermolecular β-sheet structures in relation to the native states, proteins in the IBs presented native-like secondary structures [5,6]. In addition, a percentage of some of the proteins in the inclusion bodies were shown to have a native tertiary structure and biological activity [7,8]. The characteristics of the proteins within the IBs were shown to be affected by the cultivation temperature of the host bacteria. The bioactivity in the IBs had an inverse correlation with the temperature of the cultivation. During cultivation at high temperatures, intermolecular interactions are favored at the expense of native intramolecular contacts, and consequently, the IBs become more resistant to chemical denaturation. These results demonstrated that the dissociation of IBs produced at lower temperatures can be performed in milder conditions. Thus, the functional states of the IBs can be released using conditions that break the network of intermolecular contacts that maintains the stability of the IBs without denaturing the native-like proteins that are embedded in these structures [9].

Traditionally, the proteins in IBs are solubilized using high concentrations of chaotropic agents, such as guanidine hydrochloride (GdnHCl) or urea, and the denatured and soluble proteins are refolded to their native states after the removal of the denaturing reagents. However, the refolding step is difficult with these

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http://dx.doi.org/10.1016/j.procbio.2012.12.017

conventional methods because of the generation of new protein aggregates during the removal of the chaotropic agents [10].

Aggregated proteins display specific volumes that are larger than those of the native states due to the presence of intermolecular cavities, which are not exposed to water. The application of high pressure favors transitions of the protein structures that reduce the volume of the system. In vitro, pressures of 1–3 kbar impair intermolecular and electrostatic interactions, thereby promoting the dissociation of oligomeric protein states and aggregates. However, a stronger hydration with the rupture of intramolecular bonds and a consequential denaturation of the proteins generally occurs at higher pressure levels, above 4–5 kbar [11,12]. The effectiveness of refolding strategies that use mild solubilization conditions to enable the preservation of secondary and tertiary structures is increased in comparison to the solubilization of the aggregates using high concentrations of denaturing agents, where the protein loses its existing native-like tertiary and secondary structures [13,14]. The application of high pressure is a mild dissociation technique for aggregated proteins that does not require the use of high levels of denaturing reagents and enables the maintenance of secondary and tertiary structures, which is useful for protein refolding.

High hydrostatic pressures have been utilized for the dissociation of aggregates and protein refolding [15,16]. However, the pressure level at which the refolding occurs is controversial. While some articles state that the dissociation and refolding occur concomitantly at pressure levels of 2.0–2.5 kbar [17,18], other authors only utilize this range of pressure to dissociate the aggregates. In those cases, it was shown that the refolding of proteins with quaternary structures occurred at atmospheric pressure [15,19].

At high pressures, the freezing point of water is lowered, which allows for studies on the concomitant effects of high pressures with low temperatures in aqueous solutions. At low temperatures, the proteins are driven to a lower entropic state that favors the interaction of non-polar amino acids with water [20]. Thus, the effect of high pressures can be potentiated by the use of subzero temperatures, which enhances the exposure of hydrophobic side chains to the solvent and the dissociation of the aggregates.

Endostatin (ES) is a 20 kDa C-terminal fragment digested from collagen XVIII, an endogenous antiangiogenic protein that shows a potent inhibitory effect on endothelial cell migration, proliferation and tumor angiogenesis; [21–24]. This protein suppresses tumor growth without toxicity or an acquired drug resistance [24], exhibits a broad spectrum and is not toxic [25]. Studies have demonstrated the difficulty of generating native ES [23] because ES easily aggregates, resulting mainly in insoluble and aggregated ES preparations [26]. Therefore, its correct refolding is essential for the assurance of structural stability and biological functions [27].

In the present study, we have analyzed the factors that are involved the dissociation of IBs and the refolding of ES under high hydrostatic pressure. The impact of the growth temperature on the quality of aggregated ES was assessed. We also studied the effect of high hydrostatic pressure (2.4 kbar) with subzero temperatures (−9 °C) on the dissociation of ES. The effects of application of HHP on conformation transitions of ES were investigated in order to determinate their influence on the ES folding. Additionally, we studied the use of small molecule additives to increase the recovery yields of native ES in association with the action of the pressure. All of these factors were investigated with the aim of increasing the efficiency of ES refolding under pressure.

2. Materials and methods

2.1. Expression of ES, growth conditions, cell fractionation, and inclusion body isolation

BL-21(DE3)pLysS was transformed with the vector pET-28 containing the DNA sequence coding for the amino acids methionine, alanine, and six histidine residues at the N terminus, which was followed by the sequence for murine ES (construct cTB01#8, from pETKH-1, ATCC number 63404). For the expression of ES, a colony was randomly picked from the transformants that were grown on Kan + LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 50 mg/L kanamycin) and inoculated in 2-HKSII rich medium [28]. Cells were grown at 37 °C, and the expression of ES was induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) at the beginning of the exponential phase (approximately 3.0 at A_{600nm}). The culture was separated into 1 L erlenmeyer flasks containing 250 mL 2-HKSII medium, which were then incubated at different temperatures (25 °C, 30 °C and 37 °C). After incubation with a constant orbital agitation (150 rpm) for a 16 h period, bacteria were collected by centrifugation at 2500 × *g* for 10 min at 4 °C. The pellet was resuspended in 50 mL of 0.1 M Tris–HCl, pH 7.5 and 5 mM EDTA. Lysozyme, at a final concentration of 50 μg/mL, was added, and the suspension was incubated for 15 min at room temperature. The suspension was sonicated in the presence of 0.1% sodium deoxycholate and centrifuged at 8000 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 0.05 M Tris–HCl, pH 7.5 with 5 mM EDTA and sodium deoxycholate, washed twice in 0.05 M Tris–HCl, pH 7.5, and stored at −20 °C.

2.2. Scanning electron microscopy

Scanning electron microscopy was performed by drying water-dialyzed insoluble aggregates onto clean polished Philips stubs and sputter coating in a SCD-040 sputter coater (Balzer) at 38 mA for 120 s. The samples were then viewed and photographed using a Philips XL-200 scanning electron microscope. The sizes of the IBs were analyzed using the software ImageTools.

2.3. Sample pressurization

Suspensions of ES IBs were diluted in refolding buffer (50 mM Tris–HCl, pH 7.5 with 1 mM EDTA) containing the final concentrations of 1.5 M GdnHCl and 0.5 mM oxidized (GSSG) and 0.5 mM reduced (GSH) glutathione. Samples of the suspension (2 mL) were placed into plastic bags, which were sealed and then placed into a larger plastic bag that was vacuum/heat-sealed. The bags were placed in a pressure vessel (R4-6-40, High-Pressure Equipment) with a oil for a pressure-transmitting fluid, and high pressure was applied (2.4 kbar). Samples were compressed to 2.4 kbar, incubated for 2 h and then decompressed to 1.2 kbar, 0.8 kbar or 0.4 kbar, which was maintained for 16 h, and was then followed by a decompression to atmospheric pressure. The samples were then centrifuged at 12,000 × *g* for 15 min. The supernatant was dialyzed against 50 mM Tris–HCl, pH 7.5 and centrifuged at 12,000 × *g* for 15 min to remove insoluble aggregates that formed during the dialysis process. To test the effects of additives on the HHP-induced refolding, 4,4'-dianilino-1,1'-binaphthyl-5, 5'-sulfonate (bis-ANS, 6.5 mM), heparin (2.5–20 mM), L-arginine (0.5 M), glycerol (2.5 M), sodium chloride (0.15 M), sucrose (1 M), Tween 20 (1 mM) or Triton X-100 (0.5 mM) were added to the refolding buffer.

2.4. Quantification of ES by SDS-PAGE

SDS-PAGE analysis was carried out on 15% SDS-polyacrylamide gels using the method described by Laemmli and stained with Coomassie Blue G-250. Suspensions of ES IBs were heated at 95 °C for 5 min in SDS-PAGE sample buffer (Tris–HCl 50 mM pH 8.5 containing 2% SDS and 1% dithiotreitol, 0.01% bromophenol blue and 10% glycerol) for complete ES solubilization and therefore the respective bands in the electrophoresis gels were used as a reference for the total amount of ES within IBs. The soluble fractions of the HHP-treated suspensions of IB were applied to SDS-PAGE gels under non-reducing conditions. Image J software (http://www.ncbi.nlm.nih.gov) was utilized for the analysis of the bands in the digital photography of the gels for the determination of the percentage of soluble ES in HHP-treated samples, in comparison to the total amount of ES in IBs. The total protein content was determined by a Bradford assay using pure bovine serum albumin as standard.

2.5. Determination of the solubilization of the IBs in the presence of GdnHCl

ES IBs, which were expressed at different temperatures (25 °C and 37 °C), were suspended in solutions containing 0–8 M guanidine hydrochloride (GdnHCl). After a 72 h incubation at 180° vertical rotation (35 rpm), the samples were centrifuged at 12,000 × *g* for 10 min and the absorbance of the supernatants were measured at 280 nm. The amount of ES in the insoluble fractions was quantified by SDS-PAGE analysis, as described above.

2.6. Fourier transform infrared spectroscopy (FTIR)

Attenuated total reflectance (ATR)-FTIR spectra were obtained from dry samples deposited directly onto the ATR crystal in a Nicolet 6700 IR spectrometer (Thermo Corp., USA). Spectra were collected with a 4 cm^{-1} resolution and are the result of the accumulation of 256 scans. Fourier self-deconvolution of the amide I band was performed with a 1.6 enhancement factor and a 20 cm^{-1} bandwidth. The peak positions and area assignments were done with the software OMNIC (Thermo Corp., USA) with a Voigt distribution for each peak.

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