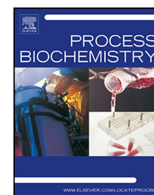
Process Biochemistry 49 (2014) 54–60

Contents lists available at ScienceDirect

Process Biochemistry

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



The effect of temperature on protein refolding at high pressure: Enhanced green fluorescent protein as a model

N.V. Malavasi [a], Y. Cordeiro [b], D. Rodrigues [a], R.M. Chura-Chambi [a], L.S. Lemke [a], L. Morganti [a,*]

[a] *Instituto de Pesquisas Energéticas e Nucleares – IPEN – CNEN/SP, Centro de Biotecnologia, São Paulo, Brazil*
[b] *Universidade Federal do Rio de Janeiro, Faculdade de Farmácia, Rio de Janeiro, Brazil*

ARTICLE INFO

Article history:
Received 27 May 2013
Received in revised form 2 September 2013
Accepted 22 September 2013
Available online 29 September 2013

Keywords:
Refolding
High hydrostatic pressure
Enhanced green fluorescent protein
Temperature
Inclusion body

ABSTRACT

The application of high hydrostatic pressure (HHP) impairs electrostatic and hydrophobic intermolecular interactions, promoting the dissociation of recombinant inclusion bodies (IBs) under mild conditions that favor subsequent protein refolding. We demonstrated that IBs of a mutant version of green fluorescent protein (eGFP F64L/S65T), produced at 37 °C, present native-like secondary and tertiary structures that are progressively lost with an increase in bacterial cultivation temperature. The IBs produced at 37 °C are more efficiently dissociated at 2.4 kbar than those produced at 47 °C, yielding 25 times more soluble, functional eGFP after the lower pressure (0.69 kbar) refolding step. The association of a negative temperature (−9 °C) with HHP enhances the efficiency of solubilization of IBs and of eGFP refolding. The rate of refolding of eGFP as temperature increases from 10 °C to 50 °C is proportional to the temperature, and a higher yield was obtained at 20 °C. High level refolding yield (92%) was obtained by adjusting the temperatures of expression of IBs (37 °C), of their dissociation at HHP (−9 °C) and of eGFP refolding (20 °C). Our data highlight new prospects for the refolding of proteins, a process of fundamental interest in modern biotechnology.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Recombinant *Escherichia coli* is usually the first system selected for the production of sufficient heterologous proteins for biochemical and structural studies and, if complex post-translational modifications are not required, for the subsequent large-scale production of economically interesting proteins.

Overproduced recombinant proteins can represent up to 90% of the total protein content produced from *E. coli*, transcending the folding capability of the bacteria and causing the bacterial quality control system to fail. As a result, misfolded proteins accumulate, leading to the formation of inclusion bodies (IBs). This process is highly protein dependent, driven by protein sequences and affected by specific folding requirements [1]. Therefore, the expression of certain proteins in heterologous systems in an insoluble form cannot be prevented, even if favorable conditions for the production of soluble recombinant proteins are used, such as cultivation of the host bacteria at a low temperature. Studies of the structural properties of IBs at the molecular level indicate that aggregated proteins embedded in IBs contain native-like secondary structures [2]. The tertiary structure and biological activity of proteins embedded within IBs were also shown to be retained to some degree [3,4]. The undesirable intermolecular β-sheet component of protein aggregates was shown to be increased at high expression levels [5]. The production of aggregated recombinant polypeptides has been described as a source of relatively pure proteins suitable for direct use in biocatalysis, possibly reaching nearly 100% activity [6,7]. IBs have also been described as a source of relatively pure target proteins that can be released in native-like conformations through mild solubilization processes [3,8]. These are indications that improved refolding yields can possibly be obtained through the expression of native-like structures in IBs, associated with the use of a mild process to dissociate the aggregates.

The dissociation of macromolecular complexes such as aggregates and oligomeric proteins is favored under high pressure [9]. Although the interactions between residues in a protein's native state and intermolecular contacts in aggregates or proteins with quaternary structures are dictated by similar forces, and thus similar responses are expected in response to the application of pressure, the pressure range able to promote the dissociation of aggregates is below 3 kbar, while most single-chain proteins only begin to suffer denaturation at pressures above 4–5 kbar [10,11]. With respect to hydrophobic solvation, high pressure and low temperature exhibit additive effects [10,12]. The use of HHP was previously described for the solubilization of aggregates and the

* Corresponding author at: Avenida Professor Lineu Prestes, 2242, CEP 05508-000, São Paulo, Brazil. Tel.: +55 11 31339695; fax: +55 11 31339709.
E-mail addresses: lmorganti@ipen.br, lmorganti@bol.com.br (L. Morganti).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.09.022

refolding of proteins [13,14]. The application of moderate pressures (up to 3 kbar) does not disrupt intra-molecular native contacts; it promotes elastic modification in protein structures [15,16] and thus can potentially be very advantageous over a widespread refolding strategy at atmospheric pressure that uses high levels of denaturing reagents. The advantage of efficient IB dissociation in the presence of harsh denaturing reagents is accompanied by the serious drawback that is the disruption of native contacts in IBs that can be important for a productive protein folding pathway [17].

Green fluorescent protein (GFP) is a 27 kDa monomeric protein that has the ability to emit bright green fluorescence upon exposure to ultraviolet light [18]. The formation of the GFP chromophore spontaneously occurs as the protein folds, furnishing a stable covalent structure. The chromophore of GFP is protected by its position near the center of a large β-barrel formed by 11 β-strands. GFP is highly resistant to high pressure-induced denaturation, which occurs at pressures above 13–14 kbar and is caused by a collapse in the β-barrel structure [19]. The enhanced form of GFP (eGFP) contains two mutations (F64L and S65T) that improve the quantum yield of fluorescence [20]. The simplicity of monitoring GFP bioactivity and the fact that the native structure must be present for emission of its characteristic fluorescence make this protein an excellent model system for protein refolding studies.

In a previous study, we used eGFP as a model and investigated the use of high hydrostatic pressure as a tool to promote the refolding of proteins from IBs [21]. We have shown that the dissociation of protein aggregates is obtained through the incubation of a suspension of IBs at high pressure (2.4 kbar). However, the refolding of eGFP is obtained at a lower pressure (0.69 kbar).

The present study provides insights into the status of the structure of eGFP within IBs. We demonstrated by infrared (FT-IR) spectroscopy in the solid phase that the secondary structure of IBs produced at 37 °C is similar to the structure of native eGFP and that the temperature of cultivation of the bacterial host interferes with the secondary structure of eGFP recombinant protein within IBs. We thus investigated whether the process of high-pressure refolding would be optimized by the use of IBs with enhanced conformational structures. Additionally, we analyzed the dissociation of aggregates in IBs and the kinetics of the maturation of the chromophore and the refolding of eGFP at HHP.

2. Materials and methods

2.1. Expression of eGFP, growth conditions, cell fractionation, and IB isolation

E. coli BL-21(DE3) strain was transformed with the vector pAE containing a DNA sequence encoding the mutant form (F64L/S65T) of the enhanced green fluorescent protein (eGFP). For the expression of eGFP, a colony was randomly picked from transformants that were grown on Kan+ LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 50 μg/L kanamycin) and inoculated in 2-HKSII rich medium [22]. Cells were grown at 37 °C, and the expression of eGFP 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 flasks that were then incubated at different temperatures (37 °C, 42 °C or 47 °C). After incubation with 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 50 mM Tris–HCl, pH 7.5, and 5 mM EDTA. Lysozyme, at a final concentration of 50 μg/mL, was added to the suspension, followed by incubation 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 50 mM Tris–HCl, pH 7.5, with 5 mM EDTA and sodium deoxycholate. The pellet was washed twice in 50 mM Tris–HCl, pH 7.5, and stored at −20 °C.

2.2. Sample pressurization

Suspensions of eGFP IBs were diluted in refolding buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM DTT). 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), and oil was used as a pressure-transmitting fluid. Samples were compressed to 2.4 kbar, incubated for 2 h, and then decompressed to 0.69 kbar, a pressure that was maintained for 16 h followed by decompression to atmospheric pressure. The samples were then centrifuged at 12,000 × *g* for 15 min, and the supernatant was saved. To evaluate the effect of pH on the refolding of eGFP, a suspension of IBs produced at 37 °C was subjected to the pressurization protocol at different pH values. Acetate was utilized to prepare buffers with pHs 4.0 and 5.0. 2-(N-morpholino)ethanesulfonic acid (MES) was utilized to prepare buffer with pH 6.0. Tris–HCl was utilized for preparing buffer with pHs 7.0, 7.5, 8.0, 8.5 and 9.0. Finally, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer was utilized for preparing buffer with pHs 10.0 and 11.0. All buffers were prepared at 50 mM and contained 1 mM EDTA and 1 mM DTT for eGFP refolding.

2.3. Fluorescence and light-scattering (LS) measurements

The LS and fluorescence measurements of the sample were recorded on a Cary Eclipse spectrofluorometer (Varian). Data were collected using a 1 cm path length cuvette at atmospheric pressure. For studies under pressure, round quartz cuvettes filled with the sample and sealed with flexible polyethylene caps were placed into a high-pressure cell equipped with three optical sapphire windows (ISS) and connected to a pressure generator (High Pressure Equipment). Ethanol was used as a pressure-transmitting fluid. The determination of the characteristic green fluorescence of eGFP was performed with an excitation wavelength of 470 nm. The excitation wavelength used to analyze the fluorescence of IBs was lower (440 nm) than the maximum excitation wavelength (470 nm), to avoid the interference exerted by IBs in the spectra due to the high levels of light scattering. The emission fluorescence spectra were collected between 450 and 600 nm at an angle of 90° relative to the incident light, using a response time of 1 s and a scan speed of 240 nm/min. For the LS measurements samples were illuminated at 320 nm, and LS was recorded from 315 to 325 nm at an angle of 90° relative to the incident light. In order to evaluate binding of 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonate (bis-ANS) to eGFP, 2.7 μM bis-ANS was added to a suspension of IBs containing 2.7 μM eGFP. The sample was excited at 360 nm and fluorescence emission was measured between 400 and 600 nm.

2.4. Quantification of eGFP by SDS-PAGE

SDS-PAGE analysis was performed on 15% SDS-polyacrylamide gels using the method described by Laemmli, and gels were stained with Coomassie Blue G-250. Suspensions of IBs were heated at 95 °C for 5 min in SDS-PAGE sample buffer (Tris–HCl 50 mM pH 8.5 containing 2% SDS, 1% dithiotreitol, 0.01% bromophenol blue, and 10% glycerol) for complete eGFP solubilization. Therefore, the respective bands in the electrophoresis gels were used as references for the total amount of eGFP within IBs. The soluble fractions of the HHP-treated suspensions of IBs were applied to SDS-PAGE gels under non-reducing conditions. Image J software (http://www.ncbi.nlm.nih.gov) was used to analyze the bands in digital photographs of the gels to determine the percentage of soluble eGFP in HHP-treated samples in comparison to the total amount of eGFP in IBs. The quantification of eGFP within IBs was obtained through a comparison with a standard curve of bovine serum albumin in the same electrophoresis gel.

2.5. 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 1.5 enhancement factor and a 20 cm^{-1} bandwidth using OMNIC software provided by Thermo Corporation.

2.6. Calculation of the rate constants of fluorescence acquisition

The slopes of the linear portions of the curves of fluorescence acquisition were used as the constants $K_{\text{folding/chromophore maturation}}$ and $K_{\text{refolding}}$. These values were calculated using the Origin 8 program.

3. Results and discussion

3.1. Influence of temperature of bacterial cultivation on the eGFP structure embedded within IBs

Aggregation is in general favored at higher temperatures due to the strong temperature dependence of hydrophobic interactions involved in the aggregation reaction [23]. The use of low temperatures for *E. coli* cultivation often affects the partitioning of proteins into soluble and insoluble fractions, resulting in higher yields and increased biological activities of soluble recombinant proteins [24]. In addition, the conformational state of the remaining insoluble

Download English Version:

https://daneshyari.com/en/article/34823

Download Persian Version:

https://daneshyari.com/article/34823

Daneshyari.com