Process Biochemistry 46 (2011) 512–518

Contents lists available at ScienceDirect

Process Biochemistry

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

Protein refolding at high pressure: Optimization using eGFP as a model

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ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form
29 September 2010
Accepted 2 October 2010

Keywords:
High hydrostatic pressure
Refolding
Green fluorescent protein
Inclusion body
Aggregation

ABSTRACT

Refolding of a mutant form of green fluorescent protein (eGFP), which only emits characteristic fluorescence when in the natively folded state, was accomplished under high hydrostatic pressure (HHP). Compression of eGFP inclusion bodies (IB) at 2.40 kbar for 30 min dissociated most of the aggregates and reduced the quantity of IBs. However, fluorescence at 509 nm indicated that eGFP did not refold under this condition. The refolding process was evaluated under various decompression conditions, following IB dissociation at 2.40 kbar. During stepwise decompression, increases in fluorescence were obtained at pressures ranging between 1.38 kbar and atmospheric pressure. The highest levels of eGFP refolding were achieved by incubation at pressure levels between 0.35 and 0.69 kbar in the absence of chaotropic reagents. The refolding was abolished when HHP was applied in the presence of 0.5–1.5 M GdnHCl. Our approach focused on monitoring the bioactivity of the recombinant protein, i.e., fluorescence, instead of solubility, which is not an ideal indicator of proper refolding. The higher yields of a bioactive product by incubation at pressure levels of 0.35–0.69 kbar without using chaotropic salts improve upon the HHP-refolding methods that have been previously described.

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1. Introduction

Heterologous recombinant proteins expressed in *Escherichia coli* tend to accumulate as insoluble and inactive protein aggregates known as inclusion bodies (IB), especially when produced at high rates. These cytoplasmic aggregates consist of elevated levels of highly enriched proteins that are trapped in insoluble particles and partially protected from proteolytic degradation. IBs form via the deposition of misfolded or partially folded polypeptides by the exposure of hydrophobic patches and the consequent intermolecular interactions [1–3]. The presence of residual native-like secondary and even tertiary structures is maintained within IBs to different extents, depending on the level and conditions of expression [4–8]. Refolding processes that use a high concentration of chaotropic agents to dissociate IBs cause the protein to completely denature. By contrast, the dissociation of aggregated proteins under mild conditions maintains the secondary structure of the protein and facilitates formation of the tertiary structure [9,10].

The application of high pressure to proteins in solution promotes structural rearrangement of the macromolecule-solvent system to accommodate the smaller volume. A decrease in the partial specific volume is predominantly attributed to a reduction in the internal cavities, voids that result from the imperfect packing of amino acids. The positive contribution to protein compressibility (β) is larger for the inner regions of the macromolecule, especially in the case of hydrophobic pockets, thereby culminating in considerably diminished mobility of the internal regions. The decreased volume of the macromolecule is partly compensated by a negative contribution to β due to water penetration and hydration of the superficial regions [11]. Proteins with high fractions of cavities unexposed to water are highly sensitive to HHP [12]. Proteins in IBs have a larger volume compared to those in the native conformation because they contain intermolecular cavities. Consequently, HHP treatment modulates the dissociation process [13]. HHP on the order of 2000–2500 bar has been shown to dissociate proteins by disfavoring hydrophobic and electrostatic intermolecular interactions under mild conditions, thereby dismissing the use of high levels of denaturing reagents to dissociate aggregated proteins. The same pressure level was also shown to be effective to refold some monomeric proteins [10,14–16].

Green fluorescent protein (GFP) is a 27-kDa monomeric protein that was initially extracted and purified by Shimomura from the jellyfish *Aequorea Victoria*. It has the ability to emit bright green fluorescence upon exposure to ultraviolet light [17]. The formation of the GFP chromophore spontaneously occurs as the protein folds, furnishing a stable covalent structure. The fact that the native

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doi:10.1016/j.procbio.2010.10.002

structure must be present for emission of its characteristic fluorescence [18] and the simplicity of monitoring GFP bioactivity make this protein an excellent model system for protein refolding studies.

In the present study, an enhanced form of GFP (eGFP) with two mutations (F64L and S65T) that improve the quantum yield of fluorescence [19] was used. The chromophore of GFP is protected by its position near the center of a large β-barrel formed by 11 β-strands. Due to its 3-D structure, GFP is resistant to high pressure-induced denaturation, which occurs at pressures above 13–14 kbar, caused by a collapse in the β-barrel structure [20]. Moreover, it has been shown that no loss of eGFP fluorescence intensity occurs at pressures as high as 9 kbar [21], a pressure level far above the 2.4 kbar that was used in the current study.

The effects of high pressure at different decompression conditions for eGFP refolding were investigated, aiming to increase the efficiency of bioactive protein production.

2. Materials and methods

2.1. IB preparation

The BL-21(DE3) strain of *Escherichia coli* was transformed with a plasmid encoding the mutant form (F64L/S65T) of the enhanced green fluorescent protein (eGFP) gene. For eGFP expression, a culture was incubated in 1000-mL shaker flasks containing 250 mL of rich culture medium (2×-HKSII) [22] supplemented with ampicillin (100 μg/mL). The flasks were inoculated with 15 mL of a pre-cultivated medium containing the *E. coli* harboring the expression plasmid and incubated on a rotary shaker at 37 °C until an optical density ($A_{600\,nm}$) of 5.0 was achieved. Protein expression was then induced by the addition of IPTG (final concentration of 0.5 mM), and the cultures were grown for 20 additional hours and harvested by centrifugation. The pellets were resuspended in 100 mL of 0.1 M Tris–HCl, pH 7.5, 5 mM EDTA and 50 μg/mL of lysozyme and the suspension was incubated at room temperature for 15 min. The suspension was sonicated in the presence of 0.1% sodium deoxycholate and centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 0.05 mM Tris–HCl, pH 7.5 and 5 mM EDTA containing 0.1% sodium deoxycholate. The centrifugation/resuspension step was repeated twice, and the resultant pellet was resuspended in 20 mL of 0.05 M Tris–HCl, pH 7.5, 0.1 M NaCl and 3 M urea and then centrifuged at 8000 × *g* for 10 min at 4 °C. The urea washing procedure was repeated four times. The pellet was then washed twice with 0.05 M Tris–HCl, pH 7.5 and 1 mM EDTA, resuspended in the same buffer for further assays and stored at −20 °C.

eGFP extracted from soluble fraction of activated *E. coli* and purified by immobilized metal affinity chromatography was used as a reference for fluorescence of the native state.

2.2. eGFP quantification

Suspensions of IB and soluble eGFP in Tris–HCl 50 mM, pH 7.5 buffer were analyzed in duplicate via SDS-PAGE. Samples of pure bovine serum albumin (BSA) at concentrations of 1–32 μg/25 μL in the same buffer were run on the same gel. Soluble eGFP was also quantified by the Bradford assay, using a curve of BSA as a standard. Protein bands in the digital images of the gels stained with Coomassie blue were quantified within a linear range using the software Image J.

2.3. IB compression

Suspensions of IBs were diluted in refolding buffer (0.05 M Tris–HCl pH 7.5, 1 mM EDTA and 10 mM DTT) to obtain an OD_{350nm} of 0.3, which corresponds to 400 μg eGFP/ml.

A 3-mL volume of the suspension was placed in bulbs of 5-mL Pasteur pipettes to ensure that a fixed volume of air (2 mL) was present in the IB suspensions to promote air oxidation. The bulbs were heat-sealed and placed in a plastic bag, which was vacuum/heat-sealed. The bag was placed in a pressure vessel with a mixture of water and oil as a pressure-transmitting fluid, and high pressure (2.4 kbar) was applied. The times employed for the pressurization and depressurization schemes are presented in the figures. The samples were then centrifuged at 12,000 × *g* for 15 min, and the supernatants were stored at −20 °C for posterior analysis. The samples compressed in the presence of GdnHCl were dialyzed to remove the GdnHCl prior to centrifugation.

2.4. Fluorescence and light scattering measurements

The fluorescence of the supernatants of the IB-compressed samples was recorded using an excitation wavelength of 470 nm in a Shimadzu F4500 spectrofluorimeter.

For spectroscopic measurements under pressure, a high-pressure cell equipped with optical sapphire windows purchased from ISS (Champaign, IL) was connected to a pressure generator. The fluorescence spectra and light scattering were recorded on an FS900 Enginburgh Instruments spectrofluorimeter. A quartz cuvette was filled with suspensions of IBs and sealed with a flexible polyethylene cap. The fluorescence was recorded using an excitation wavelength of 470 nm. Light scattering (LS) at 320 nm was measured at an angle of 90° relative to the incident light, and the emission was recorded from 315 to 325 nm.

2.5. Scanning electron microscopy

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

2.6. High performance liquid chromatography

High performance liquid chromatography (HPLC) was carried out in a Shimadsu system using a prepacked Superdex 75 10/300 GL column. The system was equilibrated with 50 mM Tris–HCl (pH 7.5) at a flow rate of 0.5 mL/min. Sample elution at 25 °C was monitored by absorption at 280 nm and fluorescence emission at 509 nm (excitation at 470 nm).

3. Results

3.1. IBs comprised of eGFP dissociate upon application of high hydrostatic pressure (HHP)

HHP is an efficient tool for dissociating macromolecular complexes because it does not cause profound changes in the secondary or tertiary structure [23]. To determine the effect of HHP on the dissociation of eGFP IBs, a suspension of IBs with an optical density of 0.3 was subjected to a pressure of 2.4 kbar (25 °C), and light scattering (LS) was monitored for 60 min (Fig. 1A). After 25 min under HHP, LS decreased to 21% of that of the initial value, suggesting dissociation of the IB into smaller particles. After decompression, LS did not return to its original value (triangle on the right), indicating irreversibility of the pressure-induced dissociation. This condition was used in the subsequent experiments to dissociate eGFP IBs.

There was a dramatic decrease in the quantity of IBs in samples subjected to 2.4 kbar for 30 min followed by direct decompression to atmospheric pressure (Fig. 1B and C). A similar reduction was observed for samples incubated for 16 h at 0.69 kbar before complete decompression (not shown). Therefore, the majority of the IB aggregates probably dissociate upon application of pressure (2.4 kbar), and the protein does not significantly re-aggregate to insoluble particles upon decompression to atmospheric pressure, as revealed by LS (Fig. 1A). The IBs consisted mostly of regular spherical particles with an average diameter of 0.8 ± 0.1 μm. The average diameter of the remaining non-dissociated particles submitted to HHP was 0.5 ± 0.1 μm, representing a 39% decrease in size. Taken together, these results suggest that the IBs that resisted HHP treatment had more compact structures. Alternatively, only the external layers of these aggregates might have dissociated under HHP.

3.2. eGFP requires resting at intermediate pressure for refolding

Upon folding, eGFP undergoes autocatalysis and forms a chromophore that emits in the green region (509 nm), hence the name given to the protein. The eGFP folding process was monitored by determination of the fluorescence intensity at 509 nm.

The fluorescence intensity of a suspension of eGFP IB at 509 nm before compression was very low, indicating that eGFP was mainly misfolded inside the IB (inset Fig. 2A – full line). Initially, a suspension of eGFP IB was subjected to 2.4 kbar for 30 min, as described in Fig. 1. At this elevated pressure, the signal at 509 nm did not increase because although the IBs had dissociated, eGFP could not refold to its native conformation under high pressure (inset Fig. 2A). Upon direct decompression to atmospheric pressure, the signal at

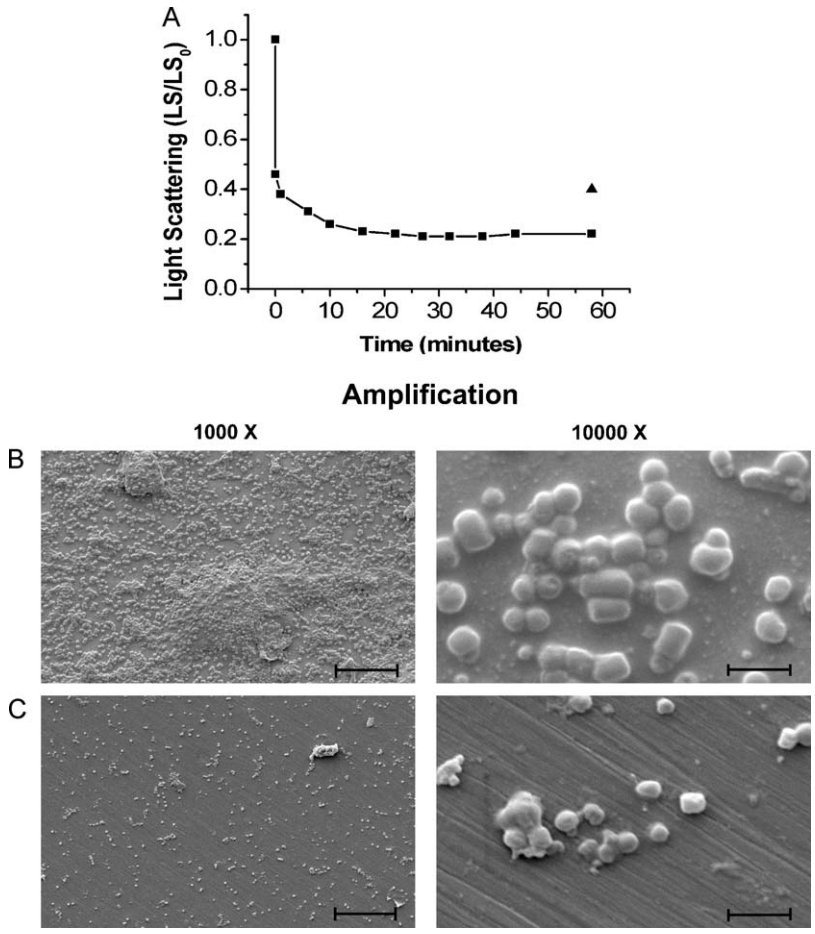

Fig. 1. HHP induces the irreversible dissociation of eGFP IBs. (A) A suspension of eGFP IBs (OD_{350nm} = 0.3) was subjected to 2.4 kbar at 25 °C, and the light scattering (LS) was followed as a function of time. The LS at each time was divided by the value at atmospheric pressure (LS_0). The excitation was set to 320 nm, and emission was collected from 315 to 325 nm. The isolated triangle corresponds to the LS value after decompression; (B and C) scanning electron microscopy of (B), untreated IB; (C) insoluble fraction of IB after compression at 2.40 kbar for 30 min and direct decompression to atmospheric pressure. Scale bars: 20 μm (original magnification 1000×), 2 μm (original magnification 10,000×).

509 nm increased 37-fold after 5 h of incubation at atmospheric pressure (Fig. 2A, circles).

It has been observed that slow decompression can improve the yield of protein refolding after HHP, which suggests that rapid depressurization rates can trap aggregation-prone species [24]. On the basis of this idea and with the goal of enhancing the eGFP refolding process, the effect of gradual decompression on the fluorescence of eGFP was investigated. The first strategy was to compress the IBs at 2.4 kbar for 30 min followed by stepwise decompression in 0.35 kbar steps and allowing the protein to remain at each pressure value for 30 min before atmospheric pressure was reached (Fig. 2A, squares). Surprisingly, the fluorescence intensity at 509 nm gradually increased from 1.38 kbar to atmospheric pressure (Fig. 2A). A 40-fold rise in fluorescence was observed using this strategy, resulting in an 80-fold increase after incubation at atmospheric pressure (inset Fig. 2A).

Due to the fact that eGFP refolding was favored under pressure levels lower than 1.4 kbar, we wondered whether it would be possible to further improve the yield of refolded eGFP by allowing the protein to rest at an intermediate pressure before total decompression. To test this hypothesis, eGFP IBs were compressed at 2.4 kbar for 30 min. Then, the protein was decompressed directly to 0.69 kbar and allowed to remain at this pressure value for 16 h before full decompression to atmospheric pressure (Fig. 2B, circles). Under these conditions, the emission at 509 nm increased 136-fold, suggesting effective refolding of eGFP to its native state. The lack of change in fluorescence upon return to atmospheric pressure also indicates that the dissociated protein was completely refolded during incubation at 0.69 kbar. Fig. 2B reveals that a stepwise decompression from 2.4 to 0.69 kbar, where the protein remained for 16 h, followed by step decompression to atmospheric pressure did not further improve the yield of eGFP refolding (Fig. 2B, squares). The inset in Fig. 2B shows that after 5 h of incubation at 0.69 kbar, the refolding process reached a maximum value. The kinetics of eGFP refolding were similar at other pressure levels (atmospheric pressure, 0.35 kbar, 1.04 kbar, and 1.38 kbar) (data not shown).

It could be argued that differences in fluorescence values may be due to variations in the kinetics of eGFP refolding at different pressures because eGFP folding may not have had enough time to reach equilibrium under slower refolding conditions. This possibility was excluded by carrying out experiments for prolonged periods, namely up to 72 h, which led to similar results to those achieved in shorter periods.

Because incubation of eGFP at 0.69 kbar after decompression from 2.4 kbar provided excellent recovery of the eGFP native conformation, we wondered whether pressure values larger and smaller than 0.69 kbar would also be as effective. Fig. 3 reveals that decompression of eGFP IB from 2.4 kbar directly to 1.38, 1.03, 0.69 or 0.35 kbar yielded increased fluorescence. However, the data suggest that pressures higher than 0.69 are not as effective for eGFP refolding. Similar results were obtained when resting at each pressure value for even longer periods (not shown).

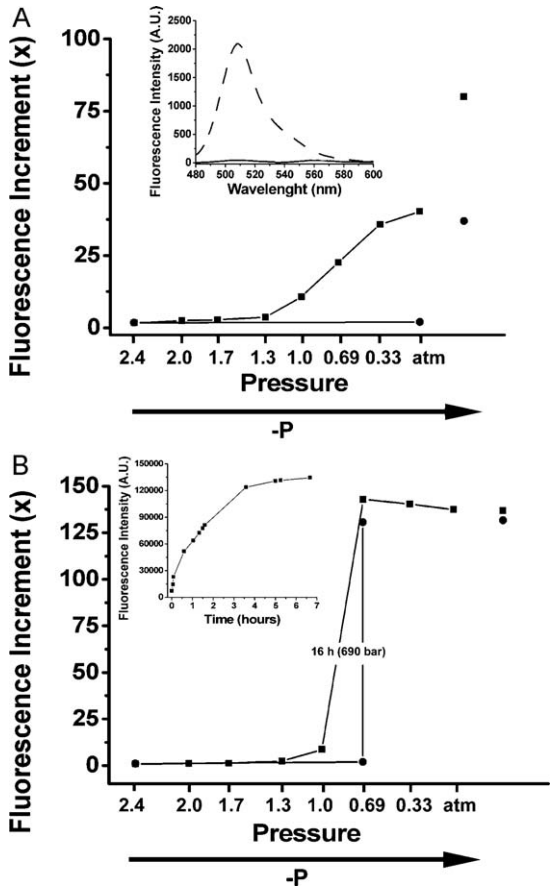

Fig. 2. Refolding of eGFP is facilitated upon incubation at intermediate pressures. eGFP IB (OD_{350nm} = 0.3) was compressed to 2.4 kbar at 25 °C for 30 min, and the fluorescence emission at 509 nm (exc = 470 nm) was monitored. In (A), the sample was decompressed (-P) directly (circles) or stepwise (squares) to atmospheric pressure. In (B), the samples were decompressed (-P) from 2.4 kbar directly (circles) or stepwise (squares) to 0.69 kbar and remained in that condition for 16 h before complete decompression to atmospheric pressure. The isolated symbols on the right correspond to the fluorescence intensity of the samples 5 h after returning to atmospheric pressure. The inset in panel (A) shows the spectra before compression (solid line), at 2.4 kbar and 5 h after returning to atmospheric pressure (stepwise decompression, long dashed line). At 2.4 kbar, the spectra profile (not shown) was the same as the atmospheric pressure profile. The inset in panel (B) shows the fluorescence intensity at 509 nm at 0.69 kbar as a function of time (direct decompression from 2.4 to 0.69 kbar).

The presence of oxygen is mandatory for chromophore formation, which is the rate-limiting step for GFP fluorescence maturation [25]. Because all the experiments performed so far had been conducted in the absence of air, a control experiment in which air bubbles were allowed in the IB sample was performed. No difference in the eGFP refolding yield was observed in this more oxidizing environment (not shown).

3.3. Refolded and misfolded states of eGFP remain soluble upon decompression

Similar amounts of soluble eGFP were obtained for samples subjected to 2.4 kbar for 30 min followed by different decompression strategies: direct decompression, stepwise decompression with resting at 0.69 kbar, and stepwise decompression with no resting (inset in Fig. 4). To obtain better quantification of the refolding process, the specific fluorescence of eGFP treated under different conditions was investigated (Fig. 4A). Direct decompression to 0.69 kbar followed by protein refolding at the latter pressure (16 h) caused eGFP to display 4.6-fold higher specific fluorescence (fluorescence emission at 509 nm/μg eGFP) than the samples subjected to direct decompression (dotted bar and empty bar). Moreover, eGFP refolded at 0.69 kbar regained 75% of the specific fluorescence of the native protein.

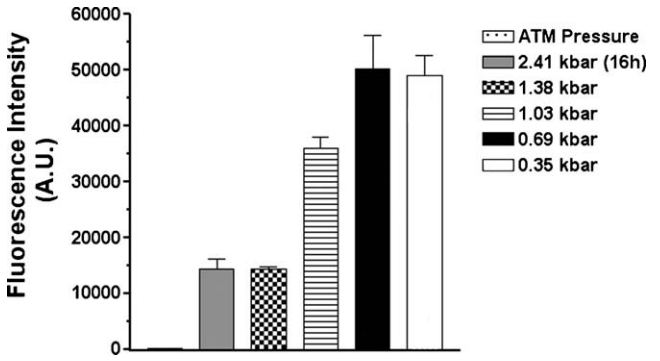

Fig. 3. The refolding yield of eGFP depends on the pressure level at which the protein remains incubated after dissociation at 2.4 kbar. eGFP IBs were compressed at 2.4 kbar for 30 min and then decompressed to 0.35, 0.69, 1.03, or 1.38 kbar, where the sample remained for 16 h before complete pressure release. The samples were centrifuged to remove insoluble material, and the fluorescence signal at 509 nm was measured. Other conditions were as described in Fig. 2. Average of triplicates.

As expected, a large increase in the yield of monomeric and fluorescent eGFP was observed for IBs compressed at 2.4 kbar followed by direct decompression with resting at 0.69 kbar (Fig. 4C) compared to the sample that was directly decompressed from 2.4 kbar to atmospheric pressure (Fig. 4B), as shown by the larger area of the fluorescent peak (509 nm) obtained by size exclusion chromatography (23.2 min – dashed line). The higher yield of native eGFP for the samples decompressed with a resting step at 0.69 kbar was accompanied by a reduction in the non-fluorescent peak obtained at a shorter retention time (17.5 min – straight line), which corresponds to soluble species with larger molecular weight. This results indicate that upon direct decompression, misfolded eGFP states re-aggregated to form larger molecular-weight soluble molecules.

3.4. eGFP refolding is impaired by the presence of GdnHCl during compression

To determine if the presence of low, non-denaturing levels of GdnHCl in combination with HHP can effectively increase the yields of soluble eGFP from aggregates, IBs were compressed in the presence of up to 2.0 M of GdnHCl.

The yields of soluble eGFP were not improved in the presence of GdnHCl (Table 1), probably because compression in the presence of GdnHCl, despite fostering a higher degree of dissociation of the aggregates, induced a more severe perturbation of the protein structure, leading to more significant reaggregation upon decompression. eGFP refolding at 0.69 kbar was impaired by the presence of GdnHCl, even at levels as low as 0.5 M, as shown by a drastic drop in fluorescence of those samples.

The yield of soluble eGFP was 79.1% of the protein content in the IB, and the specific fluorescence of the protein was 75.0%, with a final refolding yield of 59.3% (Table 1).

Table 1
Refolding yields of eGFP refolded at HHP (2.4 kbar for 30 min and 0.69 kbar for 16 h).

GdnHCl (M)	Solubility (%)	Specific fluorescence (%)	Final yield (%)
0	79.1 ± 1.76	75.0 ± 1.10	59.3
0.5	18.3 ± 0.06	0.18 ± 0.04	0.03
1.0	41.1 ± 0.04	0.90 ± 0.04	0.36
2.0	47.0 ± 0.03	0.88 ± 0.01	0.41

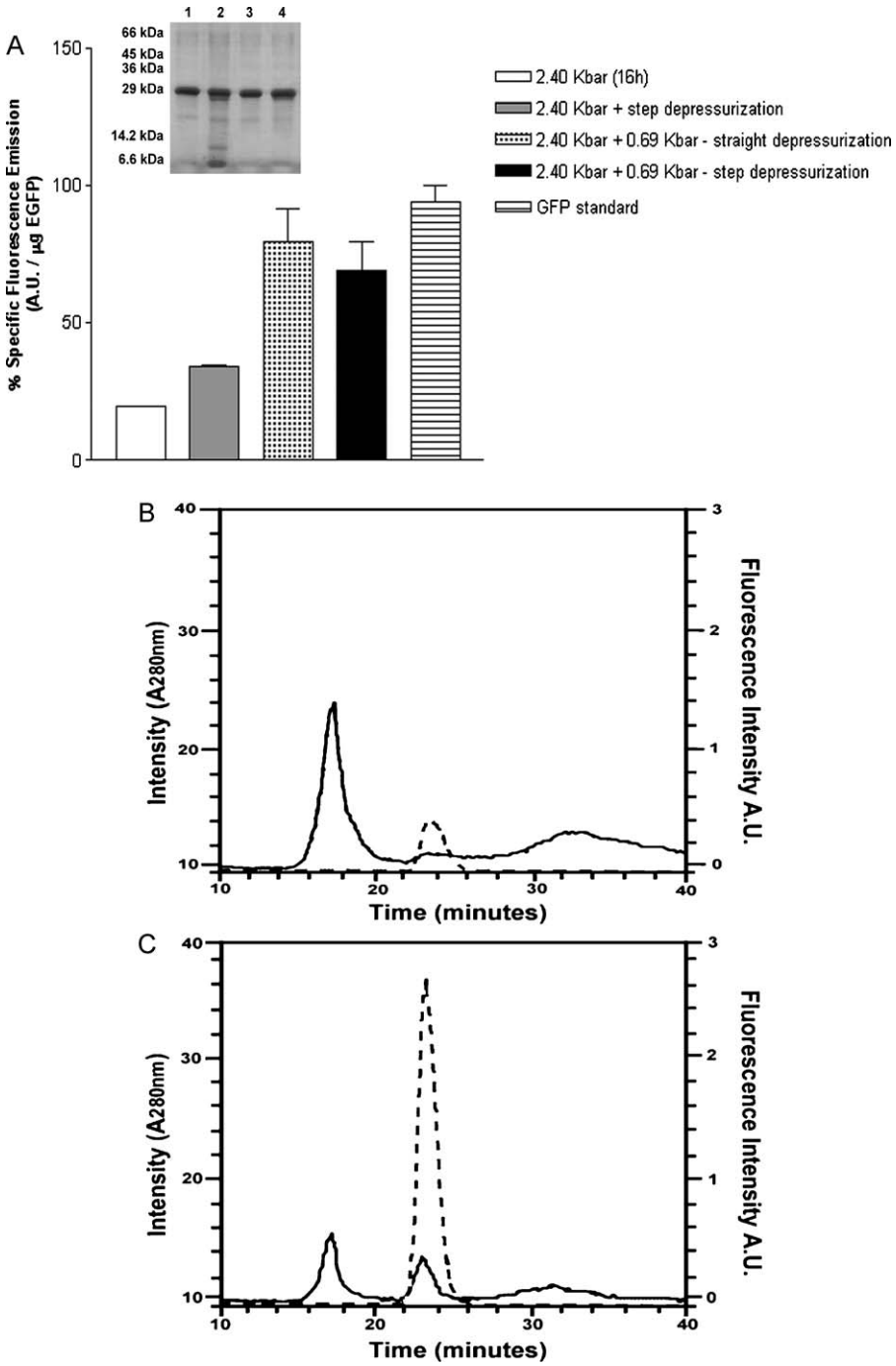

Fig. 4. Misfolded eGFP remains soluble. (A) Specific fluorescence emission (fluorescence intensity/µg GFP) of soluble fractions of IB compressed at 2.4 kbar and decompressed using different strategies. Inset: SDS-PAGE; lane 1: step decompression (0.35 kbar steps remaining at each step for 30 min); lane 2: direct decompression; lane 3: direct decompression with resting at 0.69 kbar for 16 h; lane 4: step decompression with resting at 0.69 kbar for 16 h; (B and C) size-exclusion Superdex 75 10/300 GL column on HPLC system. Peaks were detected by absorbance at 280 nm (solid lines) or by fluorescence at 509 nm (excitation at 470 nm – dashed lines). (B) Soluble fraction of IB compressed at 2.4 kbar and directly decompressed; (C) soluble fraction of IB subjected to 2.4 kbar with resting at 0.69 kbar for 16 h.

4. Discussion

An achievement of this study was to improve the use of high pressure to induce protein dissociation from aggregates and subsequent refolding. Our results indicate that pressures of 0.3–0.7 kbar are effective for refolding and may represent a general method that can be used for different proteins. Therefore, it will be interesting to test this condition for other proteins in the near future. Another important lesson was to learn the practicality of using eGFP from IBs as a model of refolding, since the prompt information of disaggregation and refolding is furnished by simple spectroscopic data.

Our results can be represented by the diagram shown in Fig. 5. IBs are stable at atmospheric pressure, and application of high pressure promotes dissociation to a state named "m*" (monomer star), represented as closed circles. The fluorescent properties of "m*" show that the protein is not in its native state, although no significant re-aggregation to the insoluble form occurs upon decompression. Nevertheless, the energy barrier necessary to reach the native state (species "m", open cycles) is sufficiently low at

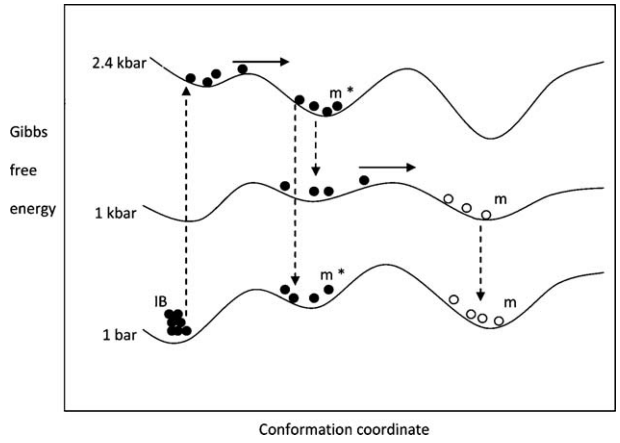

Fig. 5. Free energy diagram of the conformational states of eGFP at different conditions. m*: monomer in conformational drift; m: native monomer. The IB dissociates to "m*", which may produce the native form "m" at intermediate pressure.

intermediate pressures (0.35–0.69 kbar), which kinetically favors this process, as observed spectroscopically under this condition. Thus, the return to atmospheric pressure furnishes the stable native form of the protein.

Recent studies have reported the refolding and purification of urea-solubilized eGFP from inclusion bodies using artificial chaperone-assisted immobilized metal affinity chromatography (AC-IMAC) [26,27] and expanded bed anion exchange chromatography [28]. In these reports, eGFP recovery and specific fluorescence yields ranged from 82 to 92% and 80 to 95%, respectively. In our study we obtained a somewhat lower recovery yield of 80% of eGFP and 75% specific fluorescence of a native eGFP, used as reference. The final recovery yield of refolding (60%) was much higher than that obtained by direct dilution of urea-solubilized eGFP (14%) [26]. The eGFP concentration used in the present study was 0.4 mg/ml, and slightly lower refolding yields (23%) were obtained for a protein concentration of 1.3 mg/ml (not shown).

It is well-known that low temperature destabilizes hydrophobic interactions [29] and that dissociation of protein states induced at high pressures is improved at subzero temperatures [30]. Thus, it is likely that utilization of lower temperatures associated with high pressure may improve dissociation of the aggregated states and consequently the refolding yield of eGFP.

Pressure breaks up inter- and intramolecular protein interactions, inducing dissociation of aggregates. The fact that high pressures (1–3 kbar) can dissociate aggregated and oligomeric protein states has been extensively described in the literature [30–32]. Previous reports have demonstrated the refolding of a variety of monomeric proteins during incubation for long periods (16–125 h) at pressures of 1.5–2.5 kbar. The dissociation of aggregates and refolding are described to occur concomitantly, at the same pressure level [10,14,15,33–35].

The novelty of our study in comparison to other reports that use HHP for protein refolding was to focus on the biological activity of eGFP, i.e. fluorescence, rather than of solubility, which is not an adequate indicator of proper refolding. The use of eGFP as a model in the present study enabled us to improve the refolding protocol for this monomeric protein. Interestingly, it was shown that eGFP refolding was not favored at pressures above 1.4 kbar. Despite being effective for aggregate dissociation, compression of eGFP IB at 2.4 kbar for long times followed by direct or even stepwise decompression to atmospheric pressure was not as effective for eGFP refolding as incubation for 5 h at 0.35–0.69 kbar. In fact, contrary to what has been described in previous studies of monomeric proteins, the pressure level that induced dissolution of eGFP IB aggregates (2.4 kbar) was 3.5–7 times higher than the ideal conditions for refolding of this monomeric protein. Analysis of our data suggests that the increase in the refolding yield obtained by slow decompression of other proteins described in the literature [24] could be related to the refolding that possibly occurs during incubation at intermediate pressures lying between 2 kbar and atmospheric pressure.

The use of low levels of GdnHCl is frequently described for HHP-refolding protocols in order to improve IB dissociation [24]. The fact that in the current study the biological activity of eGFP was almost completely abolished for samples compressed in the presence of 0.5–2 M of GdnHCl shows that the dissociation of aggregates and increased solubility may not be accompanied by an adequate refolding of the protein to its native state.

Tertiary and quaternary protein structures are stabilized by the same kinds of interactions [36–38], and high pressure is effective at destabilizing both structures, promoting partial unfolding of the polypeptide toward less compact forms and dissociation of aggregates. Hence, pressure levels that cause dissociation of protein aggregates can also affect interactions involved in the maintenance of tertiary structure. It has been shown by phosphorescence emission of tryptophan, an intrinsic probe of protein structure, that application of relatively low pressures (1–2 kbar) can induce structural alterations in the native tertiary structure of monomeric proteins [39,40], as well as conformational changes preceding subunit dissociation of oligomeric proteins [41]. Thus, compression of eGFP at pressure levels above 1.4 kbar probably hampers interactions involved in maintaining protein structure, thereby hindering refolding to the native state.

The tailspike protein of Salmonella phage P22 is a viral adhesion protein that exists in a homotrimeric dome-like structure [42]. The folding and aggregation pathways of tailspike have been well characterized, and aggregation of the chains occurs by specific interactions. In contrast to the results previously described for monomeric proteins, studies have shown that nonspecific aggregates of the P22 tailspike protein dissociated upon HHP treatment (2 kbar for 90 min) only refold by oligomerization to the trimeric, native state after decompression to atmospheric pressure [16,43,44]. Rhodanese is a heterodimeric protein that is folded into two independent, equally sized domains containing α-helices and β-sheets as secondary structure elements. These domains are tightly bound to each other by non-covalent, mainly hydrophobic, interactions [45]. Partially denaturated rhodanese proteins readily aggregate via interdomain hydrophobic interactions. It has been demonstrated that rhodanese aggregation is completely reversed at 0.5 kbar or at higher pressure values. Interestingly, 0.5 kbar is also the minimum pressure capable of preventing the precipitation of an aggregation-prone state of rhodanese [46].

Based on our data as well as previous findings, we believe that incubation for long times at pressures between 0.3 and 0.7 kbar after dissolution of the aggregates by application of higher pressures (2–2.5 kbar) can be used to avoid re-aggregation while concomitantly favoring folding to the native states of monomeric and also of oligomeric proteins, thereby increasing refolding yields. This method is especially of interest for improving the refolding yield of proteins with biopharmaceutical interest. The refolding yield of the monomeric protein endostatin from IBs submitted to HHP has already been reported [47] to be increased by application of low pressure levels (0.35 and 0.69 kbar) following IB dissociation (data to be published).

Further studies on other proteins are being performed by our group to demonstrate whether this approach can be widely applied.

Acknowledgments

This work was supported by grants from the State of São Paulo Research Foundation – FAPESP (Process 07/54624-4) and

National Council for Scientific and Technological Development – CNPq (Process 479816/2007-7). We thank Juliana Mattoso for technical assistance.

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