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

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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.

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

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 $K_{folding/chromophore\ maturation}$ and $K_{refolding}$. 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
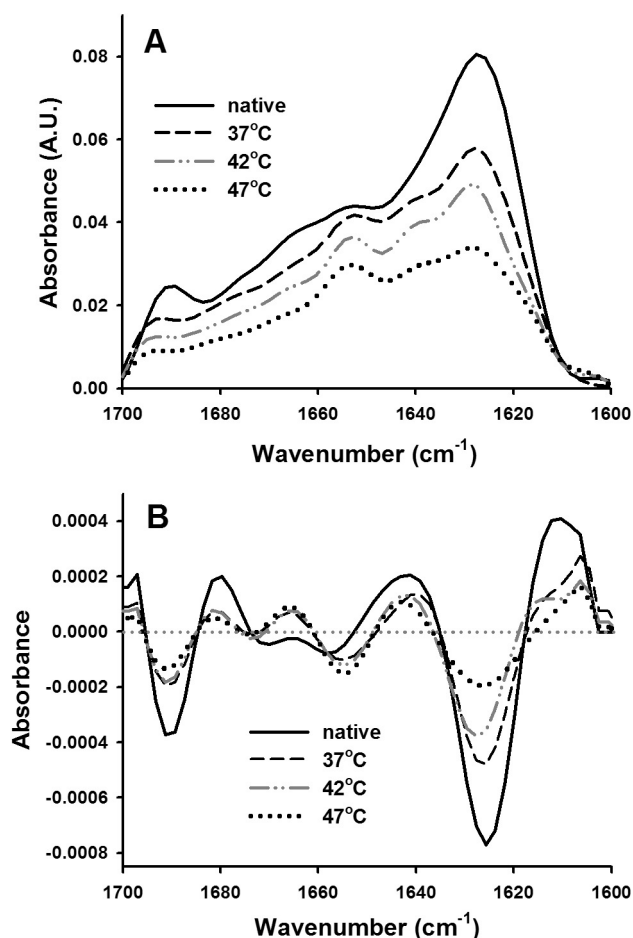
Fig. 1. ATR-FITR spectra in the amide I region of purified and lyophilized eGFP and of IBs produced at 37 °C, 42 °C, and 47 °C. (A) Fourier self-deconvoluted ATR-FTIR spectra. (B) Second derivative of the spectra shown in A.

Fig. 2. (A) Dependence on the temperature of bacterial cultivation of the fluorescence emission of suspensions of eGFP IBs containing 181 μg/ml eGFP. Excitation was set at 440 nm. (B) Dependence on the temperature of bacterial cultivation on the binding of bis-ANS to suspensions of eGFP IBs containing 2.7 μM eGFP and 2.7 μM bis-ANS. Excitation was set at 360 nm. Buffer: 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM DTT. A.U., arbitrary units. The data are representative of 3 independent experiments.

fraction can be affected by the temperature of protein expression that is used [25]. The major fraction of the recombinant eGFP accumulates as insoluble IBs at temperatures of cultivation ≥37 °C, but lower temperatures induce the production of eGFP primarily in the soluble fraction of the cytoplasm. To determine whether the temperature of cultivation influences the conformational states of eGFP embedded in IB aggregates, samples of eGFP expressed at temperatures ranging from 37 °C to 47 °C were analyzed.

GFP is a protein formed by an 11-stranded β-barrel wrapped around a single central helix containing 46% β-sheets and 11% α-helices [18]. Analysis of the secondary structure of the dried protein was performed by ATR-FT-IR. Analysis of the amide I region in the IR spectrum of lyophilized native eGFP (Fig. 1A) shows the presence of a primary peak at 1628 cm^{-1} that can be assigned as intramolecular β-sheet structures [26]. Disordered structures are attributed to the peak at 1641 cm^{-1}. The peak at 1654 cm^{-1} is assigned to contribution from α-helices and also random coil, while the peak at 1666 cm^{-1} was interpreted as corresponding to turns [26,27]. The high frequency peaks at 1678 cm^{-1} and 1691 cm^{-1} are attributed to β-sheets with an antiparallel orientation, which is in agreement with the crystallographic structure of native GFP [18] and with the described infrared spectrum of wild-type GFP [28]. A comparison among the FT-IR spectra of the IBs produced at temperatures ranging from 37 °C to 47 °C shows that alterations in the temperature of bacterial expression induced changes in the amide I region of the spectra (Fig. 1A). The most prominent feature that arises from a comparison of the spectra is that the rise in temperature of IB
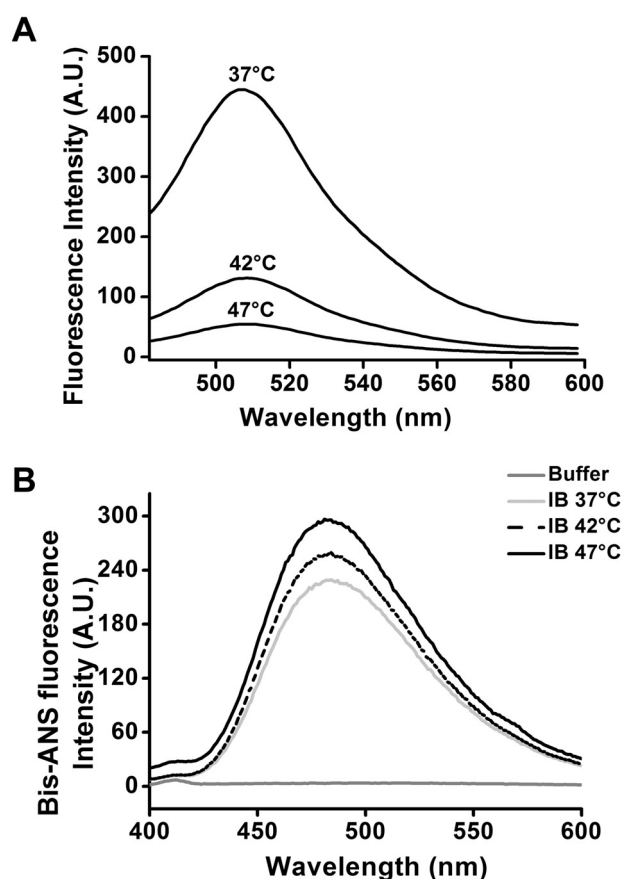
production induced a decrease in the peak at 1628 cm^{-1} (corresponding to native β-sheets), which suggests that lower levels of native β-sheets are present in the IBs expressed at the higher temperature tested. This feature is evidenced when second derivative spectra are compared (Fig. 1B). However, the percentage of the peak that corresponds to disordered structures (1641 cm^{-1}) remains relatively constant. The increase in the intensity of the 1654 cm^{-1} peak, usually attributed to α-helices, that was found for IBs produced at the higher temperatures, was also described by Scheyhing and colleagues [28] in the temperature-induced denaturation of a mutant of GFP (S65A, V68L and S72A). Due to the difficulty in discriminating between the two peaks, the authors attributed this rise to an increase in the amount of unordered structures. It is possible that this rise is due to the increased level of other structures that are exhibited at unusual positions in the infrared spectrum or that IBs possess different hydration levels. Interestingly, the peak at 1618 cm^{-1}, which is characteristic of intermolecular antiparallel β-sheet aggregation, is particularly prominent in the spectrum of IBs produced at 47 °C (Fig. 1A). Overall, a comparison of the spectrum of native eGFP with the spectra of IBs indicates that the aggregated protein produced at the lowest temperature tested (37 °C) presents a spectrum most similar to that of the native protein.

Suspensions of eGFP IBs present characteristic eGFP fluorescence with a maximal emission peak at 509 nm, indicating the existence of a certain percentage of native protein in these structures (Fig. 2A). The fluorescence measured was inversely
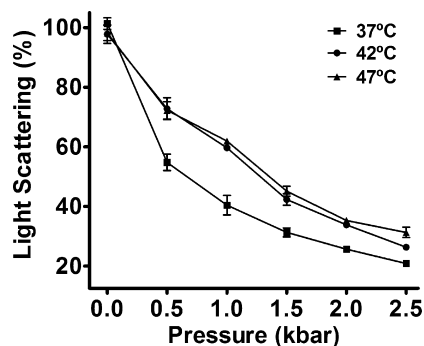
Fig. 3. Dissociation of suspensions of IBs of eGFP by high pressure. Suspensions of IBs containing 181 μg/ml of eGFP were subjected to increasing pressures, and light scattering (excitation at 320 nm and LS collected from 315 to 325 nm) was determined after 15 min incubations at each pressure level. Buffer: 50 mM Tris–HCl, pH 7.5 with 1 mM EDTA and 1 mM DTT. Data are representative of independent measurements.

Fig. 4. Fluorescence of eGFP refolded from IBs produced at different temperatures. Suspensions containing 181 μg/mL eGFP were pressurized at 2.4 kbar for 30 min and refolded by incubation at 0.69 kbar for 16 h. The suspensions were centrifuged and the fluorescence emission of soluble fractions was monitored with excitation set at 470 nm. Buffer: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1 mM DTT.

proportional to the temperature of cultivation of the host bacteria, which shows that there is a higher percentage of eGFP in its native tertiary structure in the IBs produced at 37 °C than in the IBs expressed at higher temperatures. The specific fluorescence of IBs produced at 37 °C is 8-fold higher than that of IBs extracted from cells grown at 47 °C. A similar result was obtained for the recombinant protein Aβ42-GFP (Alzheimer related Aβ42 gene fused upstream of the GFP sequence) expressed at temperatures ranging from 18 °C to 42 °C in cytoplasmic IBs [29]. In that study, the fluorescence emission of the IBs formed at 18 °C was 16-fold higher than that exhibited by IBs extracted from cells cultivated at 42 °C.

Bis-ANS is a hydrophobic probe that fluoresce upon binding to hydrophobic regions of proteins [30]. Fig. 2B shows a progressive enhancement in the fluorescence emission of bis-ANS along with the increase in the temperature of production of the IB. The total area of the fluorescence emission spectrum obtained for IB produced at 47 °C was increased by 11% in comparison to the area of the IB fluorescence spectrum produced at 42 °C and by 31% in comparison to the area of the IB produced at 37 °C. These results show that bis-ANS binding to eGFP is increased at higher temperatures, likely due to a higher exposure of hydrophobic regions of eGFP embedded within IB at 47 °C. Thus, eGPF conformation within the IBs is modified depending on the temperature of IBs production.

3.2. The temperature of IB production affects the dissociation of eGFP and the yield of refolding

The effects of expression temperature on the solubility of IBs, as monitored by the light scattering of suspensions of IBs incubated at increasing pressure levels, are shown in Fig. 3. The decrease in the LS levels indicates dissociation of the aggregates. The solubilization of IBs produced at 47 °C and 42 °C occurs at higher pressures than that of IBs produced at 37 °C. The fact that IBs of eGFP produced at 37 °C are more easily dissociated than those produced at higher temperatures indicates that higher levels of intermolecular interactions are present in the aggregates produced at the higher temperature at the expense of native intramolecular contacts, which is in agreement with the data shown in Figs. 1 and 2.

The enhanced solubility and the increase in eGFP native-like secondary and tertiary structures in IBs produced at 37 °C in comparison to IBs produced at higher temperatures suggest that higher yields of refolding would be obtained when applying high pressure to IBs expressed at 37 °C, which was confirmed (Fig. 4). The intensity of green fluorescence (509 nm) obtained from IBs produced at
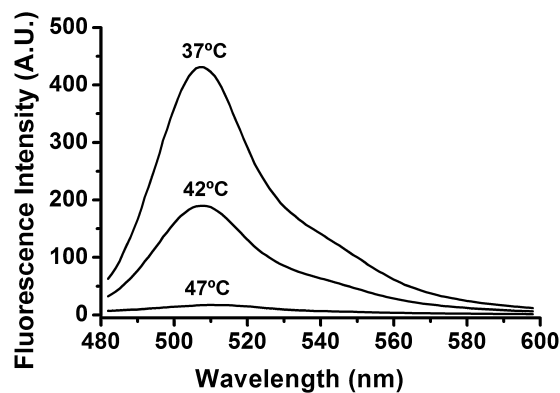
37 °C was 25 times higher than that obtained from IBs produced at 47 °C.

3.3. Effect of the association of high pressure and sub-zero temperature on the dissociation of IBs

The freezing point of water is lowered under high pressure, which permits studies of the effects of the application of high pressures at temperatures below 0 °C in aqueous solutions. At temperatures below the freezing point of water, the hydrophobic effect, which is considered the main driving force for protein folding and for intermolecular protein interactions that lead to aggregation, is weakened. Low temperatures favor the interaction of non-polar amino acids with water [12]. Cold denaturation is the process whereupon the native state of the protein loses its stability by cooling due to the hydration of non-polar residues [31]. We investigated whether the effect of high pressure on the dissociation of aggregates can be enhanced by the use of negative temperatures. With the objective of determining whether the application of high pressure at low temperatures causes irreversible denaturation of eGFP, pure and soluble protein was subjected to high pressure and low temperature and the resulting fluorescence at 509 nm was monitored. The fluorescence of native eGFP subjected to 2.4 kbar decreased slightly (7%) at 20 °C, and a temperature reduction to −9 °C resulted in a 53% decrease in green fluorescence. However, the fluorescence intensity returned to 97% of its initial value upon returning to 1 bar and 20 °C, indicating that the unfolding of eGFP that occurs at low temperature is reversible (results not shown). This result is in agreement with the literature, which indicates that the structural changes in proteins resulting from the application of low temperatures, as well as those that occur by increasing the pressure up to 5 kbar, are generally reversible [10,32]. The use of low temperatures (−9 °C) along with the application of HHP (2.4 kbar) to the eGFP IBs led to increased dissociation, indicated by a decrease in the scattering of visible light (Fig. 5A), and a 40% increase in the fluorescence at 0.69 kbar (Fig. 5B and C). While the application of HHP at 2.4 kbar at 20 °C recovered 73.2% of the soluble eGFP from the IBs, 96.0% recovery was obtained when IBs were dissociated at a negative temperature (−9 °C) at the same pressure. Additionally, the specific fluorescence of the refolded eGFP also improved from 89.8% to 96.7%, which shows that the increase in fluorescence was the result of a higher efficiency of dissociation of the IBs at the lower temperature and a higher refolding yield of the eGFP dissociated at a negative temperature.

Similar results have been described for the dissociation of Tobacco mosaic virus that occurs at high pressure (2.5 kbar) and
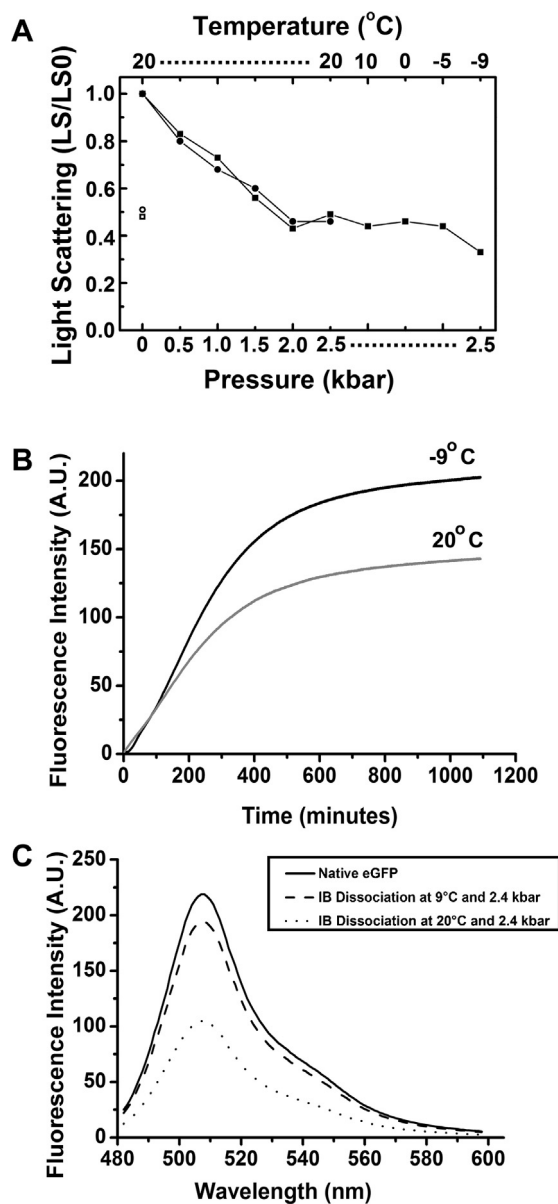
Fig. 5. (A) Effects of pressure and temperature on the dissociation of eGFP IBs and on the refolding of this protein. Suspensions of IBs containing 181 μg/ml eGFP were dissociated by the application of pressure. The compression time used was at least 5 min or until the light scattering value stabilized. Suspensions subjected to negative temperature (■ and □); suspensions not subjected to negative temperature (● and ○); values of LS obtained after decompression (□ and ○); B, Effects of refolding, verified by the fluorescence intensity (509 nm) of eGFP from a suspension of IBs subjected to high pressure. The suspension of IBs was dissociated by the application of 2.4 kbar pressure for 60 min, during which the suspension was held for 15 min at −9 °C (black line) or incubated for 60 min at 20 °C (gray line), after which the pressure was reduced to 0.69 kbar and the suspension was incubated for refolding for 18 h; C, Fluorescence of native eGFP (181 μg/ml) and of refolded eGFP from suspensions containing 181 μg/ml eGFP. Buffer: 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM DTT.

that is enhanced by low temperature (−20 °C) [33]. The yield of refolding of endostatin from IBs at high pressure was also shown to be increased by 11% by association of negative temperature (−9 °C) and HHP [34]. The use of subzero temperatures in association with high pressure can be an interesting tool to increase the dissociation of IBs, minimizing or avoiding the necessity of using denaturing reagents to solubilize the aggregates and resulting in a substantial increase in the yield of protein folding.
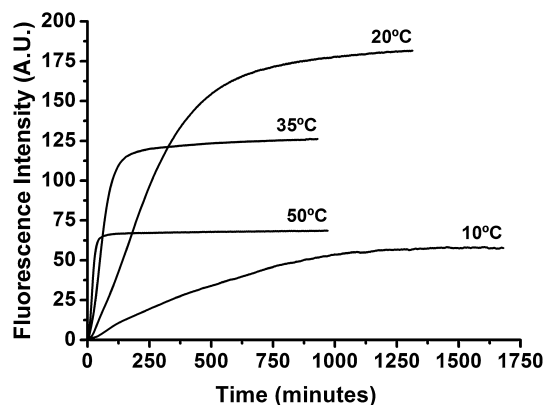

Fig. 6. Kinetics of the folding of eGFP at 0.69 kbar. Suspensions of eGFP IBs were subjected to 2.4 kbar pressure for 30 min and then decompressed to 0.69 kbar. Fluorescence was monitored at 509 nm during incubation at different temperatures. Buffer: 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM DTT. Data are representative of 3 independent measurements.

3.4. Effect of temperature on the kinetics and yield of eGFP folding

To determine the kinetics of eGFP folding at temperatures ranging from 10 °C to 50 °C, IBs were dissociated by compression of a suspension at 2.4 kbar for 30 min at −9 °C, followed by a temperature increase as indicated in Fig. 6 and decompression to 690 bar, a pressure level adequate for eGFP refolding. The lower intensity of fluorescence obtained from samples incubated at 35 °C and 50 °C is likely due to eGFP unfolding and possibly due to re-aggregation at these high temperatures. This indication is supported by the fact that at 50 °C the maximal fluorescence intensity was lower than the value obtained when the suspension was incubated at 35 °C (Fig. 6A) and by the fact that the aggregation of wild-type GFP that, as described, occurs at 35 °C and was shown to be minimized at 25 °C [35].

The folding of GFP is a slow process. After protein folding is completed, the chromophore motif, residues Ser65, Tyr66 and Gly67 in wild type GFP (Thr65, Tyr66 and Gly67 in eGFP) become buried in the central helix. The three-dimensional conformation of the protein presumably promotes the next step in the maturation of the GFP chromophore, covalent rearrangement, resulting in the cyclization of the tripeptide motif. GFP only emits fluorescence upon oxidation and dehydration of the chromophore structure [36]. The mature chromophore remains chemically intact in the GFP denatured state [37], but there is a loss of green fluorescence upon denaturation due to quenching when the buried chromophore becomes exposed to an aqueous environment. The rate of GFP refolding from an unfolded state containing a previously matured chromophore was shown to be faster than the rate of folding of a protein that had never acquired the native conformation. This occurs because the rate-limiting step of the folding process is the intermediate stage of maturation of the chromophore, oxidation, rather than the folding of the native structure of the protein [38].

The time course of fluorescence acquisition shown in Fig. 6 reflects the folding of eGFP from IBs, mainly from a state that has never been folded. Therefore, the rate of fluorescence acquisition ($K_{\text{folding/chromophore maturation}}$) depends on both factors in the following sequence: folding of the protein (faster step) and maturation of the chromophore (slower step). The fact that $K_{\text{folding/chromophore maturation}}$ increases with an increase in temperature (Table 1) indicates that the kinetics of the folding of eGFP and/or of the maturation of the chromophore are faster at higher temperatures.

To determine whether the rate of refolding of eGFP around the previously matured chromophore is affected by temperature, the native protein was unfolded by incubation at −9 °C and 2.4 kbar,

Table 1
Rates of acquisition of fluorescence from suspensions of eGFP IBs (181 μg/ml), incubated at 0.69 kbar following dissociation of the aggregates at 2.4 kbar.

Temperature (°C)	$K_{\text{folding/chromophore maturation}}$ (eGFP IBs)	R^2	$K_{\text{refolding}}$ (denatured eGFP)	R^2
10	$0.18 \times 10^{-4} \pm 0.0040 \times 10^{-4} \text{ s}^{-1}$	0.99365	$0.41 \times 10^{-4} \pm 0.0017 \times 10^{-4} \text{ s}^{-1}$	0.99174
20	$0.39 \times 10^{-4} \pm 0.0032 \times 10^{-4} \text{ s}^{-1}$	0.99926	$0.67 \times 10^{-4} \pm 0.0035 \times 10^{-4} \text{ s}^{-1}$	0.98784
35	$1.76 \times 10^{-4} \pm 0.0197 \times 10^{-4} \text{ s}^{-1}$	0.99777	$1.54 \times 10^{-4} \pm 0.0072 \times 10^{-4} \text{ s}^{-1}$	0.99648
50	$5.90 \times 10^{-4} \pm 0.0542 \times 10^{-4} \text{ s}^{-1}$	0.99899	–	–

conditions previously shown to induce fluorescence loss but not likely to chemically affect the chromophore. The protein was subsequently refolded at 0.69 kbar at temperatures ranging from 10 °C to 50 °C. We did not observe an increase in fluorescence at 50 °C, likely because of eGFP unfolding and/or aggregation. Table 1 shows that the rates of refolding ($K_{\text{refolding}}$) of the denatured protein containing the mature chromophore were faster than the rates of folding of eGFP from IBs at 10 °C and 20 °C, indicating that folding around the mature chromophore is likely to be the faster stage of eGFP folding at these temperatures. However, at 35 °C, the rate of maturation of the chromophore ($K_{\text{refolding}}$) reaches the rate of folding, as shown by the similar values of $K_{\text{folding/chromophore maturation}}$ and $K_{\text{refolding}}$ obtained at this temperature. The folding of eGFP and the maturation of the chromophore are closely linked. Our results suggest that the kinetics of chromophore maturation are influenced more by an increase in temperature than by the rate of refolding but that both factors affect the kinetics of maturation.

3.5. Effect of pH on the refolding of eGFP

To determine whether the refolding of eGFP is affected by the solution pH, we performed the high-pressure protocol (pressurization at 2.4 kbar for 1 h and 0.69 kbar for 16 h) with a suspension of eGFP in solution with pH values ranging from 4.0 to 11.0 and the fluorescence intensity (at 509 nm) of the soluble fractions was determined (Fig. 7). The observed optimal pH for eGFP refolding was found between pH 7.5 and 8.5. The fact that the observed fluorescence was low at acidic pH can be explained by the fact that native and soluble eGFP is unstable at these pH values, losing almost all fluorescence emission at 509 nm (99.9%) by incubation at pH 4.0–5.0; half of the fluorescence emission (54.7%) at pH 6.0 and 17.7% of the 509 nm emission at pH 7.0. No loss of fluorescence emission was seen when native eGFP was incubated at pHs ranging from 7.5 to 11.0. As native eGPF does not suffer fluorescence emission suppression from pH 7.5 to 11.0, we can conclude that the refolding procedure when performed at pHs 9.0–11.0 (Fig. 7) did
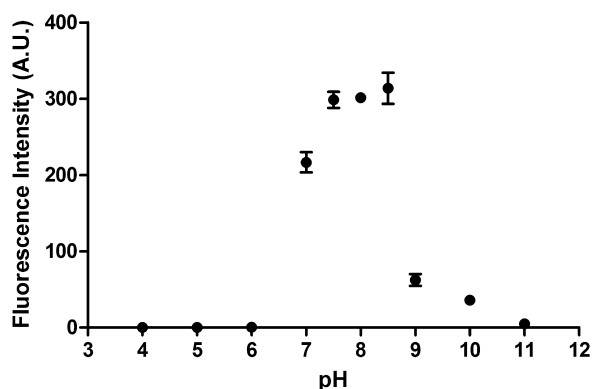
not yield natively folded eGFP. Thus, we can conclude that the refolding of eGPF was successful at pH values close to neutral (7.5–8.5) (Fig. 7).

4. Conclusions

In the present study, we showed that IBs produced at lower temperatures present secondary and tertiary structures similar to those of their native counterparts and that these structures are gradually lost through increases in the temperature of bacterial cultivation. Importantly, the IBs of eGFP produced at lower temperatures are more readily solubilized by high pressures than those produced at higher temperatures. Based on these results, and with the aim of obtaining high-quality IBs for efficient high-pressure refolding, we propose that bacterial culture be performed only at mild temperatures.

The use of temperatures below 0 °C (−9 °C) was shown to improve the dissociation of IBs induced by high pressure (2.4 kbar), and an increase in the refolding yield from 47.5% to 88.3% was observed as a result of the increased solubility of eGFP with higher specific activity. Based on these results, we propose the following changes to the folding process: the use of 2–3 kbar pressure at temperatures below 0 °C for the dissociation of the IBs, followed by incubation at higher temperatures (20–35 °C) and lower pressures (0–0.7 kbar) for efficient refolding.

The forces that keep proteins in their native/denatured/aggregated states are universal. Therefore, although this is a specific study on the refolding of eGFP, it is likely that the results described in this study will be useful for obtaining native proteins from IBs in general.

Fig. 7. eGFP refolding after pressure treatment depends on the pH. Fluorescence intensity at 509 nm (with excitation at 470 nm) of the supernatant of the suspensions of eGFP IB subjected to HHP (2.4 kbar for 1 h and 0.69 kbar for 16 h) in the following buffers: pH 4 and 5: buffer acetate; pH 6.0: buffer MES; pH 7.0–9.0: Tris; pH 10.0 and 11.0: buffer CAPS. All buffers were prepared at 50 mM concentration and contained 1 mM EDTA and 1 mM DTT.

References

[1] Tartaglia GG, Vendruscolo M. Proteome-level interplay between folding and aggregation propensities of proteins. J Mol Biol 2010;402:919–28.
[2] Curtis-Fisk J, Spencer RM, Weliky DP. Native conformation at specific residues in recombinant inclusion body protein in whole cells determined with solid-state NMR spectroscopy. J Am Chem Soc 2008;130:12568–9.
[3] Jevsevar S, Gaberc-Porekar V, Fonda I, Podobnik B, Grdadolnik J, Menart V. Production of nonclassical inclusion bodies from which correctly folded protein can be extracted. Biotechnol Progr 2005;21:632–9.
[4] Garcia-Fruitos E, Aris A, Villaverde A. Localization of functional polypeptides in bacterial inclusion bodies. Appl Environ Microbiol 2007;73:289–94.
[5] Ami D, Natalello A, Taylor G, Tonon G, Maria Doglia S. Structural analysis of protein inclusion bodies by Fourier transform infrared microspectroscopy. Biochim Biophys Acta 2006;1764:793–9.
[6] Tokatlidis K, Dhurjati P, Millet J, Beguin P, Aubert JP. High activity of inclusion bodies formed in Escherichia coli overproducing Clostridium thermocellum endoglucanase D. FEBS Lett 1991;282:205–8.
[7] Nahalka J, Patoprsty V. Enzymatic synthesis of sialylation substrates powered by a novel polyphosphate kinase [PPK3]. Org Biomol Chem 2009;7:1778–80.
[8] Peternel S, Grdadolnik J, Gaberc-Porekar V, Komel R. Engineering inclusion bodies for non denaturing extraction of functional proteins. Microb Cell Fact 2008;7:34.
[9] Robinson CR, Sligar SG. Hydrostatic and osmotic pressure as tools to study macromolecular recognition. Energ Biol Macromol 1995;259:395–427.

[10] Silva JL, Weber G. Pressure stability of proteins. Annu Rev Phys Chem 1993;44:89–113.

[11] Mozhaev VV, Heremans K, Frank J, Masson P, Balny C. High pressure effects on protein structure and function. Proteins: Struct Funct Genet 1996;24:81–91.

[12] Tsai CJ, Maizel Jr JV, Nussinov R. The hydrophobic effect: a new insight from cold denaturation and a two-state water structure. Crit Rev Biochem Mol Biol 2002;37:55–69.

[13] St John RJ, Carpenter JF, Randolph TW. High pressure fosters protein refolding from aggregates at high concentrations. Proc Natl Acad Sci U S A 1999;96:13029–33.

[14] Chura-Chambi RM, Genova LA, Affonso R, Morganti L. Refolding of endostatin from inclusion bodies using high hydrostatic pressure. Anal Biochem 2008;379:32–9.

[15] Kamatari YO, Kitahara R, Yamada H, Yokoyama S, Akasaka K. High-pressure NMR spectroscopy for characterizing folding intermediates and denatured states of proteins. Methods 2004;34:133–43.

[16] Cioni P, Strambini GB. Pressure effects on protein flexibility monomeric proteins. J Mol Biol 1994;242:291–301.

[17] Jahn TR, Radford SE. Folding versus aggregation: polypeptide conformations on competing pathways. Arch Biochem Biophys 2008;469:100–17.

[18] Ormo M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ. Crystal structure of the *Aequorea victoria* green fluorescent protein. Science 1996;273:1392–5.

[19] Herberhold H, Marchal S, Lange R, Scheyhing CH, Vogel RF, Winter R. Characterization of the pressure-induced intermediate and unfolded state of red-shifted green fluorescent protein—a static and kinetic FTIR, UV/VIS and fluorescence spectroscopy study. J Mol Biol 2003;330:1153–64.

[20] Tsien RY. The green fluorescent protein. Annu Rev Biochem 1998;67:509–44.

[21] Malavasi NV, Foguel D, Bonafe CFS, Braga CACA, Chura-Chambi RM, Vieira JM, et al. Protein refolding at high pressure: optimization using eGFP as a model. Process Biochem 2011;46:512–8.

[22] Jensen EB, Carlsen S. Production of recombinant human growth hormone in *Escherichia coli*: expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol Bioeng 1990;36:1–11.

[23] Schellman JA. Temperature, stability, and the hydrophobic interaction. Biophys J 1997;73:2960–4.

[24] Scharnagl C, Reif M, Friedrich J. Stability of proteins: temperature, pressure and the role of the solvent. Biochim Biophys Acta Protein Proteonomics 2005;1749:187–213.

[25] Vera A, Gonzalez-Montalban N, Aris A, Villaverde A. The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. Biotechnol Bioeng 2007;96:1101–6.

[26] Cordeiro Y, Kraineva J, Suarez MC, Tempesta AG, Kelly JW, Silva JL, et al. Fourier transform infrared spectroscopy provides a fingerprint for the tetramer and for the aggregates of transthyretin. Biophys J 2006;91:957–67.

[27] Cordeiro Y, Kraineva J, Gomes MPB, Lopes MH, Martins VR, Lima LMTR, et al. The amino-terminal PrP domain is crucial to modulate prion misfolding and aggregation. Biophys J 2005;89:2667–76.

[28] Scheyhing CH, Meersman F, Ehrmann MA, Heremans K, Vogel RF. Temperature–pressure stability of green fluorescent protein: a Fourier transform infrared spectroscopy study. Biopolymers 2002;65: 244–53.

[29] de Groot NS, Ventura S. Effect of temperature on protein quality in bacterial inclusion bodies. FEBS Lett 2006;580:6471–6.

[30] Hawe A, Sutter M, Jiskoot W. Extrinsic fluorescent dyes as tools for protein characterization. Pharm Res 2008;25:1487–99.

[31] Dias CL, Ala-Nissila T, Wong-ekkabut J, Vattulainen I, Grant M, Karttunen M. The hydrophobic effect and its role in cold denaturation. Cryobiology 2010;60:91–9.

[32] Winter R, Lopes D, Grudzielanek S, Vogtt K. Towards an understanding of the temperature/pressure configurational and free-energy landscape of biomolecules. J Non-Equilib Thermodyn 2007;32:41–97.

[33] Bonafe CF, Vital CM, Telles RC, Goncalves MC, Matsuura MS, Pessine FB, et al. Tobacco mosaic virus disassembly by high hydrostatic pressure in combination with urea and low temperature. Biochemistry 1998;37: 11097–105.

[34] Chura-Chambi RM, Cordeiro Y, Malavasi NV, Lemke LS, Rodrigues D, Morganti L. An analysis of the factors that affect the dissociation of inclusion bodies and the refolding of endostatin under high pressure. Process Biochem 2013;48: 250–9.

[35] Fukuda H, Arai M, Kuwajima K. Folding of green fluorescent protein and the cycle3 mutant. Biochemistry 2000;39:12025–32.

[36] Rosenow MA, Huffman HA, Phail ME, Wachter RM. The crystal structure of the Y66L variant of green fluorescent protein supports a cyclization–oxidation–dehydration mechanism for chromophore maturation. Biochemistry 2004;43:4464–72.

[37] Ward WW, Cody CW, Hart RC, Cormier MJ. Spectrophotometric identity of the energy-transfer chromophores in renilla and aequorea green-fluorescent proteins. Photochem Photobiol 1980;31:611–5.

[38] Reid BG, Flynn GC. Chromophore formation in green fluorescent protein. Biochemistry 1997;36:6786–91.