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# An Aggregation-Prone Intermediate Species Is Present in the Unfolding Pathway of the Monomeric Portal Protein of Bacteriophage P22: Implications for Portal Assembly<sup>†</sup>

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**ABSTRACT:** The head of the P22 bacteriophage is interrupted by a unique dodecameric portal vertex that serves as a conduit for the entrance and exit of the DNA. Here, the *in vitro* unfolding/refolding processes of the portal protein of P22 were investigated at different temperatures (1, 25, and 37 °C) through the use of urea and high hydrostatic pressure (HHP) combined with spectroscopic techniques. We have characterized an intermediate species, *I<sub>U</sub>*, which forms at 25 °C during unfolding or refolding of the portal protein in 2–4 M urea. *I<sub>U</sub>* readily forms amorphous aggregates, rendering the folding process irreversible. On the other hand, at 1 °C, a two-state process is observed ( $\Delta G_f = -2.2$  kcal/mol). When subjected to HHP at 25 or 37 °C, the portal monomer undergoes partial denaturation, also forming an intermediate species, which we call *I<sub>P</sub>*. *I<sub>P</sub>* also tends to aggregate but, differently from *I<sub>U</sub>*, aggregates into a ring-like structure as seen by size-exclusion chromatography and electron microscopy. Again, at 1 °C the unfolding induced by HHP proved to be reversible, with  $\Delta G_f = -2.4$  kcal/mol and  $\Delta V = 72$  mL/mol. Interestingly, at 25 °C, the binding of the hydrophobic probe bis-ANS to the native portal protein destabilizes it and completely blocks its aggregation under HHP. These data are relevant to the process by which the portal protein assembles into dodecamers *in vivo*, since species such as *I<sub>P</sub>* must prevail over *I<sub>U</sub>* in order to guarantee the proper ring formation.

In all dsDNA<sup>1</sup> phage systems, DNA packaging involves the insertion of the genomic DNA into a preformed protein “container”, the prohead. During the maturation of P22, the conversion of the proheads into mature capsids is marked by several structural and morphological modifications, such as an increase in capsid diameter, the exit of the scaffolding proteins from the prohead interior, the entrance of the genomic DNA through a unique conduit present in 1 of the 12 vertexes of the capsid, and the final attachment of up to six trimers of the tailspike protein (gp9), resulting in the infectious phage particle (*I*).

In the case of bacteriophage P22, the conduit used for DNA entrance and exit is the product of gene 1 (gp1), known as portal protein. A ring-like arrangement of 12 copies of the portal protein (2, 3) forms a channel, through which DNA

is pumped into the capsid in a process that involves two virally encoded proteins (gp2 and gp3). The driving force for this reaction is provided by ATP hydrolysis. For phage  $\phi$ 29, it is estimated that one ATP molecule must be hydrolyzed for every two base pairs that are packaged (4). A terminase complex recognizes a specific DNA sequence to ensure the packaging of the complete virus genome. This terminase complex binds to the portal protein complex, delivering the DNA to the packaging site (5). The binding of the minor proteins gp4, gp10, and gp26 closes the portal (6–8).

Despite the available information, the precise mechanism involved in the encapsidation/ejection of the DNA through this dodecameric structure during the viral life cycle is not completely understood. Nevertheless, new insights into how the process might occur were revealed by the recent structural description of the whole P22 tail machine (including the portal, gp4, gp10, gp26, and gp9) and of the C-terminally truncated form of the portal protein by the use of cryoEM and image reconstruction (8). The structural analysis (Figure 1) revealed that gp4 and gp10 attach to the portal protein after packaging to form a cylindrical tube with a central channel that serves as the conduit for the DNA exit. After that, the gp26 trimer attaches to gp10, forming a plug that prevents the packaged DNA from leaking (Figure 1A). The portal protein can be divided in three domains, namely, the crown, the wing, and the stalk (Figure 1B). In the isolated

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<sup>1</sup> Abbreviations: gp1, product of gene 1; dsDNA, double-stranded DNA; phage, bacteriophage P22; cryoEM, cryoelectron microscopy; HHP, high hydrostatic pressure; bis-ANS, bis(8-anilino-1-naphthalene-sulfonate); IPTG, isopropyl  $\beta$ -D-thiogalactoside.

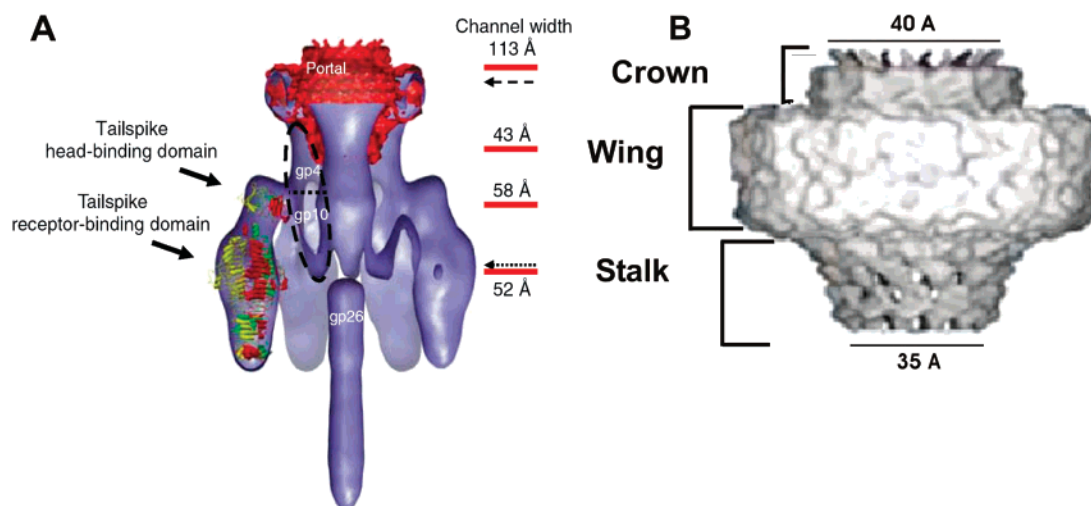


FIGURE 1: Structure of the C-terminally truncated P22 portal and of the tail machine obtained by cryoEM and image reconstruction (from Tang and collaborators, ref 8). Panel A shows the cutaway view of the tail machine with the cryoEM map of the truncated portal protein superimposed (red) and the X-ray structures of both domains of the docked tailspike (ribbon diagrams). The numbers on the right correspond to the channel width in each of the portions of the tail machine. Panel B shows the cryoEM image of the portal protein with its three domains marked. This figure adapted from ref 8.

portal protein, the outer diameter of the portal varies from 35 Å at the bottom opening (stalk) to 40 Å at the top opening (crown, Figure 1B).

As previously described, during phage assembly, the portal complex interacts with several components such as the coat and the scaffolding proteins, the terminase complex, and the DNA during its packaging. These diverse interactions suggest that the portal is a highly versatile protein with multiple domains that can switch between different conformational states (8, 9).

The *Salmonella typhimurium* bacteriophage P22 has been used as a model for the understanding of the morphogenesis of important viruses related to human diseases such as adenovirus and herpes virus (10, 11). Thus, since the formation of the portal complex is essential for virus viability, elucidation of the mechanism involved in its assembly as well as an understanding of how it works is crucial (12). In previous studies, it has been shown that during monomer  $\rightarrow$  dodecamer association, there are large changes in the local environment of amino acid residues' side chains, as well as some small changes in the secondary structure content (7, 12). However, there is no report in the literature describing the folding pathway of the monomeric portal protein of P22, and this was the aim of the present study.

Here we have studied the in vitro unfolding/refolding processes of the isolated monomers of the bacteriophage P22 portal protein at different temperatures (1, 25, and 37 °C) through the use of urea and high hydrostatic pressure (HHP) combined with spectroscopic techniques. At 25 °C, the urea-induced unfolding exhibits two well-defined transitions, with the accumulation of an intermediate species in the 2–4 M urea range, which we call  $I_U$ .  $I_U$  has part of its tertiary content compromised, as seen by the partial exposure of tryptophan (Trp) residues to the solvent, but most of its secondary structure is still preserved. This species tends to form large, amorphous aggregates as shown by size-exclusion chromatography (SEC) and by electron microscopy (EM).  $I_U$  is not observed when the experiment is performed at 1 °C, and at this temperature, the unfolding process is

reversible ( $\Delta G_f = -2.2$  kcal/mol) and exhibits only one transition between two stable states. At 25 °C, the folding process investigated upon urea dilution is irreversible and leads to the formation of  $I_U$ , which evolves into amorphous aggregates. When subjected to HHP at 25 and 37 °C, the portal monomer denatures into another intermediate species,  $I_P$ , which seems to be competent for dodecamer formation, as seen by SEC, sucrose gradient (data not shown), and EM, after decompression. At 1 °C, on the other hand, although the unfolding process of the portal monomers is more pronounced, no aggregates are detected, and the process is reversible ( $\Delta G_f = -2.4$  kcal/mol and  $\Delta V_f = 72$  mL/mol). The native portal protein binds the hydrophobic probe bis-ANS, which destabilizes the monomeric portal protein and also completely inhibits its aggregation under HHP. Taken together, the data show that, at physiological temperatures, in the absence of any other cellular or viral component, the folding process of the monomeric portal protein is irreversible, ending in the formation of intermediate species, namely,  $I_U$  and  $I_P$ . The latter can assemble into ring-like structures while the former always forms amorphous aggregates.

## EXPERIMENTAL PROCEDURES

**Chemicals.** All reagents were of analytical grade. Bis(8-anilino)naphthalene-1-sulfonate (bis-ANS) was purchased from Molecular Probes (Eugene, OR). Distilled water was filtered and deionized through a Milli-Q water purification system (Millipore Corp., Bedford, MA). The high-pressure and urea-induced denaturation experiments were performed in 50 mM Tris-HCl and 100 mM NaCl, pH 7.5 (buffer A). We emphasize that Tris buffer was chosen for pressure experiments because its pH does not change significantly under high pressure. The  $\Delta V$  (volume change) for the protonic ionization of Tris is positive and equal to 4.3 cm<sup>3</sup>/mol (13). Spectroscopic grade urea was purchased from Sigma Chemical Co.

**Sample Purification.** Portal protein monomers were purified as previously described (12) from a P22 phage strain

carrying the C-terminal His<sub>6</sub>-tagged portal protein. A pCR-Blunt plasmid (Invitrogen, Carlsbad, CA) was electroporated into *Escherichia coli* strain BL21(DE3). Cells were grown in LB media until the OD reached 0.7 at 600 nm; expression was induced by 1 mM IPTG for 5–6 h at 28 °C. The cells were harvested by centrifugation, resuspended in  $1/20$  volume of nickel binding buffer (20 mM imidazole, 20 mM Tris-HCl, 500 mM NaCl, pH 7.9) containing 0.2 mg/mL lysozyme, and frozen at –80 °C overnight. The cells were then thawed at room temperature, and PMSF (phenylmethanesulfonyl fluoride) was added as a protease inhibitor. The suspension was placed on ice, allowed to lyse, sonicated to reduce viscosity, and centrifuged. Twenty-five milliliters of the lysate was loaded to a 5 mL nickel column (HiTrap chelating; Amersham Pharmacia Biotech, Piscataway, NJ), washed with 40 mL of 65 mM imidazole (in binding buffer), and eluted with 10 mL of 500 mM imidazole (in binding buffer) (12). The protein concentration was determined by using an extinction coefficient of 99740 M<sup>–1</sup> cm<sup>–1</sup> at 280 nm with the sample diluted in 6 M Gdn-HCl.

**Urea-Induced Denaturation.** To monitor the denaturation induced by urea, the monomers (3 μM) were incubated with the desired urea concentrations during 18 h at 25 or 1 °C. After incubation, the samples were analyzed by fluorescence spectroscopy, bis-ANS binding, circular dichroism, or size-exclusion chromatography (SEC).

The refolding experiments were performed by incubating 30 μM portal monomer in the presence of 6 M urea for 18 h. Then, this sample was diluted at 25 or 1 °C to the desired concentrations of urea, shown on the abscissa of Figures 5 and 6, keeping the protein concentration fixed at 2 μM. Since unfolding was not protein concentration-dependent, this concentration was chosen to achieve lower urea concentrations after dilution. Three hours after dilution, the emission spectrum of the tryptophan and the light scattering were recorded as stated below.

**Bis-ANS Experiments.** The fluorescent dye bis-ANS (Molecular Probes, Eugene, OR) was prepared as a stock solution in water, and the concentration was determined spectrophotometrically after dilution in methanol ( $\epsilon_{395} = 23000$  M<sup>–1</sup> cm<sup>–1</sup>) (Molecular Probes, Eugene, OR). In the experiments in which bis-ANS binding to the protein was monitored, monomers (2 μM) were incubated in buffer A in the absence or in the presence of 5 or 10 μM bis-ANS. Bis-ANS spectra were recorded by exciting the sample at 360 nm and collecting emission from 400 to 600 nm. The spectral area in each condition was used to evaluate the extent of bis-ANS binding to the portal protein. The experiments were conducted at 25 °C.

**Spectroscopic Measurements.** Fluorescence spectra were recorded on an ISS K2 spectrofluorometer (ISS Inc., Champaign, IL). The high-pressure cell equipped with optical windows has been described (14) and was purchased from ISS (Champaign, IL). The pressure was increased in 200 bar steps. At each step the sample was allowed to equilibrate for 15 min prior to making measurements. There were no time-dependent changes in fluorescence spectra between 10 and 60 min.

Tryptophan emission spectra were obtained by setting the excitation at 280 nm and collecting the emission in the 300–400 nm range. The mean energy of the fluorescence emission under any condition was evaluated by calculating the center

of spectral mass  $\langle \nu \rangle$ :

$$\langle \nu \rangle = \sum \nu_i F_i / \sum F_i$$

where  $F_i$  is the fluorescence emitted at wavenumber  $\nu_i$ .

All experiments were performed at least twice using different batches of protein, and a representative result is shown.

**Thermodynamic Parameters.** The free energy change of folding was calculated from the experiments performed at 1 °C, where reversibility was observed according to

$$\Delta G_f = -RT \ln K_f$$

where  $K_f$  is calculated from the slope of the curve of  $\ln[\alpha/(1 - \alpha)]$ , where  $\alpha$  is the degree of denaturation, calculated as

$$\alpha = (\nu_i - \nu_0)/(\nu_d - \nu_0)$$

where  $\nu_i$  is the center of spectral mass of tryptophan emission in the presence of each concentration of urea and  $\nu_0$  and  $\nu_d$  are respectively the center of spectral mass of the folded and of the denatured protein.

The volume change of folding ( $\Delta V_f$ ) was calculated according to

$$\ln[\alpha/(1 - \alpha)] = \Delta V_f/RTp + \ln K_u$$

where  $R$  is the gas constant,  $p$  is high pressure,  $T$  is the temperature in which the experiment was performed, and  $K_u$  is the unfolding constant (14). The  $\Delta V_f$  is extracted from the slope of the curve while  $K_u$  is the intercept in the y-axis.

**Light Scattering (LS).** Light scattering measurements were recorded on an ISS K2 spectrofluorometer (ISS Inc.). Scattered light (320 nm) was collected at an angle of 90° to the incident light by integrating the intensity in a 315–325 nm window.

**Circular Dichroism Measurements.** Circular dichroism (CD) measurements were performed in a Jasco-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) using a 1.0 mm path-length quartz cuvette. For spectral determinations, 3 μM portal monomer was prepared in 10 mM Tris-HCl and 100 mM NaCl, pH 7.4. Data were averaged for two scans at a speed of 50 nm/min, collected in 0.2 nm steps. The baselines (buffer alone) were subtracted.

**Size-Exclusion Chromatography.** High-performance liquid chromatography was carried out on a GPC300 column using a HPLC system (Shimadzu SPD-10A). The system was equilibrated with buffer A. A flow rate of 0.3 mL/min was utilized, and the volume of sample applied to the column was 50 μL. Sample elution was monitored by fluorescence at 330 nm (excitation at 280 nm) and by the absorbance at 280 nm.

**Electron Microscopy.** After treatment with HHP or urea, the portal monomers (3 μM) were adhered to a carbon-coated Formvar layer supported on a copper grid, blotted to remove excess material, and stained for 3 min with a 2% solution of uranyl acetate prepared in water. Images were collected on film with an electron microscope Morgagni 268 (FEI Co.), operating at 100 kV, or in an FEI Tecnai G2 Spirit.

## RESULTS

**Urea-Induced Denaturation of the Portal Protein Monomer: Formation of an Intermediate Species at 25 °C.** The



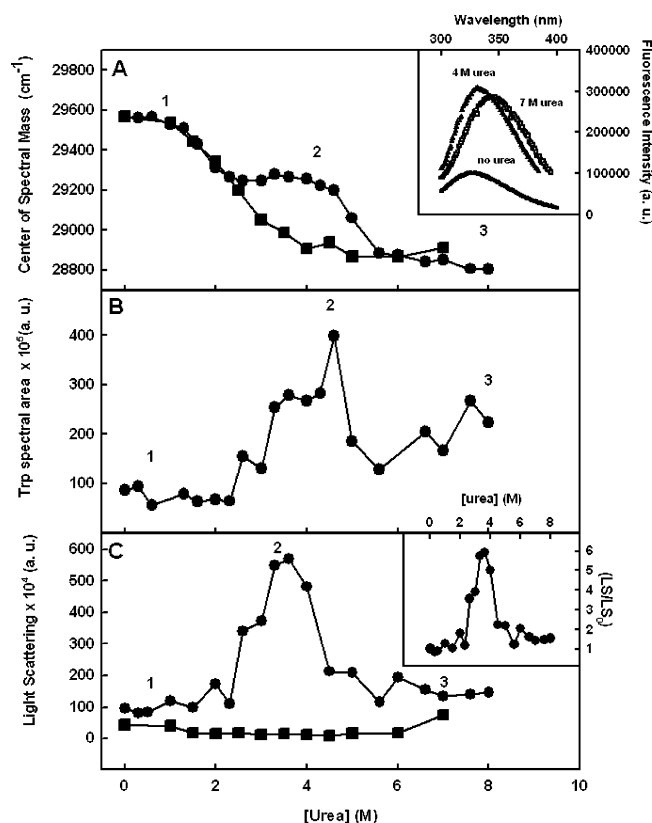


FIGURE 2: Urea-induced unfolding and aggregation of the portal monomers at 25 °C (circles) or 1 °C (squares) as monitored by tryptophan fluorescence and light scattering (LS). Changes in the center of spectral mass (A), fluorescence intensity (B), or LS (C) as a function of increasing concentrations of urea. The inset of panel A shows the fluorescence emission spectra of the species populated at the indicated concentrations of urea at 25 °C. In panels A and B, the samples were excited at 280 nm, and emission was collected from 300 to 400 nm. The LS (panel C) was measured by exciting the samples at 320 nm and collecting the scattered light from 315 to 320 nm. Inset of panel C: The area of each spectrum (LS) was normalized to the initial values ( $LS_0$ ). The three regions observed in the urea-induced unfolding curve at 25 °C are marked in numbers in each panel. The protein concentration was 3  $\mu$ M in all cases, and the samples were incubated for 18 h in the presence of urea at the indicated temperatures.

portal protein has 11 Trp residues; all but one are confined to the N-terminal half of the sequence (15). As shown previously by Raman spectroscopy, significant conformational adjustments seem to occur in the N-terminal segment of the portal protein during its oligomerization into dodecamers (rings), since one of the bands related to the indolyl group of Trp shifts from 1554 to 1551  $\text{cm}^{-1}$  (7). Thus, changes in Trp fluorescence emission might be useful for structural studies during folding and oligomerization of the portal protein.

Figure 2A shows the shift in the center of spectral mass of the Trp emission as a function of urea addition at 1 (squares) or 25 °C (circles). It is important to stress here that the samples used for these experiments were previously characterized by size-exclusion chromatography and shown to contain only the monomeric protein (see Figure 5A, continuous line). As seen, at 25 °C the unfolding process exhibits two well-defined transitions. The center of spectral mass for the fluorescence emission spectrum of the folded, native monomer of the portal protein is 29570  $\text{cm}^{-1}$  (338 nm). The addition of urea shifts the center of spectral mass

progressively to 29250  $\text{cm}^{-1}$  (342 nm) at 2.3 M with no further change on increasing the urea to 4.6 M. From 4.6 to 8 M urea, a second transition takes place, and the center of spectral mass reaches a value of 28800  $\text{cm}^{-1}$  (347 nm) at the end of the unfolding process. These two transitions suggest the presence of an intermediate species in the unfolding of the portal monomer of P22 at 25 °C. In each panel of Figure 2, the numbers 1, 2, and 3 indicate the regions where the folded monomers (region 1), the intermediate species (region 2), or the unfolded monomers (region 3) prevail. The inset in panel A shows the fluorescence emission spectra of the species that accumulate in these three regions. The intermediate species (formed in the presence of 2–4 M urea) will be called  $I_U$ .

Figure 2B shows the changes in the fluorescence intensity of Trp that take place upon urea addition at 25 °C. As seen, from 0 to 2.3 M urea (region 1), Trp emission remains unaltered; it increases approximately 4-fold in region 2, where  $I_U$  builds up, and it decreases again with the complete unfolding of the portal protein (region 3). This suggests that the Trp residues are probably partially protected from the solvent but quenched by the neighboring residues in the folded state, and the quenching is released upon partial unfolding of the protein. Above 4.6 M urea, the decrease in Trp emission is probably related to the complete exposure of these residues to the aqueous environment and the concomitant quenching by the solvent.

In order to investigate whether  $I_U$  displays a propensity to aggregate, light scattering (LS) measurements were performed. As seen in Figure 2C and in the inset, at 25 °C (circles) the LS increased almost 6-fold only in the concentrations of urea in which  $I_U$  accumulates (region 2). Thus, aggregation of the portal protein might account for the stabilization of the center of spectral mass values that characterize region 2 in the urea-unfolding curve (Figure 2A). However, the two transitions observed upon urea addition also occur in the presence of high salt concentrations (500 mM NaCl or 500 mM KCl), where aggregation is greatly inhibited (not shown; manuscript in preparation). This observation gives us confidence that the plateau (region 2) observed at intermediate concentrations of urea is not an artifact of the fluorescence measurements. Besides, since the two transitions are also observed in the presence of high salt concentrations with the concomitant inhibition of the aggregation, we reason that there is indeed an intermediate species present in the unfolding process of the portal protein, which is aggregation-prone under specific conditions.

Since at 25 °C the unfolding of the portal protein exhibits two well-defined transitions, we decided to investigate whether  $I_U$  can be formed at low temperatures. Curiously, when the experiment is performed at 1 °C (Figure 2, squares), the unfolding process fits a two-state model, with only one transition and no detectable intermediate species. We also do not observe any significant variation in fluorescence emission intensity (not shown) at this temperature. As expected, since no intermediate species is detected at 1 °C, no aggregation (Figure 2C, squares) is observed in the presence of urea. This suggests that  $I_U$  is destabilized by the low temperature, consistent with a contribution of hydrophobic interactions to its stability.

To obtain additional information on the tertiary structure content of  $I_U$ , bis-ANS binding experiments were performed

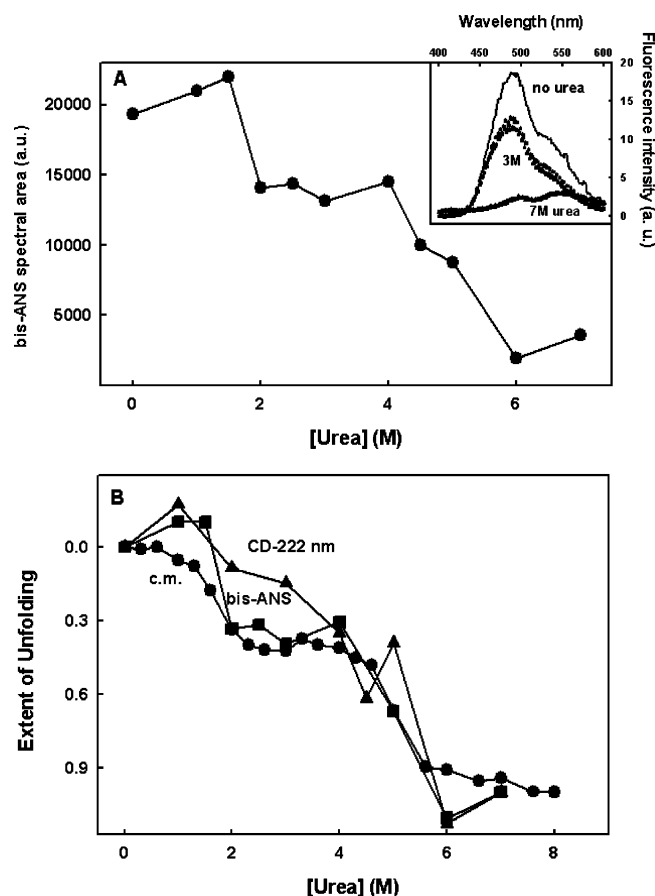


FIGURE 3: Changes in the tertiary structure content of the portal protein induced by urea, as monitored by bis-ANS binding. (A) Bis-ANS ( $5 \mu\text{M}$ ) was added to each sample and incubated in the presence of urea ( $25^\circ\text{C}/18 \text{ h}$ ). The area of its fluorescence emission spectrum (from 400 to 600 nm) was obtained by exciting the samples at 360 nm. Inset: Spectra of bis-ANS bound to the portal monomers in the absence or in the presence of 3 or 7 M urea as marked. The protein concentration was  $1 \mu\text{M}$ . (B) Extent of unfolding calculated from the changes in the center of spectral mass (●), bis-ANS binding (■), and ellipticity at 222 nm (▲) using the data presented in Figure 2A and this figure, respectively. All of the conditions are described in the legends of the corresponding figures.

(Figure 3). Bis-ANS exhibits a very weak fluorescence emission in polar solvents due to solvent quenching (16). When bound to accessible hydrophobic segments (especially in the vicinity of positive charges) such as those commonly observed in folding intermediates, bis-ANS fluorescence is greatly enhanced. This allows it to be useful as a sensor of these partially folded species (16–18). Figure 3 (inset) shows that the portal protein in its native state binds bis-ANS, as do some DNA-binding proteins (19). Binding probably occurs near the positively charged sites (12). As seen, at  $25^\circ\text{C}$  where  $I_U$  accumulates, the unfolding of the portal protein induced by urea also causes two well-defined transitions in bis-ANS binding that coincide with the center of spectral mass changes seen in the absence of bis-ANS (see also Figure 4B). In the concentrations of urea where  $I_U$  accumulates (2–4 M urea, region 2), bis-ANS binding is constant and  $\sim 30\%$  lower than that displayed by the native state. In general, folding intermediates bind ANS and related compounds on the accessible hydrophobic surfaces (20, 21). Considering that at these urea concentrations this intermediate form aggregates (Figure 2C) and that the hydrophobic contacts

are probably buried in protein–protein interactions, a decrease in bis-ANS binding capacity is expected. In the presence of high salt concentrations, the intensity of bis-ANS increases by 11–12-fold (manuscript in preparation). At high urea concentrations, where the aggregates do not exist and the portal protein seems to be denatured, bis-ANS binding is absent. The inset of Figure 3 shows the spectra of bis-ANS in the presence of the species present in regions 1 (no urea), 2 (3 M urea), and 3 (7 M urea).

In order to characterize the secondary structure content of the portal monomers in urea-induced unfolding at  $25^\circ\text{C}$ , circular dichroism (CD) spectra were recorded (data not shown but see the curve with triangles in Figure 3B). Although in the range of 2–4 M urea most of the portal protein forms aggregates, the solutions are not turbid, thus allowing CD measurements. In the presence of urea up to 2 M, the secondary structure of the protein does not change significantly while in this same urea range, the Trp emission has already shifted  $250 \text{ cm}^{-1}$  (4 nm) to the red (Figure 2A). Increasing the concentration of urea to 8 M causes a progressive loss of the signal at 222 nm, compatible with complete denaturation of the portal protein (not shown).

Figure 3B compares the extent of unfolding when the changes in the center of spectral mass of Trp emission (circles), the CD signal at 222 nm (triangles), and the bis-ANS binding (squares) are used as sensors of the structural changes induced by urea addition at  $25^\circ\text{C}$ . In the concentrations of urea where  $I_U$  accumulates (2–4 M), there is an  $\sim 40\%$  loss of tertiary content, as measured by the change in the center of spectral mass of Trp emission, with less significant changes in the secondary structure content (Figure 3B). This implies that the portal protein in the presence of 2–4 M urea loses part of its tertiary structure, exposing hydrophobic segments to the aqueous environment. These may serve as sites for intersubunit contacts, leading to the formation of aggregates. Within these aggregates, however, the protein is not completely unfolded, and most of its secondary structure is still preserved (Figure 3B).

*Characterizing the Aggregates Formed during the Folding/Refolding Processes of the Portal Protein at  $25^\circ\text{C}$ .* In order to obtain insights regarding the hydrodynamic properties of the aggregate derived from  $I_U$ , SEC experiments were performed (Figure 4A). The portal protein was incubated with different concentrations of urea at  $25^\circ\text{C}$  for 18 h and then injected into a GPC 300 column preequilibrated with buffer A (without urea) (Figure 4A). The native portal monomer elutes as a single peak at 8.8 min, while the native dodecamer elutes at 7.5 min. The protein samples incubated in the presence of 2, 3, or 4 M urea at  $25^\circ\text{C}$ , where  $I_U$  accumulates (region 2), coelute in a shorter time (6.8 min), indicating that their hydrodynamic radius is even larger than that of the dodecamers. Their elution peaks are very broad, suggesting heterogeneity. It has to be mentioned that a solution with purified dodecamers, when it is aged, displays an additional elution peak at 6.6 min (not shown), an elution time very similar to that displayed by the aggregates derived from  $I_U$ . The protein treated with 8 M urea elutes at 7.8 min, faster than the native monomer and compatible with an extended, unfolded protein. Since the elution buffer does not contain urea, the observation of an unfolded protein still present in this buffer suggests that the unfolding of the portal

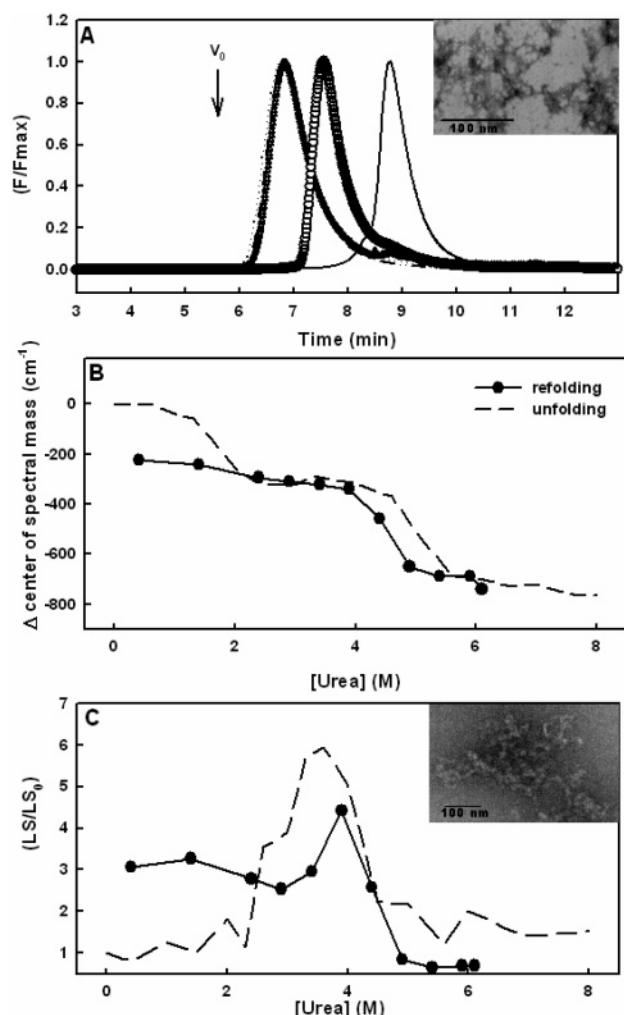


FIGURE 4: The urea-induced unfolding of the portal protein at 25 °C is irreversible: characterizing the size of the aggregates formed in the presence of urea. (A) The portal protein (3  $\mu$ M) was incubated in the presence of 2, 3, or 4 M urea at 25 °C for 18 h and then injected into a GPC 300 column preequilibrated with buffer A with no added urea (superimposed peaks eluting at 6.8 min). For comparison, the elution profiles of the native monomers (continuous line) and dodecamers (circles) are shown. Elution was followed by fluorescence emission at 330 nm with excitation set at 280 nm. The arrow indicates the void volume which is at 5.57 min. The inset shows the electron microscopy image of the protein incubated in the presence of 4.5 M urea where only amorphous aggregates are observed. The urea concentration was 3  $\mu$ M in all cases. (B and C) The reversibility of the urea-induced denaturation at 25 °C (filled circles) was monitored by the changes in the center of spectral mass (B) and by light scattering (C). For the measurements of panels B and C, the portal monomers (30  $\mu$ M) were incubated in the presence of 6 M urea at 25 °C during 18 h and then diluted to 2  $\mu$ M at the concentrations of urea shown on the abscissa for 3 h prior to the spectroscopic measurements. For comparison, the urea-induced denaturation at 25 °C from Figure 1 is reproduced in dashed lines. The inset of panel C shows the electron microscopic image of the aggregates formed upon urea dilution (0.4 M urea) at 100000 $\times$  magnification.

protein may be irreversible, at least within the time range of these experiments.

To obtain further insight into the morphology of the aggregates derived from I<sub>U</sub>, electron microscopy experiments were performed and are presented in the inset of Figure 4A. As seen, there are only large, amorphous aggregates in the sample treated with 4.5 M urea at 25 °C, which is consistent

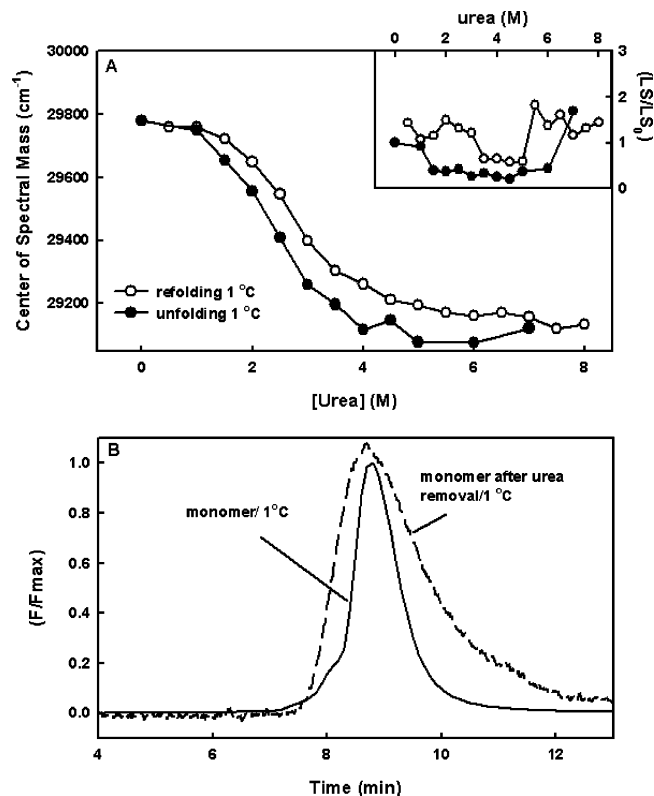


FIGURE 5: The urea-induced unfolding of portal protein monomers is reversible at 1 °C. Panel A shows the changes in the center of spectral mass of Trp emission induced by urea unfolding (filled circles) or refolding (hollow circles). The samples of monomers (30  $\mu$ M) were incubated in the presence of 6 M urea for 18 h at 1 °C and then diluted to the urea concentrations indicated on the abscissa of panel A, keeping the protein concentration at 2  $\mu$ M. The inset shows the size-exclusion chromatography of the portal monomers kept at 1 °C in the absence of urea (continuous line) and 50 min after dilution of urea to 0.4 M (dashed line) where only monomers are observed. Other conditions are as in this figure.

with its shorter elution time when compared to the dodecamers (Figure 4A).

In order to investigate the reversibility of the urea-induced unfolding of the portal protein, a concentrated sample of unfolded monomers (30  $\mu$ M) was diluted to the concentrations of urea shown on the abscissa of Figure 5B,C (circles), and the center of spectral mass of Trp emission (panel B) and the LS (panel C) were evaluated 3 h later, when equilibrium was already achieved. For comparison, the unfolding profile at 25 °C from Figure 2 is also presented (dashed lines).

It is interesting to note that the unfolding of the portal monomer is irreversible at 25 °C, as revealed by the center of spectral mass of Trp emission (Figure 4B), and the value attained after dilution to 0.4 M urea is the same as that presented by I<sub>U</sub>. This value is 200 cm<sup>-1</sup> shifted to the red in relation to that observed for the native monomer. The LS (Figure 4C) also increases 3–4-fold in concentrations of urea below 4 M and remains elevated even in the presence of 0.4 M urea (circles in Figure 4C). The inset of Figure 4C shows the EM image of the aggregates formed during refolding of the portal protein. This protein has an amorphous appearance, much like the unfolded protein (Figure 4A). The formation of these irretrievable aggregated species during refolding of the portal monomer, even at this low protein concentration



(2  $\mu$ M), may explain why this process is irreversible. In both situations (unfolding and refolding), no ring particles are observed on the EM grids.

We note that even when the refolding is performed with an elevated protein concentration (500  $\mu$ M, 25  $^{\circ}$ C), a condition that, as shown before, favors the conversion of folded monomers into dodecamers *in vitro* (12), we do not recover dodecamers (not shown). Clearly, ring formation is not limited solely by the use of a low protein concentration.

*The Folding Pathway of the Portal Protein Is Reversible Only at 1  $^{\circ}$ C.* Since the folding of the portal protein was irreversible at 25  $^{\circ}$ C, we decided to investigate whether at low temperatures (1  $^{\circ}$ C) this process would be reversible, since at this temperature the unfolding process exhibited no detectable intermediate or aggregated species (Figure 2). Figure 5 shows that when the refolding is performed at 1  $^{\circ}$ C, the center of spectral mass value returns completely to its original position (panel A) upon urea dilution, while the LS remains low (inset of panel A). These data suggest that at 1  $^{\circ}$ C the folding of the portal protein is reversible. When injected into a gel filtration column, this refolded protein elutes at 8.8 min, confirming the recovery of native, folded monomers (Figure 5B).

Since at 1  $^{\circ}$ C the folding process of the portal protein proved to be reversible, the free energy change of folding was calculated as described in Experimental Procedures. This value was  $-2.2$  kcal/mol.

*HHP-Induced Denaturation of the Portal Protein Monomer: Formation of an Intermediate Species That Aggregates into a Ring-like Structure.* In previous studies by our group and others, it has been shown that HHP is a mild perturbing agent that, in several cases, leads to the formation of partially folded states which also play an important role in amyloid diseases (23, 24). Thus, we decided to compare the effect of HHP on the structure of the portal monomers with the effects induced by urea described above.

Figure 6A shows the changes in the center of spectral mass of Trp residues of the portal protein when HHP is applied to the portal protein at 1 (squares), 25 (circles), or 37  $^{\circ}$ C (triangles). As seen, the denaturation profiles are similar at 25 and at 1  $^{\circ}$ C, suggesting comparable stability of the protein against HHP at these two temperatures. However, the final values of the center of spectral mass of the Trp emission at 3000 bar are slightly different. At 25  $^{\circ}$ C, the curve reaches 29200  $\text{cm}^{-1}$  (343 nm), while at 1  $^{\circ}$ C the final value is 29100  $\text{cm}^{-1}$  (344 nm). These values, although suggestive of extensive solvent exposure of the Trp residues, are not the same as that observed in the presence of 8 M urea (347 nm, Figure 2A), which suggests that HHP treatment, even at 1  $^{\circ}$ C, is not sufficient to promote the complete denaturation of portal monomers.

Figure 6A also shows the changes in the center of mass of Trp emission at 37  $^{\circ}$ C. At this temperature and atmospheric pressure, the portal protein undergoes spontaneous aggregation over time (further discussed in relation to Figure 7), which prevents us from analyzing urea-induced unfolding at this temperature (see Experimental Procedures). In order to allow high-pressure studies at this temperature, the sample was prepared and immediately subjected to increasing pressures. As seen, at 37  $^{\circ}$ C the portal protein is very unstable, and the total change in the center of spectral mass, although small (300  $\text{cm}^{-1}$  or  $\sim 3$  nm), is achieved at very

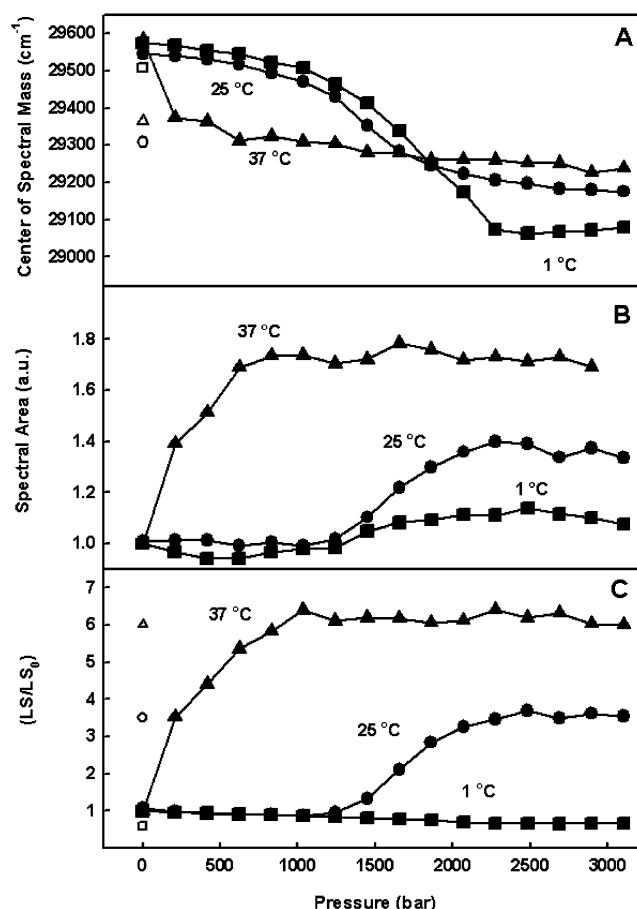


FIGURE 6: High-pressure-induced denaturation of the portal monomers at different temperatures, monitored by fluorescence spectroscopy. (A) Changes in the center of Trp spectral mass, (B) normalized Trp fluorescence intensity, or (C) normalized LS as a function of increasing pressures. The experiments were performed at 1 (■), 25 (●), or 37  $^{\circ}$ C (▲). Isolated symbols on the left represent the values obtained after returning to atmospheric pressure. The protein concentration was 3  $\mu$ M. Other conditions are as in Figure 2.

low pressures (500 bar). The low stability of the portal protein at 37  $^{\circ}$ C is expected, since in previous studies by Moore and Prevelige (12) at this temperature, the portal protein appeared to be slightly unfolded.

Taken together, these results suggest that HHP up to 3000 bar is unable to completely denature the portal protein in this range of temperatures. The center of mass values observed under pressure at 25 and 37  $^{\circ}$ C are very similar to that found for the intermediate  $I_U$  (342 nm), which might suggest that HHP converts the portal protein into a partially unfolded species, as observed (reviewed in refs 24 and 25).

As seen in Figure 6A, when pressure is released at 25 or 37  $^{\circ}$ C, the center of mass of the emission spectrum of the Trp does not come back to the original value (empty symbols at left), showing that the structural changes induced by HHP are only reversible at low temperature.

Figure 6B shows that the changes in the center of spectral mass promoted by HHP are accompanied by an increase in Trp emission, as shown earlier (Figure 2B) for the first transition of the urea-induced unfolding of the monomers, where  $I_U$  accumulates. At 37  $^{\circ}$ C, this increment levels off at very low pressures ( $\sim 500$  bar) similar to the profile observed for the shift in the center of mass (Figure 6A). The



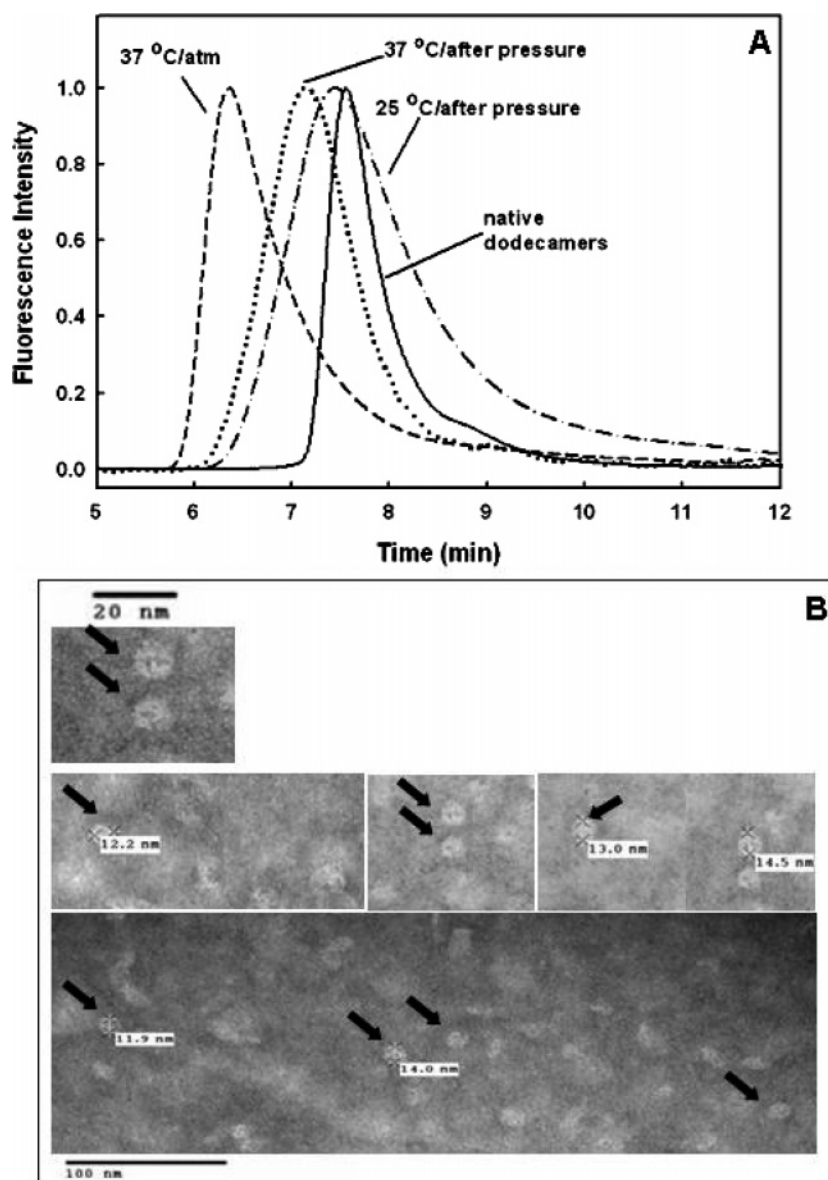


FIGURE 7: Determining the hydrodynamic radii and the morphology of the species formed after pressure treatment. (A) Elution profiles in a GPC 300 column of the protein incubated at 37 °C at atmospheric pressure for 8 h (37 °C/atm, dashed line) or after pressure treatment at 37 °C (dotted line) or at 25 °C (dash-dot-dash line). Pressure-treated samples were subjected to 3000 bar for 60 min before analysis. For comparison, the elution profile of the dodecamers is shown by a solid line. The elution of the proteins was followed by fluorescence at 330 nm. In panel B, the monomers (3  $\mu$ M) were pressure-treated at 25 °C before EM imaging at 42000 $\times$  or 67000 $\times$  magnification. Different fields are shown. The scale bar = 20 or 100 nm, as indicated.

intermediate species formed under pressure will be called  $I_P$ , and the next experiments were designed to investigate its similarity with  $I_U$ .

In order to evaluate whether  $I_P$ , like  $I_U$ , can form aggregates, LS measurements were performed (Figure 6C). Curiously, in spite of the fact that the protein is under pressure, where aggregation is not expected (17, 23, 24), an oligomeric species is formed at 25 and 37 °C, resulting in a 4- and 6-fold increase in LS (panel C), respectively, even at 3000 bar. After decompression (isolated symbols at left), the LS values remain elevated, suggesting that the aggregates are stable even at atmospheric pressure. At 1 °C, as expected, aggregation is completely inhibited (Figure 6C, squares), and upon pressure release the center of spectral mass returns almost completely to its original position (isolated square in panel A), suggesting reversibility of the unfolding process. The calculated free energy change and the volume change

of folding at 1 °C were  $-2.4$  kcal/mol and 72 mL/mol, respectively.

In terms of propensity to aggregate,  $I_P$  and  $I_U$  are quite similar, although LS does not provide any information about the size and morphology of the aggregates. To investigate the hydrodynamic properties of the aggregates formed after pressure treatment, SEC experiments were performed (Figure 7A). As mentioned before, incubation of the portal monomers at atmospheric pressure and 37 °C leads to a slow (in hours) increase in LS (not shown), suggesting aggregation of the protein. However, at 25 °C there is no spontaneous increase in the LS at atmospheric pressure at this low protein concentration (not shown). Figure 7A shows that a sample incubated at atmospheric pressure at 37 °C for 8 h elutes as a broad peak at 6.3 min (long dashes), indicating formation of a species larger than the native dodecamers (7.5 min, continuous line). Strikingly, the oligomers produced after

HHP treatment at 37 or at 25 °C exhibit retention times of 7.1 (dotted line) and 7.45 min (dash-dotted line), respectively, which are close to that of the native dodecamers (7.5 min). This result suggests that HHP induces the formation of an oligomer with a size compatible with that of the native dodecamers. We recall that the aggregates formed in the presence of intermediate urea concentrations elute at 6.8 min (Figure 4A) and, thus, are different from the aggregates formed by the pressure treatment.

The aggregates formed under pressure at 25 °C were chosen for the next experiments, where their size and morphology were investigated. Figure 7B shows the electron microscopic images of the sample recovered from HHP treatment at 25 °C. As seen, this sample, although very heterogeneous, reveals numerous “ring-like” structures, marked by arrows. These annular structures have diameters ranging in size from 12 to 14 nm, a value that is compatible to that measured before for the dodecamers observed by cryoEM, where it was observed that the external diameter of the portal protein ranges from 8 to 16 nm (8). In some of the rings in Figure 7B it is also possible to see the internal channel through which the DNA passes during virus morphogenesis. These observations suggest that HHP induces the formation of aggregates that have a ring-like morphology and other physical characteristics similar to the native dodecamer, as seen by SEC and EM images. However, it is possible that this annular structure formed after pressure treatment is composed by a different number of subunits. Further studies are necessary to address this issue.

In order to learn more about the tertiary structure content of the portal protein under HHP at 25 °C, bis-ANS binding experiments were performed (Figure 8). Curiously, when the portal protein is incubated in the presence of bis-ANS in a 1:2.5 and 1:5 molar ratio (protein:bis-ANS), its stability decreases (Figure 8A, circles) as seen by the displacement of the center of mass curves to lower pressure values with increasing concentrations of bis-ANS. The finding that bis-ANS renders the portal protein more unstable indicates a change in conformation and is consistent with an earlier observation that bis-ANS makes it more susceptible to protease digestion (12). Another important observation is the fact that bis-ANS completely inhibits the HHP-induced aggregation of the portal protein, as seen in the LS measurements of Figure 8B. Bis-ANS has been shown to be effective as an inhibitor of the aggregation of the prion protein (27) as well as the  $\beta$  amyloid peptide (28). Bis-ANS also inhibits the polymerization of P22 capsid (29).

Figure 8C shows the changes in bis-ANS binding by portal protein subjected to HHP. As mentioned, the native, soluble portal protein binds bis-ANS. As shown, at low-pressure values there is a sharp decrease in the bis-ANS fluorescence signal that is compatible with its release from its binding site in the portal protein. However, as pressure increases, there is a second transition where the bis-ANS fluorescence signal increases considerably, suggesting that under high pressure the portal protein is not completely unfolded but instead preserves part of its tertiary structure. Since bis-ANS inhibits the pressure-induced aggregation of the portal protein, we assume that its binding profile reflects structural modification of the monomeric, soluble protein. The inset of Figure 8C shows the elution profile for the monomer of the portal protein before and after decompression in the

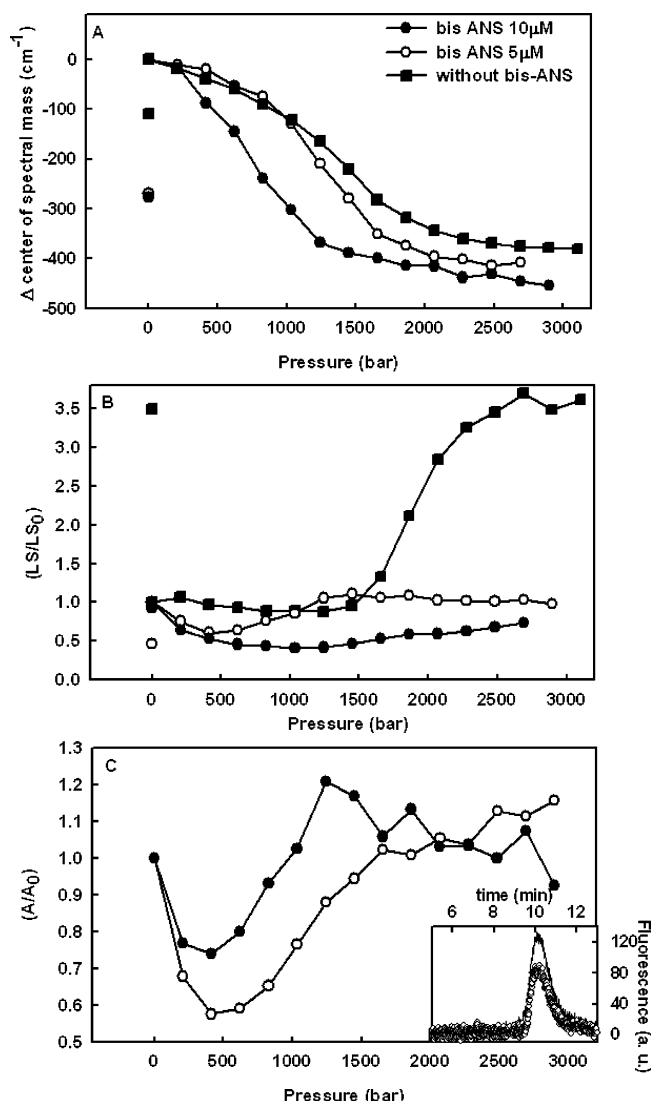


FIGURE 8: High-pressure-induced denaturation of the monomers of the portal protein in the presence of bis-ANS. The monomers (2  $\mu$ M) were incubated in buffer A in the absence (■) or in the presence of 5 (○) or 10  $\mu$ M (●) bis-ANS. Panel A shows the changes in the center of spectral mass of Trp emission at each pressure value, and panel B shows the variations in LS values normalized for better comparison. In panel C, the binding of bis-ANS under pressure was monitored by measuring the area of its fluorescence emission spectra (A), which was divided by the initial area ( $A_0$ ). The inset shows the SEC profile of the monomers (2  $\mu$ M) in the presence of bis-ANS (10  $\mu$ M), before (continuous line) or after being subjected to 3000 bar for 60 min (hollow circles). The protein elution was monitored by following the emission at 330 nm with the excitation set at 280 nm.

presence of bis-ANS (10  $\mu$ M). As shown, in the presence of bis-ANS the portal protein elutes at 10 min instead of 8.9 min, suggesting that, upon binding, this probe induces the compactness of the protein as suggested before by Moore and Prevelige (12). Here it is possible to see refolded monomers after decompression, suggesting reversibility of the unfolding process under these conditions. However, the center of spectral mass of Trp emission does not come back to their original values (isolated symbols on panel A). It is possible that the bis-ANS that remains bound to the protein might be interfering with tryptophan fluorescence.

## DISCUSSION

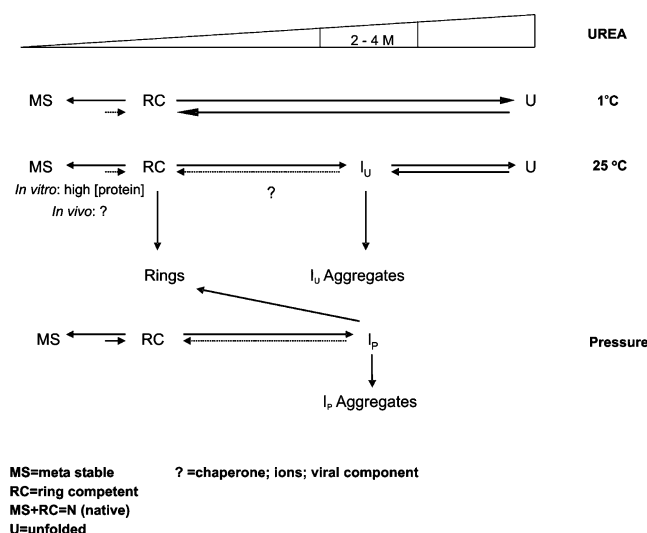
An important observation derived from the present study is that the unfolding process of the portal monomer, whether induced by a chemical or by a physical agent, is only reversible at a low temperature (Figures 5 and 6) or in the presence of ligands such as bis-ANS (Figure 8). At 1 °C, the unfolding process fits a two-state model, with no detectable intermediate present. Intermediate species that are formed at 25 or 37 °C tend to aggregate, suggesting that the formation of these aggregates compromises the correct folding of this protein, which has a natural tendency to form physiologically relevant oligomers (dodecamers). However, two different types of aggregates are described here: one that has an amorphous appearance (Figure 4), while the other, although heterogeneous in shape, reveals a substantial population of a ring-like structure (Figure 7). These aggregates originate from different intermediate species,  $I_U$  and  $I_P$ , respectively. The amorphous species forms in the presence of intermediate concentrations of urea (2–4 M), while the annular species is observed under HHP.

It is not known whether the dodecamers formed *in vivo* arise from an intermediate species present in the folding pathway of the portal protein (such as  $I_P$ ) or from the native monomeric protein. On the other hand, *in vitro*, ring formation has been reported to occur slowly (in hours) at 25 °C (12) in the absence of any added cofactor, but at extremely high protein concentrations ( $\sim 500 \mu\text{M}$ ). This time frame (hours) is much longer than that required for phage assembly, implying that protein–protein collision is not the only rate-limiting step for dodecamer formation *in vivo*. Obligatory structural transformations have to take place to drive assembly, and indeed, it has been observed that there is a change in the  $\alpha$ -helical content in going from monomers to dodecamers as well as a red shift in Trp emission (7, 12).

It has also been postulated (12) that the monomers of the portal protein of P22 exist in a metastable conformation (MS) unable to form dodecamers. Changes in the secondary and tertiary structures might take place in order to convert this metastable conformation into a ring assembly competent conformation (RC). Thus, at least *in vitro*, dodecamer formation can start from the native monomer. However, as we have shown here, the completely unfolded species (U) obtained in the presence of high urea concentrations at physiological temperatures does not fold spontaneously back to its native conformation; instead, it evolves into an aggregated amorphous species. These observations suggest that *in vivo* there might be a cellular or viral component that guarantees the correct folding of the portal monomers, avoiding this deleterious intermediate ( $I_U$ ). Another possibility is that dodecamers are assembled *in vivo* from an intermediate species with properties similar to  $I_P$ , which forms during the folding of the monomeric protein.

All of the species described thus far in the folding/dodecamerization of the portal protein are summarized schematically (see Scheme 1). We place RC on the pathway to U, although the only evidence we have on this issue is the fact that RC is less folded than MS, possibly representing the first step to the complete unfolding of the portal protein.  $I_U$  and  $I_P$  are shown as the species that give rise to aggregates (or ring-like structures as in the case of  $I_P$ ), since they can be trapped in the presence of high salt concentrations devoid

Scheme 1



of aggregates (manuscript in preparation). Of course, several questions remain open in this pathway, and further studies will be necessary for its complete elucidation.

*In vivo*, what factor(s) could be involved with the conversion of the metastable conformation into a ring-competent species ( $\text{MS} \rightleftharpoons \text{RC}$ )? As previously shown, the  $\text{MS} \rightleftharpoons \text{RC}$  conversion is separated by a high-energy barrier (12 kcal/mol of subunits) (12). It is possible that the binding of any viral component to the portal protein during phage morphogenesis would shift this equilibrium in the direction of RC, favoring ring formation as previously suggested (5, 30, 31). We have shown here that HHP seems to mimic somehow the effects of such factors to produce the structural changes that are necessary to convert at least a fraction of the population of MS into a ring assembly competent species ( $I_P$ ). Thus,  $I_P$  might share properties with RC, at least in terms of the aggregates they can form. Previous work with phage  $\phi 29$  has suggested the interaction of chaperonins with the connector of this phage (31). This interaction would facilitate the interaction of connector protein with the scaffold and capsid proteins during capsid assembly.

In the case of the connector of the bacteriophage SPP1, it has been shown that magnesium ions aid in the assembling of this portal protein into rings (32), but no information is available regarding the effects of ions on the folding of the portal protein of P22.

The formation of correctly folded monomers was observed in our study at low temperature or in the presence of bis-ANS (Figures 5 and 8). This implies that *in vivo* there might be a cellular or viral cofactor (a specific ion or nucleic acid, a cellular chaperone, the scaffold protein of P22, etc.), which aids in portal folding, avoiding these nonproductive intermediates that is aggregation prone and favors the formation of intermediates such as  $I_P$  which funnels into dodecamer formation.

Under the conditions where folding is reversible (1 °C), the thermodynamic parameters were calculated. Interestingly, the free energy change of folding for the monomeric portal protein is quite small ( $-2.2/-2.4$  kcal/mol), regardless of the perturbing agent employed. This low stability probably is related to the plasticity of the portal protein, which has to interact with several different ligands, such as the coat and



the scaffolding proteins, the terminase complex, and the DNA during its packaging. Also, the monomeric portal protein has to undergo structural adjustments in order to make intersubunit interactions during dodecamer formation. All of these interactions require malleability, which is probably achieved with this low thermodynamic stability.

There are very few examples in the literature of monomers successfully denatured by HHP (33, 34). The portal monomer constitutes a new example. In fact, at 37 °C the protein proved to be even more unstable than at 25 or 1 °C, denaturing at very low pressure values. It is possible that physiologically, in the temperature range in which the bacterial cells grow, the portal protein assumes this very unstable conformation with a strong tendency to aggregate, thereby shifting from the productive to the nonproductive pathway. Again, cellular or viral cofactors might impede this deviation, funneling this species into the dodecamer formation pathway. In our experiments, HHP had this effect.

The data presented here also suggest that the portal protein has a specific bis-ANS binding site that, when occupied, decreases the stability of the protein (Figure 8). By evaluating the bis-ANS binding profile under pressure, it is possible to distinguish two regions: the first region is observed at low-pressure values, being characterized by the release of this probe from its binding site(s); in the second region, there is a progressive recovery of the bis-ANS binding, and at elevated pressures, the species formed are still able to accommodate this probe, suggesting persistence of tertiary structure elements. Since the portal protein seems to have two domains (12), it is tempting to suggest that these two regions observed in the high-pressure-induced denaturation curve in the presence of bis-ANS are associated with the unfolding of each one of these domains. It has to be recalled that, since bis-ANS inhibits the pressure-induced aggregation of the portal protein, we are assuming here that the bis-ANS binding observed under pressure does not reflect the presence of the aggregated species but, instead, the persistency of the tertiary contacts of the portal protein that seem to be preserved under pressure.

## CONCLUSION

Our data show that, at intermediate urea concentrations (2–4 M) or under HHP at adequate temperatures, the monomers of the portal protein of P22 are converted into two different intermediate species,  $I_U$  and  $I_P$ , respectively, which both exhibit a strong tendency to undergo aggregation. However, the aggregates formed exhibit different morphologies, being amorphous in the case of  $I_U$  and at least in part an ordered, ring-like structure, in the case of  $I_P$ . The folding of the monomeric portal protein of P22 proved to be irreversible at physiological temperatures but reversible at 1 °C, where a two-state folding process is observed. This suggests that external factors might be involved in vivo to guarantee the formation of the folded state of the portal protein.

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