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High-pressure applications in medicine and pharmacology

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Abstract

High pressure has emerged as an important tool to tackle several problems in medicine and biotechnology. Misfolded proteins, aggregates and amyloids have been studied, which point toward the understanding of the protein misfolding diseases. High hydrostatic pressure (HHP) has also been used to dissociate non-amyloid aggregates and inclusion bodies. The diverse range of diseases that result from protein misfolding has made this theme an important research focus for pharmaceutical and biotech companies. The use of high pressure promises to contribute to identifying the mechanisms behind these defects and creating therapies against these diseases. High pressure has also been used to study viruses and other infectious agents for the purpose of sterilization and in the development of vaccines. Using pressure, we have detected the presence of a ribonucleoprotein intermediate, where the coat protein is partially unfolded but bound to RNA. These intermediates are potential targets for antiviral compounds. The ability of pressure to inactivate viruses, prions and bacteria has been evaluated with a view toward the applications of vaccine development and virus sterilization. Recent studies demonstrate that pressure causes virus inactivation while preserving the immunogenic properties. There is increasing evidence that a high-pressure cycle traps a virus in the 'fusion intermediate state', not infectious but highly immunogenic.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Proteins play the major functions in cells either as isolated molecules or forming macromolecular complexes held together by noncovalent interactions. Life depends on macromolecular

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recognition, especially at the level of protein folding and protein–nucleic acid (NA) interactions. The mechanism by which unstructured proteins spontaneously fold to their native functional form is still not completely understood (Radford 2000). To this end, it is necessary to isolate and describe the intermediate structures that occur during folding. Here, it is reviewed that in several cases these intermediates have been trapped under pressure and their dynamics and structure have been characterized by fluorescence, light scattering, NMR and hydrodynamic methods. These intermediate structures are key elements in the pathogenesis of many diseases.

Hydrostatic pressure is a robust tool to study the thermodynamics of protein folding, protein–protein interactions and protein–nucleic acid interaction (Silva *et al* 1996, Mozhaev *et al* 1996, Silva *et al* 2001b, Balny *et al* 2002). In recent years, high pressure has emerged as an important tool to tackle several problems in medicine and biotechnology. One of the main innovations of using high pressure is the stabilization of folding intermediates, which makes it possible to characterize their structures and dynamics by different methodologies (Silva *et al* 2001b). More recently, misfolded proteins, aggregates and amyloids, derived from partially folding intermediates at the junction between productive and off-pathway folding, have been studied as well, which points toward the understanding of the protein misfolding diseases (Ferrão-Gonzales *et al* 2000, 2003, Torrent *et al* 2003, Ishimaru *et al* 2003a). High hydrostatic pressure (HHP) has also been used to dissociate non-amyloid aggregates and inclusion bodies (Foguel *et al* 1999, Webb *et al* 2001). The competition between correct folding and misfolding, which in many proteins leads to formation of insoluble aggregates, is an important problem in the biotechnology industry and in human diseases such as amyloidosis, Alzheimer's, Parkinson's, prions and tumour diseases. All this recent application of HHP has opened new avenues for a plethora of biotechnological applications. The great number of pathologies caused by protein misfolding has originated an incessant search for new approaches to combat these diseases. The use of high pressure promises to contribute to identify the mechanisms behind these defects and create therapies against these diseases.

The high-pressure approach has also been applied to viruses and other infectious agents for the purpose of sterilization and in the development of vaccines (Silva *et al* 1992a, Oliveira *et al* 1999, Tian *et al* 2000, Pontes *et al* 2001, Gaspar *et al* 2002, Gomes *et al* 2003). Using small icosahedral viruses, the pressure studies reveal that a gradient of partially folded (molten globule) conformations is present between the unfolded and fully folded structure of several bacteria, plant and mammalian viruses. Pressure has permitted us to detect the presence of a ribonucleoprotein intermediate, where the coat protein is partially unfolded but bound to RNA. These intermediates are potential targets for the development of lead antiviral compounds. Pressure studies on viruses have direct biotechnological applications, especially concerning their potential to conduct into new vaccines. The ability of pressure to inactivate viruses, prions and bacteria has been evaluated with a view toward the applications of vaccine development and virus sterilization. Recent studies demonstrate that pressure causes virus inactivation while preserving the immunogenic properties. This apparently occurs because high pressure traps the virus particle in the fusogenic state, not infectious but highly immunogenic (Gaspar *et al* 2002, Gomes *et al* 2003).

2. Protein folding and protein–protein interactions

Pressure affects the equilibrium between denatured or dissociated and native forms in the direction of the form that occupies a smaller volume (Weber and Drickamer 1983, Silva and Weber 1993, Mozhaev *et al* 1996, Silva *et al* 1996, Frye and Royer 1997). The structural region of the protein that is most sensitive to pressure is the hydrophobic core, especially when cavities are present (Silva *et al* 1996, Frye and Royer 1997, Royer 2002). The unique

characteristic of pressure to isolate molten globules or to favour the state that appears to have a segment of the protein in a partially folded state is remarkable. The mechanism by which one-dimensional information (linear sequence of amino acid residues) is transferred to four dimensions (3D structure and dynamics) remains one of the major challenges in modern biology. Most of the thermodynamic studies in the last 30 years have been done with conventional perturbing agents, such as high temperature, urea, guanidine, etc. However, these agents provide limited information because they cause drastic changes in the protein structure, not likely to occur at physiological conditions. Folding of a protein, or association of one protein with another, is accompanied by an increase in volume because of the additive effects of the formation of solvent-excluding cavities and the release of bound solvent. Water is released as non-polar amino acid residues are buried, as well as when salt linkages are formed (Silva and Weber 1993). The Gibbs free energy (and the equilibrium constant) for an interprotein or intraprotein interaction will depend on the standard volume change (ΔV) of the reaction:

$$\Delta G(p) = \Delta G(0) + p\Delta V \tag{1}$$

$$\ln(\alpha_p^n/(1-\alpha_p)) = p(\Delta V/RT) + \ln(K_{d0}/n^n C^{(n-1)}) \tag{2}$$

where $\Delta G(p)$ and $\Delta G(0)$ are the free energies for association/folding at pressure p and at atmospheric pressure respectively; K_{d0} is the equilibrium constant for dissociation or denaturation at atmospheric pressure; ΔV is the volume change, α_p is the extent of reaction at pressure p, and n is the number of dissociating subunits.

The packing among the different components plays a major role in stabilizing proteins. Because of the large number of atoms, packing defects cannot be avoided, which leads to formation of cavities (Frye and Royer 1997, Royer 2002). Cavities are also related to the metastability of some proteins and macromolecular assemblages (Lee *et al* 2000, Foguel *et al* 1995, De Souza *et al* 1999, Gaspar *et al* 2001) and high pressure has proved to be a useful tool to investigate packing and cavities. Coiled-coil proteins are excellent models to evaluate the balance between packing and hydrophobic interactions. Studies with the large two-stranded coiled-coil protein tropomyosin show that, under pressure, this protein is substantially denatured (Suarez *et al* 2001), but that enough residual coiled-coil region remains to maintain a 'denatured dimer'. On the other hand, the short coiled-coil dimer, formed by the 31-amino-acid polypeptide chains of the leucine zipper GCN4-p1, dissociated completely under pressure to unfolded monomers, with the decrease in volume reflecting the rupture of the hydrophobic interactions (Silva, unpublished results).

3. Protein folding intermediates isolated under pressure

It has been generally accepted that the understanding of protein folding requires the isolation of intermediate structures. Some of these intermediates have been trapped under pressure and their dynamics and structure have been characterized by different methods (Silva *et al* 2001b). Several model proteins have been used for studies of protein folding and dimerization, including the E2 DNA-binding domain (E2-DBDD) from human papillomavirus (Foguel *et al* 1998, Lima *et al* 2000), LexA repressor (Mohana-Borges *et al* 2000) and Arc repressor (Silva *et al* 1992b, Peng *et al* 1993, Foguel and Silva 1994, Oliveira *et al* 1994). The pressure-induced population of partially folded intermediates has also been found for lysozyme (Nash and Jonas 1997), ribonuclease A (Zhang *et al* 1995), apomyoglobin (Bondos *et al* 2000), the Ras binding domain of RalGEF (Inoue *et al* 2000) and DHFR (Kitahara *et al* 2000) as determined by high resolution NMR. The partially folded conformation of pressure-denatured proteins binds a

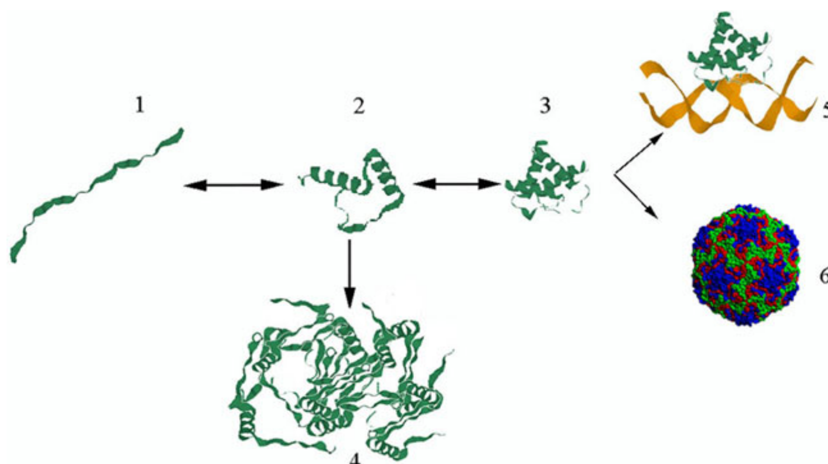

Figure 1. Protein folding/misfolding diagram. Representation of the folding and assembly of protein and multimolecular complexes: (1) unfolded protein; (2) folding intermediate; (3) tertiary structure; (4) protein misfolding—aggregate; (5) protein–DNA complex; (6) virus.

substantial amount of water (Oliveira *et al* 1994) and pressure denaturation is prevented by the lack of water. The crucial participation of water in protein denaturation has been explained by a theoretical approach that postulates the infiltration of water into the protein matrix (Hummer *et al* 1998, Hillson *et al* 1999).

4. Misfolding, off-pathway aggregation and amyloidogenesis

The isolation of folding intermediates is crucial to the understanding of protein misfolding and protein aggregation. In the last decade, several diseases, such as Alzheimer's disease and other amyloidogenic diseases, spongiform encephalopathies (caused by prions), inherited emphysema, cystic fibrosis and probably many cancers have been found to be caused by protein misfolding (Dobson 1999, Horwich 2002, Sacchettini and Kelly 2002, Caughey and Lansbury 2003). Biotechnology companies also face many problems with proteins aggregating into inclusion bodies when they are expressed in bacteria (Clark 2001). Figure 1 shows how functional proteins and macromolecular assemblies may divert from the folding pathway to a misfolding conformation. To control misfolding and aggregation, we have to understand the energetics, the dynamics, and the mechanisms of correct assembly. As described below, pressure has been useful not only to investigate the correct pathways (leading to products 3, 5 and 6–figure 1), but also to the incorrect, misfolded form (product 4).

The competition between correct folding and misfolding, which in many proteins leads to formation of insoluble aggregates, is an important problem in the biotechnology industry and in human diseases. The off-pathway aggregation of proteins often occurs *in vivo* with heterologous proteins that are over-expressed in *Escherichia coli*, resulting in the formation of inclusion bodies or amorphous aggregates within the cell (King *et al* 1996, Clark 2001, Carrio and Villaverde 2002). Pressure may affect the two pathways (aggregation/misfolding and correct folding) as recently demonstrated for the tailspike protein of bacteriophage P22 (Foguel *et al* 1999, Lefebvre and Robinson 2003), rhodanese (Gorovits and Horowitz 1998), myoglobin (Smeller *et al* 1999, Meersman *et al* 2002) and the amyloidogenic proteins transthyretin (Ferrão-Gonzales *et al* 2000, 2003) and synuclein (Foguel *et al* 2003).

5. Dissociation of amorphous aggregates and inclusion bodies

High-pressure treatment dissociated tailspike aggregates and resulted in formation of monomers and native folded trimers, without changes in buffer, dilution of reagents, or modification of reaction conditions (Foguel *et al* 1999). Our results also indicated that aggregation intermediates have similar specific chain recognition events as those that are involved in proper folding events, and suggest that an increased understanding of this specificity will lead to improved folding methodologies. On the practical side, the use of high pressure appears to be an efficient tool for combating aggregation in a variety of research and industrial settings (Foguel *et al* 1999, St John *et al* 1999). St John *et al* (1999) combined pressure in the range 1–2 kbar with low, non-denaturing concentrations of guanidine hydrochloride (GdmHCl) to dissociate aggregates from lysozyme and recombinant human growth hormone (rhGH) as well as IB from β-lactamase. When these aggregates were incubated at 2 kbar for 24 h in the presence of 0.75 M GdmHCl, the yield of properly folded hormone was 100%, in contrast to the low yield obtained at atmospheric pressure. Aggregates of lysozyme were also dissociated by incubation in 0.8 M GdmHCl at 2 kbar for 48 h in the presence of oxidized glutathione and DTT concomitantly. Compared with the samples that remained at atmospheric pressure, the samples subjected to HHP presented an increase up to 100% of the fraction of soluble protein with a regaining of 70% of catalytic activity.

6. Pressure studies on amyloidogenic proteins

Besides being an important problem for biotechnology industries, protein aggregation is also a problem for human health (Dobson 1999, Horwich 2002, Sacchettini and Kelly 2002, Caughey and Lansbury 2003). Several soluble cellular proteins undergo fibrillogenenesis under special conditions and the accumulation of fibrils in specific organs and tissues can ultimately lead to death. Among these proteins, Aβ-peptide, α-synuclein, prion protein and transthyretin wt and mutants respond for important diseases such as Alzheimer's, Parkinson's, prions and senile sytemic amyloidoses or familial amyloidotic polyneuropathy, respectively.

In recent years, we have studied the folding and aggregation processes of transthyretin (TTR) (Ferrão-Gonzales *et al* 2000, 2003, Foguel *et al* 2003). Hereditary amyloidosis, which encompasses adult age onset autosomal disorders such as familial amyloid polyneuropathy, cardiomyopathy and senile systemic amyloidosis, has been associated with a large number of point mutations (over 80 to date) in a protein called transthyretin (TTR), previously known as pre-albumin (Kelly 1998). The hallmark of these diseases is the accumulation of insoluble protein fibrils made up of TTR. This protein is a 127-amino-acid β-sheet tetramer that circulates in plasma and is known to bind retinol binding protein and thyroxine. TTR fibril formation is thought to depend upon tetramer dissociation (Kelly 1998). Pathological mutations have been shown to decrease tetramer stability, and fibril formation can be inhibited by molecules that bind to and stabilize the TTR tetramer (Lashuel *et al* 1998). Treatment of TTR by high pressure leads to the population of partially unfolded monomers and pressure release results in altered tetramers with weaker subunit interactions and with amyloidogenic properties (Ferrão-Gonzales *et al* 2000, 2003).

When the tetramers of TTR were subjected to HHP, they dissociated into monomers with a partially folded structure (Ferrão-Gonzales *et al* 2000). Under 3.0 kbar, the tryptophan emission presented a large redshift, indicating substantial denaturation, but the protein could bind *bis*-ANS, suggesting persistency of some tertiary contacts. Thus we have proposed that TTR forms a partially folded conformation under pressure. The most surprising result arising from these studies was the observation of fibril formation immediately after pressure release

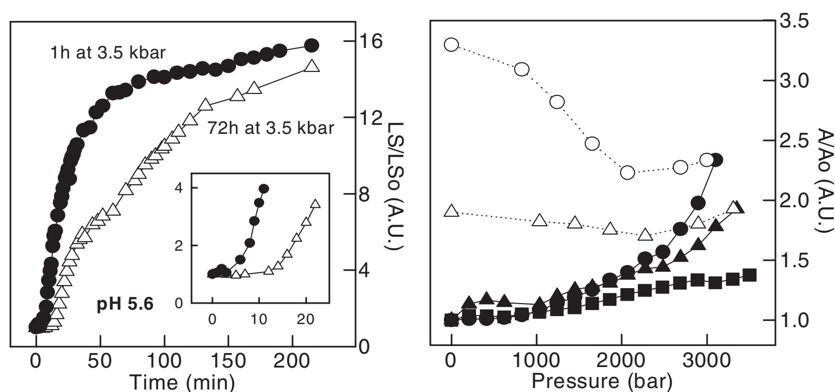

Figure 2. Pressure-induced amyloidogenesis of transthyretin. Left-hand panel: aggregation of TTR after a cycle of compression–decompression as followed by light scattering (LS) increase. At pH 5.6, 3.5 μM TTR was compressed at 3.5 kbar for 1 h (●) or 72 h (△). Then pressure was released and the LS was followed and normalized by the initial value (LS_0). Right-hand panel: *bis*-ANS binding as a function of pH: filled squares, pH 7.5; filled triangles, pH 5.6, and filled circles, pH 5. The hollowed symbols represent the decompression pathways. [TTT] = 1 μM and [*bis*-ANS]= 10 μM. Adapted from Ferrão-Gonzales *et al* (2000).

under conditions that are close to the physiological ([TTR] = 3.5 μM; pH 5–5.6; 37 °C) (figure 2). The oligomeric state of the protein that gives rise to the appearance of these fibrils is a 'loose' tetramer in equilibrium with a small fraction of monomers, as visualized by gel filtration chromatography (Ferrão-Gonzales *et al* 2000).

These studies led us to propose that there is an altered tetramer, called T_4^*, in equilibrium with monomeric species, also able to aggregate into fibrils. Thus, by taking advantage of the tenuous effect of HHP in proteins we were able to populate a partially folded tetramer of TTR (T_4^*, preaggregated state), which appears to be physiologically relevant. Recently, we have expanded our studies with TTR by comparing the thermodynamic stability among wt TTR and the aggressive amyloidogenic mutants (L55P and V30M) and the non-amyloidogenic mutant T119M (Ferrão-Gonzales *et al* 2003). Our data show that there is an inverse correlation between amyloidogenesis and thermodynamic stability where L55P was the least stable variant with the highest propensity for fibrillogenesis.

Another advantage of the use of HHP is drug screening (Foguel *et al* 2003). Since aggregation of TTR occurs few minutes after decompression (∼30 min), in a day it is possible to scan several compounds with potential for inhibiting fibril formation. In the case of TTR, this represents an enormous improvement, since under optimal conditions at atmospheric pressure TTR aggregation takes 72 h to be completed.

In contrast to the previous mentioned studies where HHP was used to dissociate aggregates of several different proteins, in the case of TTR high pressure induced the formation of an amyloidogenic intermediate with high tendency to undergo aggregation when the pressure was released. Recently, by the use of HHP, Webb *et al* (2001) studied the dissociation–unfolding and aggregation of the homodimeric IFN-γ in the presence of different concentrations of sucrose. They also observed that HHP increased the aggregation rate through increased solvation of the protein, which exposes more surface area, thus shifting the equilibrium to the transition state, which has a lower volume than the native state. Thus the equilibrium favours the transition state at high pressures, which increases the formation of monomers that are competent for aggregation. The addition of sucrose (0.125–1 M) inhibited aggregation under pressure by shifting the equilibrium in favour of the dimeric, native species.

Smeller *et al* (1999) also observed a strong tendency of horse heart metmyoglobin to aggregate after a cycle of compression–decompression at high temperatures (45 and 60 °C). They showed that there were two types of pressure-induced aggregate: one that could be dissociated by moderate pressures (<3 kbar) and one that was pressure insensitive.

The pressure-induced aggregation of proteins may seem counterintuitive, because pressure often dissociates multimeric proteins. Thus, for each protein one has to investigate whether pressure populates a 'sticky' folding intermediate with lower partial molar volume. If so, aggregation is the preferential route.

7. Aggregation properties of tumour suppressor protein p53: how to design new strategies to combat cancer

The tumour suppressor protein p53 is a 393-amino-acid-residue transcriptional factor with an important role in cell cycle control, especially after cellular stresses such as genotoxic damage, cytokines, hypoxia and alterations of ribonucleotide pools (Hall and Lane 1997). Most human cancers (≈50%) result from mutations in the p53 protein, mainly at its DNA binding domain (p53C), affecting the DNA binding ability and/or the protein stability. We found recently that p53/DBD undergoes denaturation when subjected to hydrostatic pressures at 37 °C, leading to a misfolded and aggregated state (Ishimaru *et al* 2003a). However, when we investigated the pressure effects at 4 °C, the protein denaturation was displaced to higher pressures and aggregation was non-cooperative with denaturation. Some neuroblastomas, carcinomas and myelomas show an abnormal accumulation of the wild-type tumour suppressor protein p53 either in the cytoplasm or in the nucleus of the cell. Recently, we demonstrated that the wild-type p53 core domain (p53C) can form fibrillar aggregates after mild high-pressure perturbation (Ishimaru *et al* 2003a). Gentle pressure denaturation of p53C induced fibrillar aggregates, as shown by electron and atomic force microscopies, by binding of thioflavin T and by circular dichroism. On the other hand, heat denaturation produced granular-shaped aggregates. Annular aggregates similar to those found in the early aggregation stages of a-synuclein and amyloid-b were also observed by atomic force microscopy immediately after pressure treatment. Annular and fibrillar aggregates of p53C were toxic to cells, as shown by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. Interestingly, the hot-spot mutant R248Q underwent similar aggregation behaviour when perturbed by pressure or high temperature. We propose that the fibrillogenesis of p53 may contribute to the loss of function of p53 and seed the accumulation of conformationally altered protein in some cancerous cells.

Many strategies for rational cancer therapy have been pursued in recent years. Among these is the development of drugs or peptides that mimic the tumour suppressor function or that re-establish and stabilize the active conformation of p53C (Bullock and Fersht 2001, Foster *et al* 1999). From this point of view, it is essential to understand the molecular basis that drives p53 folding and stabilizes its native conformation in order to understand why some mutations, or even some alternative conformations of the wild-type protein, lead to loss of protein function, and hence to tumours. To seek partially folded states of the wild-type p53 core domain (p53C) we used high hydrostatic pressure (HP) and subzero temperatures. Aggregation of the protein was observed in parallel with its pressure denaturation at 25 and 37 °C. However, when HP experiments were performed at 4 °C, the extent of denaturation and aggregation was significantly less pronounced (Ishimaru *et al* 2003b). On the other hand, subzero temperatures under pressure led to cold denaturation and yielded a non-aggregated, alternative conformation of p53C. Nuclear magnetic resonance ($^{1}H^{15}N$-NMR) data showed that the alternative p53C conformation resembled that of the hot-spot oncogenic mutant R248Q.

This alternative state was as susceptible to denaturation and aggregation as the mutant R248Q when subjected to HP at 25 °C. Together, these data demonstrate that WT p53C adopts an alternative conformation with a mutant-like stability, consistent with the dominant-negative effect caused by many mutants. This alternative conformation is probably related to inactive forms that appear *in vivo*, usually driven by interaction with mutant proteins. Therefore, it can be a valuable target in the search for ways to interfere with protein misfolding and hence to prevent tumour development (Ishimaru *et al* 2003b).

8. Reversibility of fibrillogenesis: potential pharmaceutical applications

The description of drugs able to dissociate preformed fibrils is aimed for by several laboratories and by pharmaceutical industries (Sacchettini and Kelly 2002). These drugs would be candidates for the treatment of the amyloidogenic diseases. In order to find or even design the ideal drug, the interactions that hold a fibril intact need to be dissected. To tackle this problem, we have used HHP to dissociate the fibrils of TTR and α-synuclein (Foguel *et al* 2003). Our data have shown that all these fibrils were very sensitive to HHP, undergoing dissociation immediately when subjected to high pressures. In the case of TTR, its fibrils were dissociated by pressure (3 kbar) into a partially folded monomer. When pressure was released, aggregation took place, giving rise to the appearance of new fibrils. Aggregates of rhodanese were also reversibly dissociated by pressure (Gorovits and Horowitz 1998, Panda *et al* 2000).

In the case of α-synuclein involved in Parkinson's disease, fibrillogenesis is very slow, taking several days. Parkinson's disease is the second most common neurodegenerative disorder in humans. It is associated with resting tremor, postural rigidity, and progressive degeneration of dopaminergic neurons in the *substantia nigra* pars compacta (Spillantini *et al* 1997). When the synuclein fibrils are subjected to HHP, they dissociate into a soluble protein that undergoes fibrillogenesis again very slowly (Foguel *et al* 2003) (figure 3). Fibrils formed by wt α-syn were less susceptible to pressure denaturation than the Parkinson's-disease-linked variants, A30P and A53T. This implies that fibrils of α-syn formed from the variants would be more easily dissolved into small oligomers by the cellular machinery. This result has physiological importance in light of the current view that the pathogenic species are the small aggregates rather the mature fibrils (Caughey and Lansbury 2003).

The high susceptibility of fibrils against HHP suggests that hydrophobic and ionic interactions might display the most important role in fibril maintenance. In conclusion, these results indicate the use of HHP as a promising tool to dissociate aggregates and IB of important proteins for human and animal consumption. Since HHP perturbs gently the structure of proteins, it allows the population and further characterization of important intermediate states present during the folding pathway of several proteins. In the case of amyloidogenic proteins, these intermediates are targets for the development of drugs that can block fibrillogenesis.

A general volume diagram for protein folding and aggregation (figure 4) has been proposed by our group (Silva *et al* 2001b). In this diagram, the population of an amyloidogenic intermediate without proceeding to aggregation is a unique property of pressure, which opens the prospect of characterizing the structure of the amyloidogenic form. In the case of transthyretin, a less stable tetramer is formed after decompression and aggregation can be prevented by maintaining the temperature at 4 °C (Ferrão-Gonzales *et al* 2000, 2003). The pressure studies on this protein led directly to the idea of a 'pre-aggregated', oligomeric state with loosened subunit interactions. There is recent evidence that the formation of intermediate assembled states may have a crucial contribution to the neurodegeneration in amyloidogenic diseases, such as Parkinson's. Overall, the results on the amyloidogenic proteins transthyretin (Ferrão-Gonzales *et al* 2000, 2003), α-synuclein (Foguel *et al* 2003)

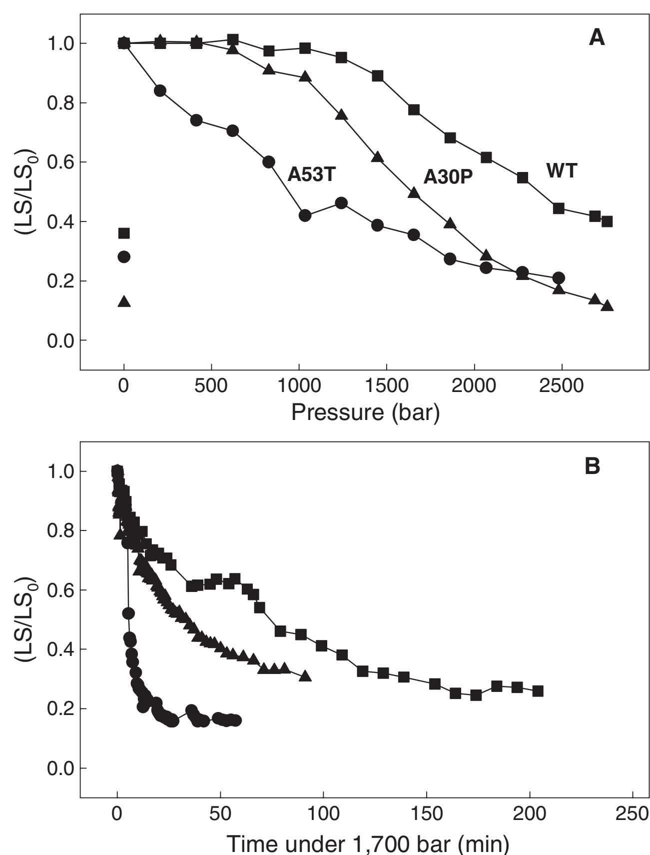

Figure 3. Pressure dissociation of synuclein fibrils. (A) *Pressure titration of α-synuclein fibrils from wt and variant proteins*. Fibrils were produced at atmospheric pressure (see text) and then subjected to increasing pressure at 37 °C while recording the LS as in figure 1. Proteins were wt (■); A53T (●) and A30P (▲). The isolated symbols on the left represent the LS values obtained after decompression. (B) *Kinetics of the pressure-induced dissociation of wt and variant fibrils of α-syn*. The fibril solutions were compressed at 1700 bar at 37 °C. The extent of fibril dissociation was recorded at intervals as a decrease in light scattering (LS) in comparison to the initial value (LS_0). Symbols as in panel (A). Adapted from Foguel *et al* (2003).

and more recently on tumour suppressor p53 protein (Ishimaru *et al* 2003a, 2003b) suggest that stable intermediates can be achieved by carefully tuning high pressure and temperature. The isolation of these intermediates provides targets for the development of lead compounds capable of blocking protein misfolding and aggregation, potential drugs against amyloidogenic and tumour diseases.

9. Use of high pressure in the development of vaccines and drugs against viruses

Virions have evolved to move their genome between cells of a susceptible host and between hosts. The virus particle is composed of either a membrane enveloped or non-enveloped protein shell and nucleic acid (Johnson 1996). The protein shell is a multifunctional nanodevice: it shields the nucleic acid; participates in chemical reactions for particle maturation; and is able to penetrate the cell and undergo disassembly. To pack an infectious genome, integral multiples of 60 subunits are required to form the shell, resulting in non-identical contacts between subunits.

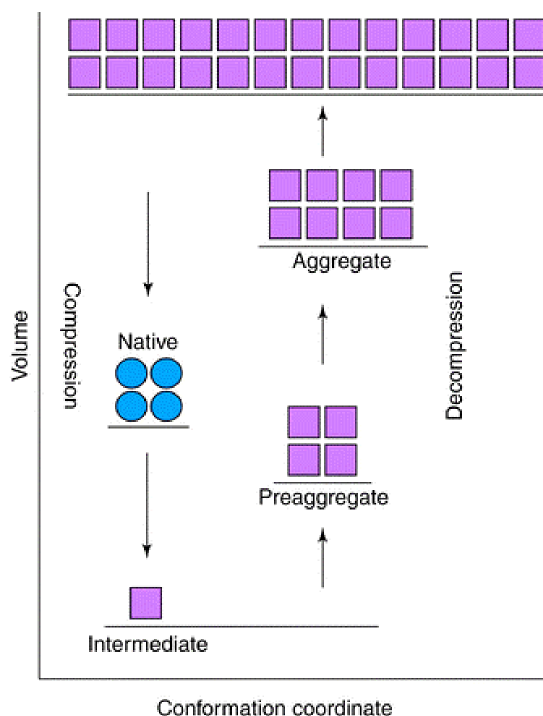

Figure 4. Volume diagram for the pressure dissociation and generation of amyloidogenic conformations. This diagram uses the tetrameric transthyretin (TTR) as an example but it can be generalized to other proteins studied. Under pressure, the tetramer (coloured blue) of TTR unfolds into a molten-globule-like conformation (pink isolated square) as measured by its ability to bind the fluorescent dye *bis*-ANS. Upon decompression, a tetramer with an altered conformation (pink squares) is formed, triggering the aggregation into fibrils (pink squares). However, as observed in the case of tailspike protein, bovine growth hormone and β-lactamase (see text), high pressure may convert previously formed aggregates into native protein. Thus, aggregates, similar to native proteins, have packing defects making them sensitive to pressure. From Silva *et al* (2001b).

Pressure studies have corroborated the idea that the plasticity of the viral proteins is required for successful assembly of a virus particle (Silva *et al* 2002). Combined thermodynamic and structural approaches have been used to try to identify the general rules that govern virus assembly. In general, it has been found that the capsid coat proteins (monomers or dimers) are much less stable to pressure than the assembled icosahedral particles (Silva *et al* 1996). The isolated capsid and the assembly intermediates assume different partially folded states in the assembly pathway (Oliveira *et al* 1999, Da Poian *et al* 1994, 1995, 2002, Gaspar *et al* 1997). Single-amino-acid substitutions in the hydrophobic core of the coat protein of icosahedral viruses produce large decreases in stability against pressure and chemical denaturants, as recently shown for MS2 (Lima *et al* 2004). High pressure has allowed us to isolate a ribonucleoprotein intermediate, which is a potential target for antiviral drugs (Oliveira *et al* 1999). Similar to the protein–nucleic acid complexes of Arc repressor (Foguel and Silva 1994), several viruses undergo cold denaturation under pressure, suggesting the entropic stabilization of the particles (Da Poian *et al* 1995, Bonafe *et al* 2000, De Souza *et al* 1999, Oliveira *et al* 1999).

A ribonucleoprotein intermediate has been detected in the pressure dissociation of several viruses (Da Poian *et al* 1994, 1995, 2002, Gaspar *et al* 1997, Oliveira *et al* 1999, 2000) as

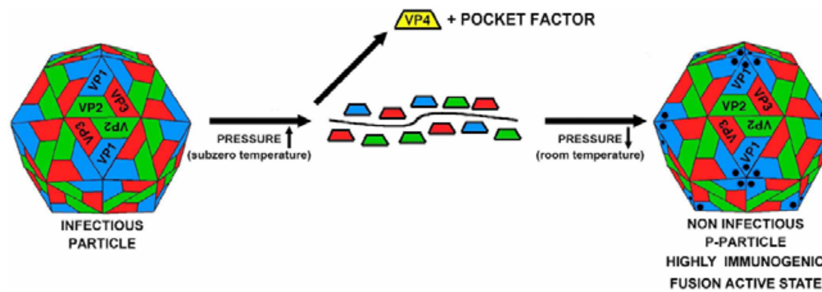

Figure 5. Picornavirus dissociation and inactivation. Proposed model for the disassembly of picornaviruses by pressure and low temperature. Pressure plus low temperature disrupt the icosahedral structure, but the capsid proteins (VP1, VP2 and VP3) still remain bound to the RNA. This particle loses infection on return to atmospheric pressure and room temperature. This infectivity loss appears to be due to release of VP4 and the 'pocket factor'. Adapted from Oliveira *et al* (1999).

shown for the case of picornaviruses (figure 5). We have postulated that the ribonucleoprotein intermediate would serve as a core for ready regeneration of the particle when the pressure is reduced. An unusual feature of this ribonucleoprotein intermediate is the presence of partially unfolded coat proteins bound to the RNA. RNA apparently plays a chaperone-like role during assembly of the capsid. In the absence of RNA the subunits drift to a disorganized structure and cannot renature when the perturbation is withdrawn. Ribonucleoprotein intermediates are potential target for antiviral drugs. The ribonucleoprotein intermediate seems to have a condensed structure, which demonstrates the high degree of plasticity of the coat-protein–RNA complex (Gaspar *et al* 1997, Da Poian *et al* 2002).

Another common feature found in the pressure studies of different viruses is a progressive decrease in folding structure in moving from assembled capsids to ribonucleoprotein intermediates (in the case of RNA viruses), free dissociated units (dimers or monomers) and finally unfolded monomers. We have proposed a gradient of molten-globule states between the fully structured coat protein in the capsid and the unfolded monomers. High pressure would affect primarily the quaternary and tertiary structure of the capsid protein, leading to partially unfolded, molten-globule conformations. In contrast, high urea concentrations would primarily disrupt the secondary structure.

To be effective, disassembly has to occur fast and at the correct time after endocytosis. The switch for this process is usually attributed to the acidic pH inside the endocytic vesicles, but *in vitro* many viruses are not uncoated by low pH, or the uncoating occurs slowly, not consistent with the requirement for rapid replication. High pressure provides a powerful tool to explore the uncoating of animal viruses. The family *Picornaviridae* includes several viruses of great economic and medical importance (Rueckert 1996). These viruses have in common a capsid structure composed of 60 copies of four different proteins, VP1 to VP4, and their 3D structures show similar general features. Oliveira *et al* (1999) have described the differences in stability against high pressure and cold denaturation of these viruses. Both poliovirus and rhinovirus are stable to high pressure at room temperature—pressures up to 2.4 kbar are not enough to promote viral disassembly and inactivation. Within the same pressure range, foot and mouth disease virus (FMDV) particles are drastically affected by pressure, with a loss of infectivity of more than four log units. The dissociation of polio- and rhinoviruses can be observed only under high pressure at low temperatures in the presence of low concentrations of urea (1–2 M). The pressure and low temperature data reveal clear differences in stability among the three picornaviruses, FMDV being the most sensitive, polio the most resistant and rhino having intermediate stability.

The changes produced by pressure and low temperature in picornaviruses are shown in figure 5. The most important feature is that after a pressure cycle there is reassociation to a non-infectious particle (named P-particle). In the case of FMDV, we further demonstrated that pressure completely abolishes the infectivity and results in a highly immunogenic particle (Ishimaru *et al* 2004). Figure 5 also shows a possible explanation for loss of infection that has previously been proposed by other authors to account for heat treatment of some picornaviruses (Rossmann 1994), where the defective particle would lose VP4 and/or small molecules (pocket factors) bound to the canyon. The pressure-inactivated picornavirus may ressemble the A-particle detected in poliovirus and rhinovirus upon interaction with the host cell, which also lacks the internal capsid protein VP4 (Rueckert 1996, Rossmann 1994). The A-particle is substantially less infectious than natural virions and has been considered an intermediate in uncoating. This form is similar to the 'fusion intermediate state' that has been found in enveloped viruses (Carr and Kim 1993, Skehel and Wiley 2000). During the fusion process, the conformations of the coat proteins and envelope glycoproteins change, which on one hand leads to non-infectious particles and on the other may lead to the exposure of previously occult epitopes, important for vaccine development. These irreversible conformational changes evoked by high pressure that resemble the changes that occur '*in vivo*' are discussed below for most of the viruses we have studied.

We have also used high pressure to evaluate the role of maturation cleavage on flock house virus (FHV) by comparing wild-type (wt) and cleavage-defective mutant (D75N) FHV virus-like particles (VLPs) (Oliveira *et al* 2000). The mature particles were less stable to pressure than the cleavage-defective particles, which can be explained by the metastability elicited upon maturation. We also have evidence that the small gamma subunit is released after a cycle of compression and decompression. A similar situation would occur with picornaviruses, with pressure-induced release of VP4 (figure 5). In both cases, pressure induces the formation of a non-infectious particle, apparently a fusogenic state of these non-enveloped viruses.

10. Fusion active states of viruses as the basis for Pascalized vaccines

Enveloped animal viruses undergo membrane fusion in order to deliver their genome into the host cytoplasm (Carr and Kim 1993, Skehel and Wiley 2000). We have recently found that high pressure inactivates the enveloped viruses influenza, Sindbis and VSV by trapping the particles in the 'fusion intermediate state' (Gaspar *et al* 2002, Gomes *et al* 2003). Altogether, we show that hydrostatic pressure triggers a conformational change in the glycoprotein of influenza and Sindbis viruses at neutral pH values that is very similar to the change triggered by the low pH values at the endosomes. Our results indicate that pressure rescues the conformation of the metastable, native state (larger volume) into the fusogenic state (smaller volume) by populating a transition-activated state, which although of high energy presents a smaller volume. This is represented in the diagram of figure 6. The use of pressure to populate fusion-active states can be utilized in the development of new antiviral vaccines and drugs.

The formation of non-infective particles after a cycle of compression and decompression has been demonstrated in many viruses such as rotaviruses (Pontes *et al* 2001), infectious bursal disease virus (IBDV) (Tian *et al* 2000), vesicular stomatitis virus (VSV) (Silva *et al* 1992a, Gomes *et al* 2003), simian immunodeficiency virus (Jurkiewicz *et al* 1995), HIV (Nakagami *et al* 1996), influenza (Gaspar *et al* 2002), lambda phage (Bradley *et al* 2001), picornaviruses (Oliveira *et al* 1999, Ishimaru *et al* 2004) and alphaviruses (Gaspar *et al* 2001, 2002). For IBDV, high pressure caused elimination of infectivity, which retained the original immunogenic properties, giving rise to high titres of virus neutralizing antibodies (Tian *et al* 2000). With the membrane-enveloped viruses, the membrane glycoprotein undergoes a conformational change

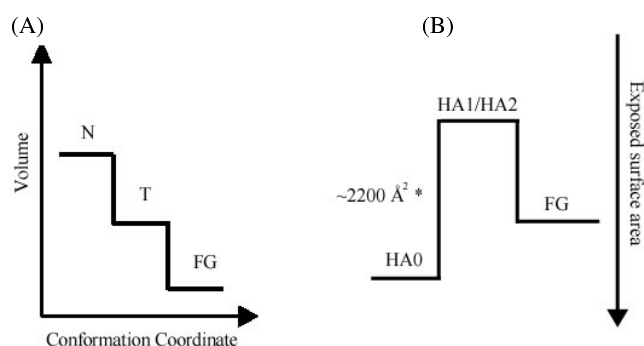

Figure 6. Fusogenic state of viruses induced by pressure. (A) Volume diagram showing the conversion between native non-fusogenic state (**N**) and fusogenic state (**FG**). The transition intermediate state is represented by (**T**). (B) Changes in volume and in the solvent-exposed surface of haemagglutinin precursor protein (HA0) when it is first converted into the cleaved complex (HA1/HA2) and then when the protein undergoes the conversion from native (N) into fusogenic (FG) conformation.

induced by pressure similar to the receptor-activated conformational change (Gaspar *et al* 2002, Gomes *et al* 2003). The finding that influenza and HIV envelope glycoproteins undergo pH- or receptor-activated conformational changes indicates that their native states are metastable. For influenza virus, this change is usually referred to as the spring-loaded model, in which the fusion-peptide region is thought to insert into the target membrane at an early step of the fusion process (Carr and Kim 1993, Skehel and Wiley 2000). For influenza, high pressure, similar to low pH, elicits the spring-loaded mechanism, leading into an increase in solvent exposure, as represented in figure 6. The structure of the ectodomain of the haemagglutinin precursor HA0 (Chen *et al* 1998) reveals that the proteolytic cleavage elicits structural rearrangements that can explain why the protein becomes labile and sensible to pressure. A water-exposed cavity in HA0 becomes filled with non-polar residues (HA2 1–10), and several ionizable residues implicated in the N $\rightarrow$ FG transition are buried (figure 6(B)). The two buried surfaces add up to 2177 Å^2, and the previously wet cavity in HA0 becomes non-polar. This change makes it sensitive to both pH and pressure. The influenza virus system has the unique property of having a water-penetrated cavity in the precursor protein, which eliminates water on proteolytic cleavage, originating the pH and pressure sensitivity (figure 6(B)).

Indeed, influenza virus subjected to pressure exposes hydrophobic domains as determined by tryptophan fluorescence and by the binding of *bis*-8-anilino-1-naphthalenesulfonate (*bis*-ANS), a well established marker of the fusogenic state in influenza virus. Pressure also produced an increase in the fusion activity at neutral pH as monitored by FRET (fluorescence resonance energy transfer) using lipid vesicles labelled with fluorescence probes (Gaspar *et al* 2002). These data indicate the change of the labile native state of the envelope complex to a more stable one, mimicking the fusion-active conformation.

Virus inactivation by hydrostatic pressure has been evaluated with a view toward two potential applications: vaccine development and virus sterilization. Concerning viral vaccines, there are basically three types of immunization strategy: use of live (attenuated) particles; use of killed (inactivated) whole-virion particles and use of subunit vaccines (hepatitis B). Immunization is the most efficient strategy to prevent infectious diseases in animals and humans (Bloom 1996). In several cases, the antibodies against pressurized virus particles were as effective as those against the intact viruses when measured by their neutralization titre in a plaque reduction assay (Silva *et al* 1992a, 1992b, Oliveira *et al* 1999, Tian *et al* 2000, Pontes *et al* 2001, Ishimaru *et al* 2003a, 2003b).

We have also used high pressure to inactivate the bacteria leptospires (Silva *et al* 2001a), the causing agent of important zoonotic and human diseases. *Leptospira interrogans* serovar hardjo was inactivated by relatively low hydrostatic pressures. Electron microscopy studies indicated the dislocation of the outer membrane, partial loss of the helical shape and extrusion of the axial filament from the cytoplasmic cylinder of the pressurized leptospires. When the pressure-treated leptospires were inoculated into rabbits they were highly immunogenic and are now under evaluation with the goal to formulate a vaccine.

The high titres of the neutralizing antibodies elicited by pressure-inactivated viruses indicate that hydrostatic pressure can be used to prepare whole-virus immunogens. Effective immunization against viruses requires presentation of the whole virus particle to the immune system. The employment of high pressure to prepare antiviral vaccines may have important advantages over other methods such as chemical inactivation, attenuation or isolated subunit vaccines. Attenuated live viruses can revert after a certain time and may cause the disease that they are intended to prevent, or worsen the real disease. Immunization with isolated subunits has several problems, especially because the immune system recognizes the isolated antigen less effectively than the whole virus. A reliable physical method, such as high pressure, to prepare killed vaccines should not have the same problems.

The reason why the pressurized viruses maintain the immunogenic potential probably resides in the fact that the structural changes are very subtle. As we discussed above pressure treatment seems to mimic the changes that are produced when viruses bind to cellular receptors. We found that pressure-inactivated VSV attaches to the cellular membrane, but it is not internalized by endocytosis (Da Poian *et al* 1996, Gomes *et al* 2003). This result can be explained by the fact that high pressure leads the envelope protein to the fusion conformation.

11. Conclusions and perspectives

The increasing use of high hydrostatic pressure in biology has ranked this approach as the most promising to tackle basic problems, such a protein folding, as well as the development of new applications in medicine and pharmacy. Pressure has permitted us to characterize the high degree of plasticity of proteins. Recent detailed studies on the kinetics of pressure unfolding and folding together with several theoretical approaches have increased our understanding of the pressure effects. Furthermore, the isolation of folding intermediates is crucial to the understanding of protein misfolding and protein aggregation, which have been recently undertaken by pressure. The population of an amyloidogenic intermediate without proceeding to aggregation is a unique property of pressure, which opens the prospect to characterize the structure of the amyloidogenic form.

The reduced stability of virus particles at high pressure and low temperature suggests the potential application to inactivate viruses of medical importance. We have found that several viruses are inactivated by pressure at room temperature. The substantial evidence that high pressure traps viruses in the 'fusion intermediate states', not infectious but highly immunogenic, is very promising for vaccine development. Pressure-induced population of fusion-active states can be utilized in the development of new antiviral vaccines and drugs. These studies are important not only as a potential approach to produce anti-viral vaccines but also to sterilize biological products, such as blood and plasma derivatives.

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