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High-Pressure Chemical Biology and Biotechnology

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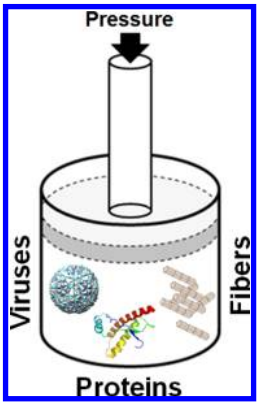

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1. INTRODUCTION

In his memorable book published in 1949, *The Physics of High Pressure*,[1] Bridgman states that "it is a well-known result of thermodynamics that a substance is only completely characterized thermodynamically when the $P-V-T$ relation is given." In the last three decades, the field of biology has taken Bridgman's advice, and hydrostatic pressure has become a robust physicochemical tool for the study of biological macromolecules. The pressure effects that we will discuss in this Review are those related to the range of pressures up to 10 kbar (1000 MPa), at which water is in a liquid state at room temperature. The highest pressure to which living organisms in the ocean depth are exposed is on the order of 1−1.2 kbar (e.g., at 10 000 m depth, the pressure is approximately 1000 bar).[2]

As pressures below 10 kbar very rarely result in direct breakage of covalent bonds, their predominant effect is on the conformation of macromolecules, which is maintained by weak forces such as hydrogen bonds, van der Waals, and hydrophobic interactions under normal pressure conditions.[3−7]

The changes in the standard free energy of a reaction associated with changes in pressure, at constant temperature, are simple and straightforward. As noted by Gregorio Weber,[8] the ideal system to be studied under pressure is one in which

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the reaction is independent of the concentration of the molecular species being examined, that is, a pseudo-first-order or unimolecular reaction. For small molecules, cis−trans or boat-chair isomerization is a good example of this type of reaction. In flexible macromolecules, the interconversion of conformational isomers might represent such a reaction, similar to a protein folding reaction. The latter case provides an interesting and illuminating example of the type of system described by Weber. In 1914, Bridgman himself used high pressure to denature egg albumin.[9]

The first step in the analysis of such a system is to apply the simple thermodynamic relationship:

$$(\mathrm{d}\Delta G(p)/\mathrm{d}p)T = \Delta V(p) \quad (1)$$

where ΔV represents the difference between the volumes occupied by the solvated products and reactants. The equilibrium constant $K(p)$ can be expressed by

$$\mathrm{d}\ln K(p)/\mathrm{d}p = -\Delta V(p)/RT \quad (2)$$

Thus, the Gibbs free energy (and the equilibrium constant) for an intramolecular (as well as for an intermolecular) interaction will depend on the standard volume change (ΔV) of the reaction:

$$\Delta G(p) = \Delta G(\mathrm{o}) + p\Delta V \quad (3)$$

where $\Delta G(p)$ and $\Delta G(\mathrm{o})$ are the free energies for folding/association at pressure p and at atmospheric pressure, respectively. If we further develop the equation for a reaction of order n, we obtain:

$$\mathrm{N}_n \leftrightarrow n\mathrm{D}$$

$$\ln(\alpha_p{}^n/(1-\alpha_p)) = p(\Delta V/RT) + \ln(K_{\mathrm{do}}/n^n C^{(n-1)}) \quad (4)$$

where K_{do} is the equilibrium constant for unfolding or dissociation at atmospheric pressure; α_p is the extent of the reaction at pressure p; C is the protein concentration; and n is the number of dissociating subunits ($n = 1$ for an isomerization reaction). This equation is usually applied to proteins but can be extended to any macromolecule, including nucleic acids, as well as to supramolecular structures such as viruses.[7] The effects of pressure on a biophysical system will always follow Le Chatelier's principle, driving the equilibrium toward a smaller volume.[34]

In the case of proteins, high-pressure approaches can reveal transient conformations that occur during the unfolding process but are not easily assessed using other methods.[7] Equilibrium and kinetic analyses of molecules under high hydrostatic pressure have resulted in great advances, allowing the appraisal of protein folding and misfolding landscapes. In vitro studies performed under equilibrium or kinetic conditions involving hydrostatic pressure have provided useful information about protein conformational changes and interactions, including those that depend on the presence of other partners, such as nucleic acids, cofactors, and ions. Applying high pressure is also an excellent approach for studying situations in which protein folding occurs incorrectly, such as in the so-called protein folding disorders, which include Alzheimer's, Parkinson's, tumoral, and prion diseases. Because partially folded intermediates, leading in some cases to misfolding and the occurrence of protein aggregates, are stabilized by pressure, the application of high pressure permits the characterization of these aggregation reactions. The wide range of diseases that result from protein misfolding has made this an important research focus for pharmaceutical and biotechnology companies. The use of high pressure as a research tool promises to further contribute to our ability to identify the mechanisms underlying these defects and develop therapies for these diseases, as in earlier studies on prion proteins, the p53 tumor suppressor protein, and transthyretin. High pressure has also been used to study viruses and other infectious agents for the purpose of sterilization (e.g., in food processing) and in the development of vaccines as well as being widely utilized for the nonthermal processing of food. The fundamental chemico-biological principles upon which a plethora of applications in biomedicine and biotechnology rest will be discussed in this Review.

2. FUNDAMENTAL EFFECTS OF PRESSURE ON MACROMOLECULES

2.1. Proteins − General Aspects

The application of pressure has opened important frontiers for understanding how polypeptides fold into highly structured conformations, how they interact with ligands and other proteins, and how they assemble into supramolecular structures such as viruses and amyloids.[3−7,10] In proteins, pressure induces changes that range from small conformational effects, compressibility effects, and changes in populations of intermediate states to complete loss of native folding.

The secondary structure of proteins (α-helices, β-sheets, and turns) is not highly sensitive to pressure per se, as hydrogen bonds are highly incompressible at pressures up to 10 kbar. In fact, in some cases, secondary structure can be stabilized by pressure, as recently shown for a 21-aa helical peptide that was stabilized by pressure.[11] Thus, pressure has the unique effect of preserving unmodified protein secondary structures that are independent of tertiary folding.[5−7] For this reason, pressure tends to convert proteins into partially folded segments or molten-globule intermediates.[7,12−19]

The first report of the effect of pressure on protein denaturation was a simple, but seminal article by Bridgman published in the *Journal of Biological Chemistry* in 1914, entitled "The Coagulation of Albumen by Pressure".[9] In his article, Bridgman not only describes the denaturing effects of pressure but also sheds light on the additive effects of high pressure and low temperature on proteins by stating that "6000 atm applied at 0 °C for 1 h produced a somewhat greater stiffening than would have been produced at 20 °C". Following Bridgman's report, the field remained dormant for half a century. In the late 1950s and early 1960s, Suzuki and co-workers described their analyses of the pressure denaturation of ovalbumin using an apparatus that allowed them to perform measurements under increased pressure.[20,21] These studies were followed by other pioneering works by the groups of Heremans, Weber, and several others.[22−27]

In the 1970s, Gregorio Weber's investigations of high-pressure protein denaturation were ground-breaking for the time. In a 1976 paper, Weber and co-workers showed that the pressure denaturation of lysozyme and chymotrypsinogen does not occur through a simple transition, but a complex one that involves a plurality of processes.[22] This provided the first evidence contradicting the generally accepted dogma that protein folding/unfolding represents a two-state reaction. In fact, we now know that in most cases, pressure drives proteins to intermediate protein conformations, as can be visualized in

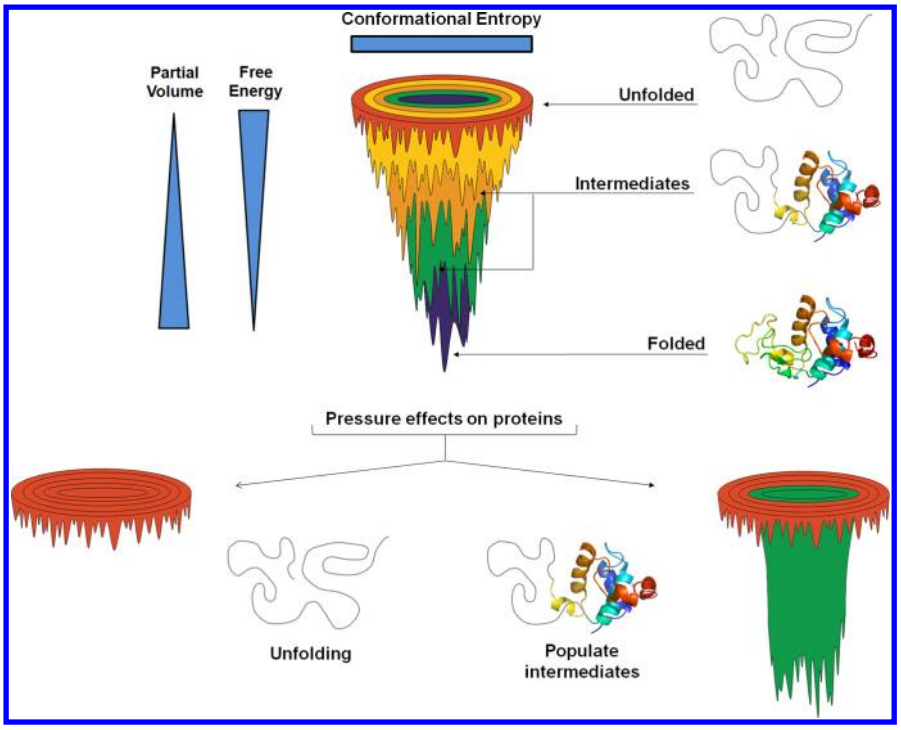

Figure 1. Schematic diagram showing a typical 3D-folding funnel for a protein. The minimal global assumed for a folded polypeptide chain (rainbow structure, PDB code: 1E8L) is colored in dark blue. Intermediate substates are colored green and yellow and exemplified by a partially folded model (gray/rainbow structure). The unfolded state (loop structure colored in gray) is represented by an increase in the free energy and a decrease in the partial volume for the molecule. General effects of pressure in the protein landscape are represented in the lower panel. Pressure may lead to protein unfolding (left) or enrichment of partially folded intermediates (right).

Figure 1. A funnel folding landscape is the best current representation of how a protein evolves from a large collection of disordered states to a restricted number of conformations that constitute its native state ensemble.[28] As shown in this figure, pressure can populate either the fully unfolded state or the partially unfolded states.[12−14,29]

Almost a century after Bridgman's publication, the consensus is that pressure affects proteins due to many factors, where in many cases water-excluded cavities can dominate. The predominant contribution of cavities for the volume change was clearly demonstrated in Royer's experiments using an SNase mutant (Figure 2B−D).[30] The packing among the various domains of proteins plays a major role in their stabilization. Because of the great number of atoms present in a protein molecule, packing defects cannot be prevented, which leads to the formation of cavities.[30−32] The volume change is the net result of the disappearance of protein cavities, the electrostriction of the broken electrostatic interactions, and the reduced volume upon additional hydration. Electrostriction is particularly important in hetero protein−protein interactions, as has been demonstrated for the interaction of cytochrome b5 with cytochrome c[33] and of myosin light chain calmodulin-binding domain with calcium-saturated calmodulin.[34] In the case of macromolecular assemblages, cavities have been particularly shown to be related to metastability,[35−37] and therefore high pressure has proved to be a key tool for exploring their packing.

In addition to making it possible to assess the thermodynamics of protein folding, hydrostatic pressure also provides a robust means of probing protein−protein, protein−nucleic acid, and protein−ligand interactions,[5,7,10,15,38−40] as will be further discussed below. Among its advantages over other approaches, high pressure allows the investigator to isolate folding intermediates and characterize their structures and dynamics using various methodologies.[7,41] Because misfolded proteins, aggregates, and amyloids are usually derived from partially folded intermediates, pressure has been widely employed to assess many of the protein misfolding diseases, as will be further discussed in this Review.[41−44]

2.2. Nucleic Acids, Lipids, and Glycosaminoglycans

As compared to proteins, there are fewer studies on the effects of pressure on nucleic acids.[45−47] Most of these studies have addressed the impact of pressure on the stability of double helical nucleic acids. Unlike proteins, the helical forms of DNA and RNA are stabilized by hydrostatic pressure. However, the degree of stabilization is quite low, mostly because their structure depends more on hydrogen bonds (which are little affected by pressure) than on changes in ionization states and solvent interactions. Recent studies examining small RNAs appear to show that some of the observed effects (especially destabilizing effects) might be due to the release of solvent-excluded cavities. Molecular dynamics simulations of the pressure effects on the small RNA oligomer showed that it is destabilized by pressure in a manner similar to that of folded proteins.[48] Studies on a short oligodeoxyribonucleotide with a repeat of the human telomeric sequence also revealed that the volume change of folding into a quadruplex conformation was positive and occurred with the release of water molecules.[49] In a more recent study, the pressure stability of G-quadruplex DNA (thrombin binding aptamer) was studied in the presence of crowding agents.[50] The studies also showed that that G-quadruplex DNA was unfolded by high pressure (folding ΔV = 54.6 mL/mol), and the crowding agents decreased the effects of pressure.[50] Interestingly, the results show how a complex DNA

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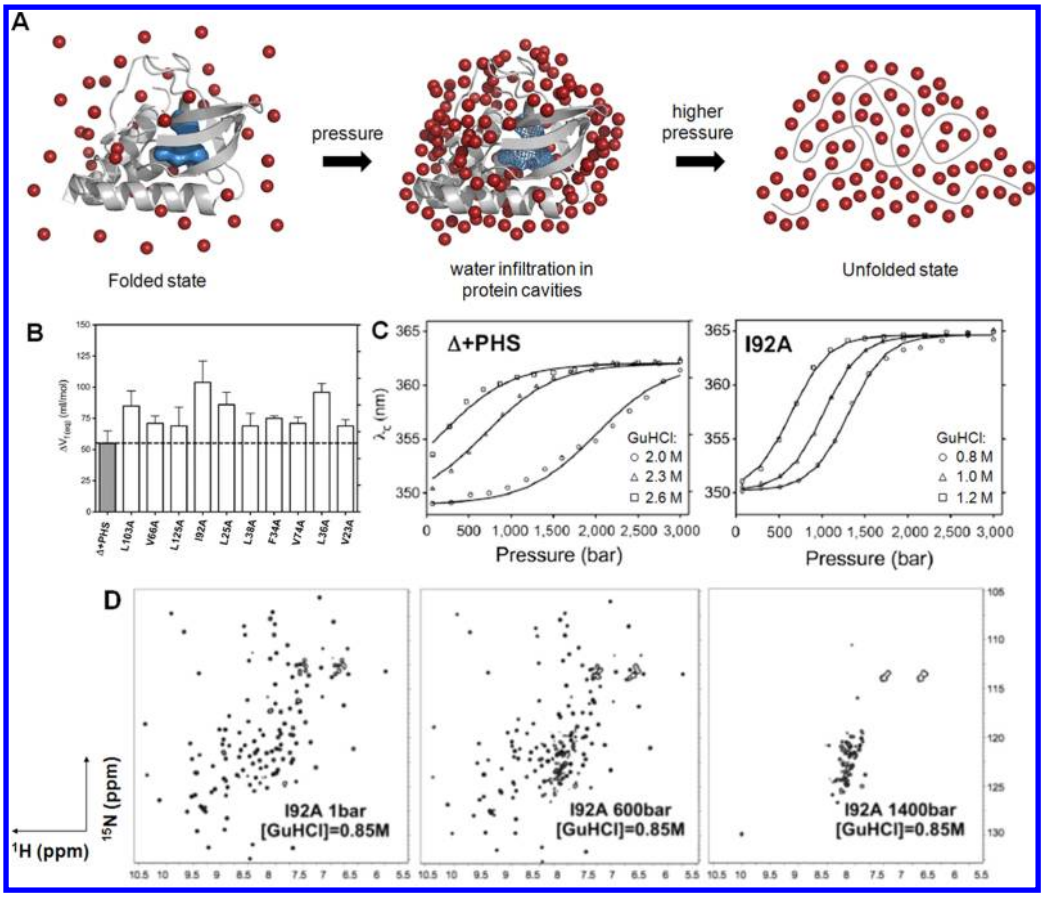

Figure 2. (A) Schematic representation showing the pressure effect in water-excluded cavities (blue surface within the protein model). SNAse I92A mutant (gray, PDB code: 3MEH) was used for illustration. Under pressure, water molecules (red spheres) infiltrate into the protein leading to cavities disassembly (blue mesh within the protein model) and unfolding. (B) Volume changes obtained from high-pressure fluorescent experiments for SNAse Δ + PHS and 10 cavity-containing mutants. (C) TRP fluorescence was used to follow pressure unfolding for SNAse Δ + PHS (left) and SNAse I92A (right). (D) HSQC spectra showing fully folded SNAse I92A (left), the midpoint of the pressure unfolding (middle), and fully unfolded (right). See ref 30 for more details. (B)−(D) were reprinted with permission from ref 30. Copyright 2012 National Academy of Sciences.

structure is dependent on the formation of water-excluded cavities and how crowding agents and osmolytes counteract the effects of pressure, similar to what has been observed with proteins.[51]

In contrast to the low sensitivity of nucleic acids to pressure, lipid membranes are extremely sensitive to pressure.[52−54] Pressure affects the fluidity and permeability of biological membranes. Most of the effects of pressure on membranes are due to phase changes from the liquid crystalline to the gel state.[52−55] As noted by Winter and co-workers, high pressure can also induce the formation of additional gel phases that are not observed under atmospheric conditions, such as the interdigitated high-pressure gel phase Lβi observed in phospholipid bilayers with acyl chain lengths of 16 carbon atoms.[52,56] Membrane proteins are usually more sensitive to pressure than water-soluble proteins. Pressure treatment of membrane proteins has been shown to cause irreversible effects in some cases.[57−59] The high barosensitivity of lipid membranes and their integral proteins explains why cellular membranes are usually disrupted at pressures between 1 and 2 kbar. However, in some cases, reversible high-pressure studies on protein−lipid interactions are possible. A remarkable example is the recent high-pressure studies on the N-Ras protein in the presence of membrane to populate low-lying excited substates, which are crucial in signaling.[60]

Glycosaminoglycans (GAGs) are complex polysaccharides that are involved in many important physiological processes, including cell growth, adhesion, differentiation, and cell signaling, among others. GAGs are linear polysaccharides composed of repeating disaccharide units of uronic acid (D-glucuronic acid or L-iduronic acid) and amino sugars (D-galactosamine or D-glucosamine) with a backbone that is negatively charged due to the presence of carboxylic acid and sulfate groups.[61] Because of their negative charges, these molecules exhibit an extended conformation in solution and are surrounded by a water shell. The electrostatic repulsion of their negatively charged groups and binding of large amounts of water confer compressive resistance. It is generally believed that under compression, the interactions with water molecules change, and GAGs occupy a smaller volume; after decompression, they return to their original volumes. This feature would be important for the biomechanical and osmotic properties of cartilage, which is rich in GAGs,[62] and for the resilience of synovial fluid and the vitreous humor of the eye.

3. PRESSURE PERTURBATION OF PROTEIN EQUILIBRIUM REACTIONS

3.1. Protein Folding

In his 1992 book "Protein Interactions",[8] Gregorio Weber dedicated two chapters to the effects of pressure on proteins.

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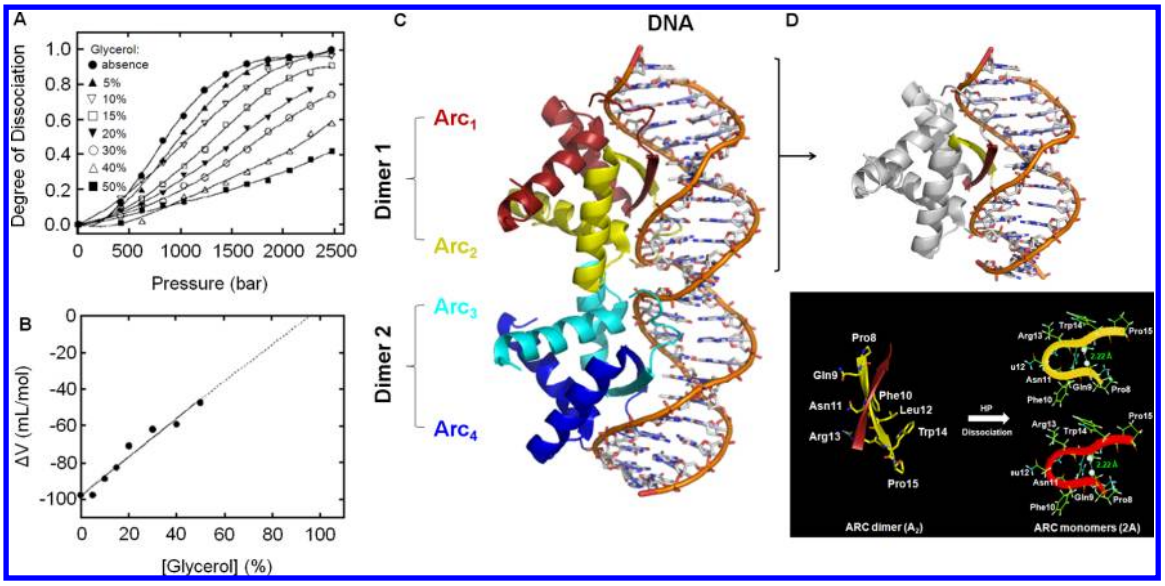

Figure 3. (A) Pressure effects on the degree of dissociation for Arc repressor in different concentrations of glycerol (see legend). (B) Volume change dependence against increasing concentrations of glycerol for Arc repressor dissociation. Dotted line shows $\Delta V = 0$ extrapolation. (C) Structural architecture for DNA-bound Arc repressor (PDB code: 1BDT). (D) High pressure on Arc repressor leads to a molten-globule state. DNA-binding motif of Arc repressor (top, residues 8−14) changes from an intermonomer β-sheet configuration, in the native dimer, to a intramonomer β-sheet (bottom), in the molten-globule substate. Parts (A) and (B) reprinted with permission from ref 51. Copyright 1994 Elsevier. Part (D) adapted from ref 13. Copyright 1993 National Academy of Sciences.

After reviewing the studies conducted prior to the publication of this book, Weber developed the hypothesis that under high pressure, proteins are penetrated by water in the same way that they are penetrated by chemical denaturants, such as urea and guanidine, when these agents are added at high concentrations at atmospheric pressure. Indeed, several studies have corroborated Weber's hypothesis that pressure produces swollen protein molecules in which short-range amino acid interactions are replaced by interactions with water. Thus, because the folded structure of a protein is extremely dependent on the formation of water-excluded cavities, manipulating pressure would be an ideal approach to tackle the problem of protein folding. In the last 20 years, water-excluded cavities in proteins have been explored with the combined use of several spectroscopic and structural tools and by varying hydrostatic pressure.[30−32,51,63−67] By shifting the equilibrium toward conformations with newly exposed amino acid residues, high pressure leads to a population of conformational states that are more solvated than the native state. The decrease in the volume of proteins that occurs upon partial or complete disruption of their native structure can be explained by contributions from the following phenomena:

(1) The loss of free volume arising from packing defects in the completely folded structure.

(2) The hydration of exposed nonpolar amino-acid residues.

(3) The electrostriction of exposed charges.

To determine why pressure leads to a disruption of the hydrophobic core of a protein and to a consequent decrease in the protein volume, several theoretical[68−70] and experimental[30,51,71,72] approaches have been employed.

Kauzmann was the first to note the apparent paradox[73] that pressure might denature proteins by destabilizing hydrophobic forces. Hummer and co-workers[68] addressed this problem theoretically and proposed that proteins can be denatured by pressures of a few kilobars due to the pressure-dependent transfer of water into a protein's interior, rather than the transfer of the protein's nonpolar residues into water. Using an information theory model for hydrophobic interactions, these authors explained the pressure-induced destabilization of hydrophobic aggregates and corroborated Weber's idea that pressure denaturation can be caused by water infiltration. Their model also supported previous experimental reports showing that Arc repressor becomes more resistant to pressure as water is withdrawn through replacement with glycerol (Figure 3A and B).[51] The volume change associated with Arc denaturation was observed to decrease linearly with the increasing glycerol concentration, whereas the free energy of the reaction increased.[51] The pressure that promoted 50% denaturation of the protein ($p_{1/2}$) increased in direct proportion to the decrease in water content. Thus, pressure denaturation of Arc repressor does not occur in the absence of water (Figure 3C and D). As discussed in the previous chapter, the structure of G-quadruplex DNA also seems to rely on the formation of water-excluded cavities, and the pressure-unfolding effects are attenuated by crowding reagents.[50]

Hillson and co-workers[69] elaborated on Hummer's model using an off-lattice minimalist model to describe the effects of pressure in slowing the folding/unfolding kinetics of proteins. According to their model, pressure would exert its effects on the contacts present in the transition state, which would result in an exponential decrease in the chain reconfigurational diffusion coefficient. Their theoretical deduction is consistent with experimental results regarding the kinetics of the pressure-induced denaturation of some proteins.[74,75] A positive activation volume would be expected if the prevalent effect arises from the fact that the transition state of the methane−methane interaction occurs at a volume larger than the associated state.[68,69] Although this situation has indeed been observed for SNase,[74] in most cases, the volumes of the transition states of protein folding lie below the volume of the folded state and are intermediate to those of the folded and unfolded states.[7,75−77] Therefore, other forces in addition to

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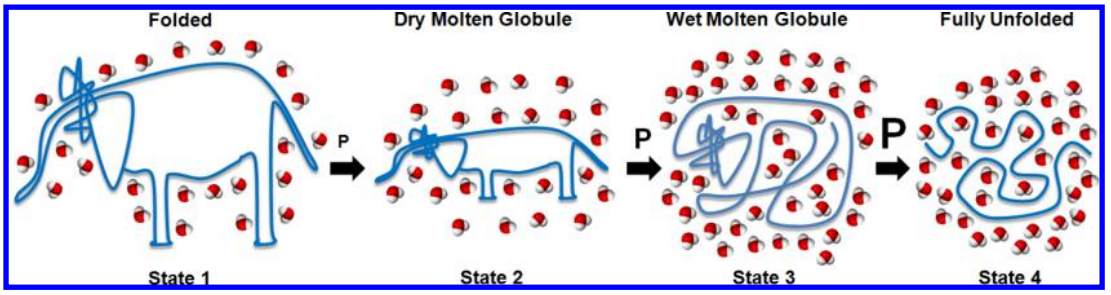

Figure 4. Schematic diagram illustrating the effects of increasing pressure on proteins. States 1−4 are populated upon pressure increase. Folded protein is shown like an elephant to represent the analogy with the Indian parable of the "six blind men and one elephant" that no single analysis will reveal the whole picture of protein behavior.

hydration, such as electrostatic interactions, must contribute to the kinetics of pressure-induced denaturation.

Elegant tests of the theory of water penetration have been performed by many groups. Using ubiquitin as a model, Day and Garcia found that the probability of cavity formation in a bulk solvent decreases more than the probability of cavity formation in the protein when pressure is increased.[78] Through analyses of molecular dynamics, these authors found that the system volume changes associated with cavity opening are approximately 12 mL/mol smaller in the protein than in the bulk solvent.[78] Thus, the transfer of water from the bulk solvent into the protein is associated with a decrease in the system volume.

However, this simple model, when contrasted to pressure experiments with ubiquitin at high-resolution NMR, is incomplete. At predenaturing pressures, pressure seems to induce changes in the structure without water penetration. Wand and co-workers have found that the conformation of ubiquitin would change into a dry molten globule.[79] Thus, in contrast to what was predicted from the molecular dynamics simulations, the interior of ubiquitin at 2.5 kbar would be dry. In a well-designed pressure/temperature study of the hydrogen-bond network of ubiquitin, Nisius and Grzesiek[80] also did not find an increase in amide proton exchange rates in this pressure range. However, as noted by these latter authors,[80] the pressure of 2.5 kbar can be used to examine only the early stages of ubiquitin unfolding, as its full unfolding only occurs at pressures greater than 5.0 kbar. Nevertheless, a pressure study using high-pressure crystallography revealed that a 160 Å^3 hydrophobic cavity created by a mutation in T4 lysozyme was penetrated by four molecules of water.[81]

The overall consensus is that pressure is a much more useful approach to protein unfolding than temperature and chemical denaturation. At low pressures, predenatured states, such as those observed for ubiquitin[79,80] and arc repressor,[82] can be sampled under high pressure. These states exhibit a conformation similar to the dry molten globule observed for ubiquitin. At higher pressures, wet molten globule, partially, and fully unfolded states are progressively populated, as shown in Figure 4.

The experimentally determined ΔV will depend on the changes of volume between the sampled states, which will depend on the probe method used and on the evaluated pressure range. As in the Indian parable of the "six blind men and one elephant" (translated into a poem by John Godfrey Saxe[83]), no single analysis will reveal the whole picture (see scheme in Figure 4). Each of our experimental approaches is evocative of a blind man examining the elephant. Analyses of changes in protein conformation, such as fluorescence, will tend to detect more global and later changes (as observed from state 2 to 3), whereas high-pressure NMR is a better method to detect earlier changes (side specific); and later changes will be detected at higher pressures by dramatic changes in the NMR spectra, such as a loss of peak dispersion and decreased signal intensity.

In a few cases of weakly stable proteins, the population of different states can be fully examined at relatively low pressures. A typical example was provided by Fuentes and Wand[72] in a pioneering work using apocytochrome b562, which has low stability due to its lack of the heme prosthetic group. Using pressure to perturb the hydrogen exchange behavior (detected by NMR), the authors found that the two central helices are the most stable domains, similar to previous results using chemical denaturants. The main novelty was that the determined volume change for these regions was highly negative (−102 mL/mol), and, in contrast to the outcome predicted by Kauzmann,[73] there was no measurable nonlinearity of the pressure effects.[72]

Recently, Royer and co-workers studied the contribution of protein cavities to the main transition from a folded to an unfolded ensemble of conformations by high-pressure NMR. Their study demonstrates that pressure causes protein denaturation primarily as a result of the presence of water-excluded cavities.[30] In their analyses, these authors utilized 10 cavity-containing mutants of staphylococcal nuclease (SNAse). As illustrated in Figure 2, the folding landscape of SNAse could be detailed on the basis of high-pressure NMR data. In an elegant recent study, the same group used pressure-jump NMR experiments to probe the effects of the internal cavities on folding routes as well on the folding kinetics.[84] Their main conclusion of this work is that wild-type SNase and some close variants fold in a linear pathway, whereas a highly stable mutant presents multiple parallel folding pathways.[84]

However, additional studies on simple systems are still needed. Grigera's group performed a study addressing the effect of pressure and temperature on the phase transitions of Lennard-Jones particles in water and, thus, of solubility.[70] Their results were in agreement with experimental findings of other researchers. The authors verified that the application of pressure leads to a decrease of the cluster size, and they proposed the application of their model to protein folding. In a study published very recently, Sarma and Paul performed a molecular dynamics simulation to explore the molecular mechanism of the protective effect of the osmolyte trimethylamine-*N*-oxide (TMAO) in opposing pressure denaturation.[85] Using aqueous solutions of *N*-methylacetamide (NMA) as a model, they found that pressure induces compression of the hydration shell of nonpolar groups and increases the hydration

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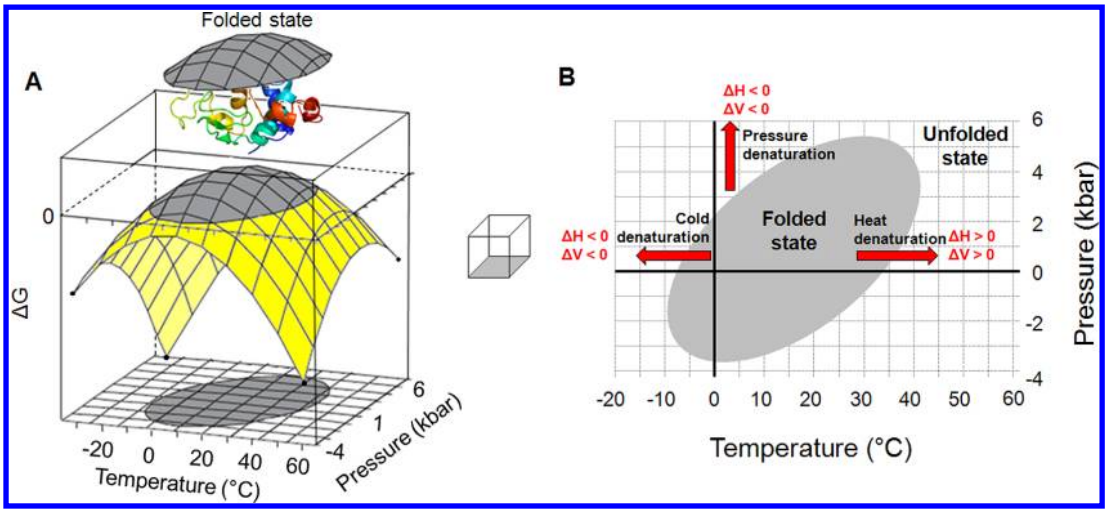

Figure 5. (A) Schematic diagram for the free-energy change of unfolding (ΔG) as a function of temperature (T) and pressure (p) for a protein gives an elliptic paraboloid distribution for ΔG. The cap (gray) shows the higher stability region ($\Delta G > 0$) in the native state of the protein (PDB code: 1E8L). $p-T$ extreme conditions (black dots) lead to a decrease in ΔG values and protein unfolding. (B) $p-T$ plane projection shows the elliptic phase diagram for a protein and physical conditions to achieve the unfolding. 3D diagram was adapted from ref 107. Copyright 2002 Elsevier.

number. Their MD studies revealed that TMAO prevents pressure-induced increases in hydration of NMA molecules.[85]

3.2. Recent Developments in the High-Pressure Structural Biology and Biophysics of Proteins

In the last 10 years, there has been a great advancement in the technologies available to study the effects of pressure on biological molecules, especially on proteins. The use of fluorescence methods (available for more than 50 years) has also undergone technical advancements. For an account of the methodological progress in the use of fluorescence with high pressure, see the review by Dellarole and Royer.[86] However, the methodology that has experienced the greatest progress is high-pressure NMR.[87] Earlier studies used the autoclave approach (for a review, see ref 88). The main advantage of these approaches was that they could achieve pressures up to 10 kbar. However, the main disadvantage was that modern 2D and 3D pulse techniques were difficult to implement, particularly to study proteins at low protein concentrations (submillimolar). The adaptation of Yamada's capillary approach by Akasaka could overcome the problem of the use of commercial probes to conduct multidimensional heteronuclear NMR.[89] The design of a high-volume NMR cell by Wand allowed for triple resonance spectroscopy at pressures up to 2.5 kbar.[90,91] Further developments have occurred in recent years, including a ceramic cell made of yttrium-stabilized ZrO_2 that is safe to be used in cryoprobes.[92] A recent technical development is the ability to obtain high-resolution NMR data using high-pressure jumps.[93] In this latter advance, a microprocessor-controlled pressure jump was employed to produce rapid pressure changes at any point in the pulse sequences.[92] These new methodologies will enable new studies to be performed to study protein unfolding, protein dissociation, and protein–ligand reactions.

Recent developments have also occurred in other spectroscopic methods used to study proteins under pressure, notably, the recent description of a high-pressure cell for circular dichroism measurements.[94] Instead of using quartz or sapphire, MgF_2 windows with a reduced aperture were used. Using CD in combination with site-directed spin-labeling EPR (SDSL-EPR), the authors measured structural changes in holo and apomyoglobin as a function of pressure. The CD measurements allowed the authors to follow the changes in secondary structure of the protein up to 2.0 kbar. The main advantage of circular dichroism is its simplicity and requirement for relatively low protein concentrations.

High-pressure crystallography has also seen advances in the past decade.[87,95,96] Several recent studies have revealed substates of a crystallized protein under pressure. For example, in the case of the L99A mutant of T4 lysozyme, a pressure of 2.0 kbar induced the water occupancy of a buried cavity (empty at atmospheric pressure).[81] A remarkable achievement was the description of the high-resolution crystal structure of bovine Cu,Zn superoxide dismutase at 5.7 kbar.[97] The structure was very similar to the structure at atmospheric pressure, but local changes in the atomic coordinates could be characterized. The changes were anisotropic and revealed regions in the protein that were flexible. The impressive structure of an icosahedral virus (CPMV) obtained under pressure by Fourme and co-workers[87] is also a notable achievement.

In summary, all of these technical developments open major avenues to new studies of the effects of pressure on proteins and macromolecular assemblies.

3.3. Coupling of Low Temperature and High Hydrostatic Pressure to Explore Protein Folding

As noted in section 2.1, Bridgman has already discussed the additive effects of high pressure and low temperature.[9] Because of the decrease of the water melting point to −20 °C at 2.0 kbar, the use of high pressure as an experimental tool has been extremely useful for exploring cold denaturation of proteins. This tool has been applied in studies on both small proteins, such as Arc repressor,[71] ribonuclease,[98] lysozyme,[99] allophycocyanin,[100] ubiquitin,[80,99,101] and large multimolecular complexes, such as phycobilisomes,[102] viruses,[35,103,104] and amyloid fibrils.[41,105]

In a pioneering study published in 1971, Hawley[106] described the pressure–temperature-reversible denaturation of chymotrypsinogen at low pH. By assuming a two-state transition and integrating the equation $d\Delta G = \Delta V\, dp - \Delta S\, dT$ from T_0, p_0 to T, p:

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$$\Delta G = \Delta G_0 + \Delta k'/2(p - p_0)^2 + \Delta\alpha'(p - p_0)(T - T_0) - \Delta C_p[T(\ln T/T_0 - 1) + T_0] + \Delta V_0(p - p_0) - \Delta S(T - T_0) \quad (5)$$

In the resulting equation of state, Δ denotes the change in the corresponding parameter upon unfolding; k' is the compressibility factor, $k' = (dV/dp)_T = -V_k$; α' is the thermal expansivity factor, $\alpha' = (dV/dT)_p = -(dS/dp)_T = V\alpha$; and $C_p = (dH/dT)_p$ is the heat capacity at constant pressure.

The transition line in Figure 5A, where the protein unfolds, is given by $\Delta G = 0$. In the region in which the protein is stable, that is, in its native state, $\Delta G > 0$.[107] The physically relevant solution of the curve in the $T-p$ plane has an elliptical shape, with $(\Delta\alpha')^2 \geq \Delta C_p \Delta k'/T_0$.

The shape of the elliptic phase boundary is defined by six thermodynamic parameters (ΔC_p, ΔV, ΔS, $\Delta k'$, $\Delta\alpha'$, and ΔG_0, which is the reference Gibbs free-energy change under unfolding), resulting in the well-known elliptical contours of constant free-energy difference in the pressure−temperature plane, as represented in Figure 5B.

The primary assumption made in applying eq 5 is that the pressure-unfolded state is similar to the cold- and heat-unfolded states. Although this is true for some proteins, in most cases, the pressure-denatured state is not similar to the heat-denatured state or to chemically denatured states. In the case of Arc repressor, the pressure-denatured state differs from the fully unfolded states obtained using high temperatures and urea.[12,13,82] The pressure-denatured state exhibited the conformation of a wet molten globule (state 3 in Figure 4) that was different from the fully unfolded states.[12,13,82] The cold-denatured state of Arc repressor is similar to its pressure-denatured state.[71] Even when Arc repressor was stabilized by binding to operator DNA, the complex underwent pressure-assisted cold denaturation.[71]

The elliptical PT approach was used recently to evaluate hydrogen-bond deformation in ubiquitin using high-pressure NMR.[80] By measuring the hydrogen-bond scalar couplings of 31 hydrogen bonds in ubiquitin as a function of pressure and temperature, the authors found that short-range hydrogen bonds (HBs) are little perturbed and that many HBs with large sequence separations underwent greater changes. The pressures utilized in these experiments were not high enough to promote ubiquitin denaturation. Under these predenatured stages (likely a transition between states 1 and 2 in Figure 4), the authors found that most of the HBs behaved similarly for the whole protein, as they were stabilized by increases in pressure and destabilized by temperature.[80] More recently, the same group used subzero temperatures under pressure to promote pressure-assisted cold denaturation of ubiquitin.[108] The NMR structural data revealed that the cold-denatured state is an unfolded ensemble with a high propensity for a first β-hairpin and α-helix that are similar to the native state, whereas the C-terminus has a high propensity to form a nonnative second α-helix. The authors clearly demonstrated that the pressure-denatured, cold-denatured, and alcohol-denatured states of ubiquitin display similar structures, as determined by NMR spectroscopy.[108] This study clearly showed how pressure and cold act similarly to reduce the hydrophobic effect.

Using an elegant computational approach, Dias[109] demonstrated that hydrophobic interactions can account for cold and pressure denaturation by forming configurations in which amino acid residues are separated by a single layer of water molecules. These authors' model would explain the elliptical PT behavior as well as the maintenance of some secondary structure in pressure- and cold-denatured states. However, the field is quite open for future exploration with other proteins.

3.4. Protein Conformation Substates Examined under High Pressure

Because pressure in the range of 1 bar to thousands of bars has rather mild effects on proteins, it can be employed to study populations of predenatured states. Akasaka and co-workers have used pressure to assess substates on the basis of the effects of pressure on the volumes of component conformers.[38,110,111] In several of their analyses, these authors applied pressure perturbation in combination with NMR to study slow, rare, and large-amplitude motions that are usually neglected using other types of perturbation, such as high temperature and chemicals.[87,111] Because high pressure increases the occupancy of excited states of a protein, the conformers can be characterized.

As discussed in section 3.1, NMR relaxation has been employed to detect pressure-induced effects on the fast internal motion of ubiquitin.[79] In this study, it was clearly shown that methyl-bearing side chains exhibit a greater and more variable pressure dependence than the main chain. The native state ensemble of ubiquitin was suggested to contain a significant fraction of conformers that exhibit the characteristics of a proposed dry molten globule. Under the studied conditions (up to 2.5 kbar), ubiquitin was still native. As previously discussed, this result contradicts the idea that pressure forces water inside the protein structure, as the authors did not observe water molecules fluctuating into the structure of the protein in the early perturbation effects. Thus, hydration would occur only when the protein began to denature. Subdenaturing pressures populate a more compact and higher-energy state, which the authors propose similarity to the static compact structures revealed by low-temperature crystallography of proteins.

In an early high-pressure NMR study of Arc repressor dimers, it was also found that the proteins assumed a "pre-dissociated" dimer conformation at pressures lower than those leading to dissociation and denaturation, and this conformation was also in a more compact state.[13,82] At the time of this early study, it was not possible to investigate the hydration state of this conformation.

3.5. High Hydrostatic Pressure and Supramolecular Structures

Multimeric proteins play key roles in various biological processes. The formation of cilia, flagella, and the cytoskeleton depends on proteins composed of multiple subunits arranged in a specific manner. Furthermore, numerous metabolic pathways depend on the activity of proteins that contain more than one subunit. It is of particular note that the diversity of functions and properties found in supramolecular protein structures is not present in their isolated subunits.

In the last three decades, a number of researchers have attempted to understand the thermodynamic principles that govern the association of multimeric proteins. In general, the inverse process, that is, the dissociation of these structures, is the process observed through experimentation. Temperature and pH, progressive dilution of samples, the addition of denaturing agents such as urea and GuHCl, or the application of high hydrostatic pressure can be used for this purpose.

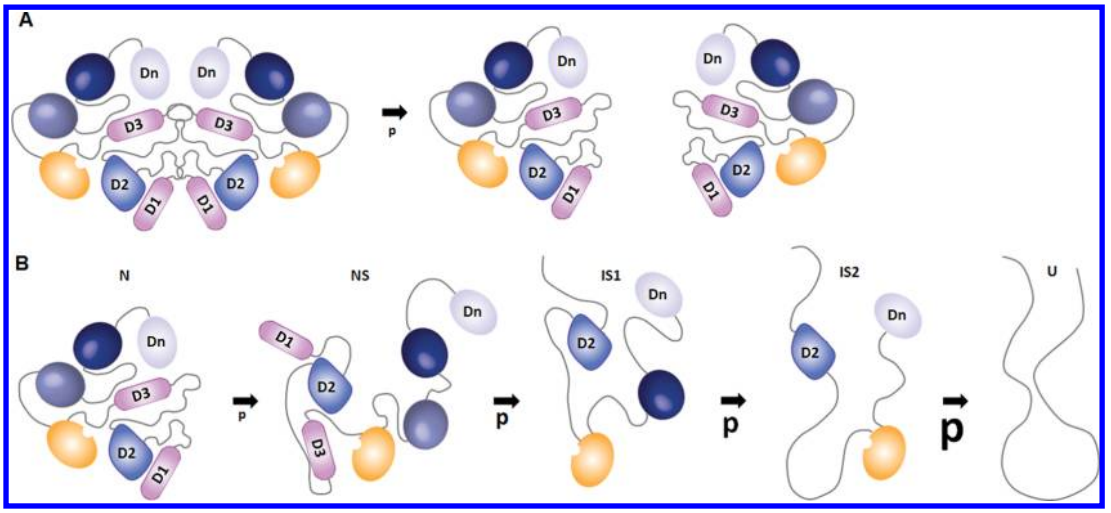

Figure 6. Schematic diagram for high-pressure effects on a multimeric protein. (A) Pressures from 0.5 to 3.0 kbar tend to alter the equilibrium between the multimeric proteins and its constituent subunits dissociation. (B) Pressure effects on dissociated native (N) multimeric proteins. Transition between native to native substate (NS) species can be explained by the conformational drift hypothesis from Weber.[117] Increased pressure at this stage populates different intermediate substates (IS1, IS2, ISn) and unfolding (U). Multiple domains are exemplified as D1, D2, Dn.

Josephs and Harrington[112] were the first to conduct seminal studies on the effects of pressure on a multimeric protein. They observed a reduction in the sedimentation coefficient of myosin when the speed of ultracentrifugation was increased and deduced that the pressure generated by rotation, despite being lower than 200 bar, promoted the dissociation of myosin into its subunits. Although other examples of this type of behavior were found, most oligomeric proteins were not affected by such low pressures. A few years later, Heremans and co-workers applied pressures much greater than 200 bar and were able to observe the dissociation of casein,[22] microtubules,[23] and ribosomes.[113] A general lesson obtained from studies examining oligomeric proteins is that pressures from 0.5 to 3.0 kbar tend to alter the equilibrium between oligomers and their constituent subunits (Figure 6). However, the folding of monomeric globular proteins is usually affected only at pressures between 5 and 12 kbar (Figure 6).[3,4] In both cases, pressure promotes dissociation and denaturation in a controlled fashion, making it possible to examine partially folded states and measure thermodynamic folding parameters. As shown in Figure 6 depending on the domain structure of the monomer, different states can be populated, ranging from a native-like to fully unfolded states. A more complex behavior is observed along the scale from dimers to larger assemblies. Additionally, as compared to temperature, which affects the system's energy content, increased pressure generally shifts the equilibrium between associated and dissociated forms of proteins toward dissociation without affecting the system's energy content.[3,4,114,115]

The use of hydrostatic pressures in the range of 0.3−3.0 kbar has contributed substantially to our understanding of the factors that control the interactions between protein subunits. Some of the most important information regarding the dissociation process, including the fact that the separation of a protein into its subunits is often accompanied by dramatic conformational changes, has emerged from studies that employed pressure as exemplified in Figure 6.[114,116−118] Increased pressure diminishes the affinity between protein subunits due to the progressive loss of the free energy of association. In the case of dimeric and tetrameric proteins, the reassociation process may be defective (anomalous), as observed for lactate dehydrogenase[116] and tryptophan synthase:[114] defective forms of these enzymes were obtained after a cycle of compression/decompression. In both cases, the recovery of function took some time, suggesting that the reassociated forms must undergo a conformational adjustment before protein activity recovers.

In large protein complexes, the loss of affinity between subunits seems to be greater. For example, the hemoglobin of *Glossoscolex paulistus*,[119,120] a gastropod hemocyanin,[121] and several icosahedral viruses[10,122−124] lose the capacity to reassociate correctly after decompression. All of these effects can be explained as a result of the process known as conformational drift, in which atomic contacts between subunits are replaced by contacts with a solvent.[114,116,117] When Weber first proposed the conformational drift hypothesis in 1982[125] and 1986,[117] the general belief was that each protein displays a unique folded conformation. At that time, we had little knowledge of the many diseases caused by the conversion of proteins into misfolded and amyloid states.[118] As we will show in more detail, the use of pressure has made it possible not only to characterize several of these proteins but also to discover new proteins that can assume amyloid conformations, such as the tumor suppressor protein p53.[126,127]

The application of hydrostatic pressure also makes it possible to assess the dissociative behavior of protein aggregates with two, three, four, or various other numbers of subunits. Dimeric proteins share a common characteristic that, in principle, is expected to occur in any dissociation process: concentration dependence. Equation 4 in section 1 permits the calculation of the standard volume change from measurements performed at a fixed protein concentration. This volume change has been designated ΔV_p. It can be deduced that a change in the protein concentration from C_1 to C_2 at a fixed pressure will result in a parallel displacement, Δp, of the curve along the pressure axis related to the volume change ΔV_C, according to the following:

$$\Delta p = (n - 1)(RT/\Delta V_C) \ln(C_2/C_1) \quad (6)$$

Under stochastic equilibrium, $\Delta V_p = \Delta V_C$. This stochastic equilibrium, in which the subunits are indistinguishable in terms of their rates of dissociation and association, is partially

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lost for some trimeric and tetrameric proteins[42,116,128] and is lacking among several larger aggregates.[119,121−123] In these cases, the dissociation process is described as exhibiting a deterministic, mechanical type of behavior where external perturbation reveals the individual characteristics of the subunits, which then begin to behave independently. In extreme cases, the dissociation process becomes completely independent of the concentration, as observed for some viruses.[10,123] In these instances, heterogeneous populations of subunits exist in terms of their free energies of association.

3.6. Free Energy and Volume Coupling between Folding and Ligand Binding

Hydrostatic pressure has been widely applied to explore the involvement of water-excluded cavities and hydration in the interactions of proteins with nucleic acids, cofactors, and ions. Hydration has been proven to be the most influential factor in discriminating between nonspecific and specific DNA binding by several transcription factors and restriction enzymes.[15] Our group has used pressure to evaluate the thermodynamics of protein−DNA complexes, such as the Arc repressor,[71,129] E2 DNA binding domain of papillomavirus,[130,131] and LexA repressor complexes (Figure 7A−C).[132] As discussed above, pressure has minimal effects on the structure of double-stranded DNA, which is highly advantageous when studying the interactions of proteins with nucleic acids. In the case of Arc repressor, the DNA recognition process is tightly coupled to the folding and oligomerization of the protein, and large differences in the effects of pressure are observed for specific and nonspecific DNA.[15,71,129] The detailed thermodynamics of the formation of a given protein−nucleic acid complex can be studied using pressure coupled with temperatures below 0 °C without freezing. For the Arc repressor complex, pressure-assisted cold denaturation of the protein−operator DNA complex was achieved using pressure at subzero temperatures.[71] The results revealed that the formation of the specific complex is followed by an increase in nonpolar interactions and dehydration, which would explain the entropy-driven character of this process.[71]

Coupling of hydrostatic pressure with osmotic perturbation has been explored in other studies.[133,134] The application of hydrostatic pressure counteracts the effects of osmotic pressure by restoring the hydration shell of an enzyme, leading to recovery of the correct recognition pattern,[133] supporting the idea that water solvation plays an important role in DNA site-specific recognition.[133,134]

The coupling between protein folding and the binding of small ligands has also been studied using high pressure. Such studies were carried out to probe the interaction of troponin C (TnC) with Ca^{2+} (Figure 8).[36,115−117] This component of the troponin complex of vertebrate skeletal muscle consists of two structurally homologous domains, N and C, connected by an exposed α-helix. Each domain includes two EF sites. The N-terminal domain of TnC contains sites I and II, which show low affinities for Ca^{2+}, while sites III and IV in the C-domain display high affinities for Ca^{2+} and also bind Mg^{2+}. Mutants of full-length TnC and of its isolated domains have been constructed using site-directed mutagenesis to replace various Phe residues with Trp.

In these studies examining TnC, high hydrostatic pressure was employed as a perturbing agent in the presence or absence of urea. On the basis of changes in Trp emission, we determined that only the C-domain apo state is denatured by

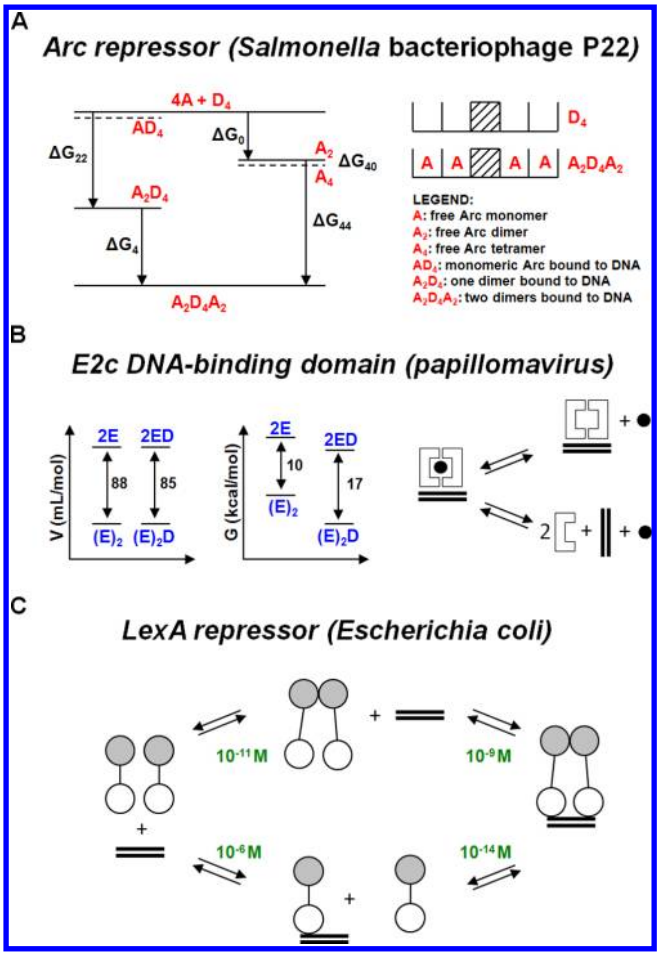

Figure 7. High-pressure studies to probe thermodynamics of protein−DNA complexes. (A) Free-energy levels between DNA binding and subunit association for Arc repressor from *Salmonella* bacteriophage p22. ΔG_{22} is the free-energy change for dimerization in the presence of DNA, ΔG_4, for formation of a second dimer bound to DNA, ΔG_0, for dimer formation in the absence of DNA, ΔG_{40}, for dimer−dimer association in the absence of DNA, and ΔG_{44}, the global free-energy change for Arc-DNA complex. (B) Volume (left) and free-energy (middle) changes for E2c DNA binding domain dissociation. D is the double-stranded DNA and E is an E2c monomer. Diagram illustrating two routes for E2c-DNA subunit dissociation (right). Monomers can dissociate and remain bound to DNA (upper), and monomers can dissociate from DNA as well (lower). (C) Model of LexA binding to an operator DNA sequence. The first route consists of LexA dimerization (K_{20}), followed by binding to DNA (K_{21}), and the second route consists of binding of a monomer to DNA (K_{01}), followed by binding of the second monomer (K_{11}). Part (A): Used and adapted with permission from ref 129. Copyright 1993 Wiley. Part (B): Adapted from ref 130. Copyright 2000 National Academy of Sciences. Part (C): Adapted with permission from ref 132. Copyright 2000 The American Society for Biochemistry and Molecular Biology.

pressure in the range of 1−1000 bar in the absence of urea. The addition of Ca^{2+} increases the stability of both the N and the C domains such that complete denaturation is achieved only when high hydrostatic pressure is combined with urea treatment.[39] High-pressure NMR spectroscopy has also been employed to follow these changes.[39] The ^{1}H NMR spectra obtained in the presence of Ca^{2+} were found to be typical of a highly structured protein and allowed us to follow the changes in the local environments of several amino acid residues as a function of pressure at a fixed concentration of urea. The

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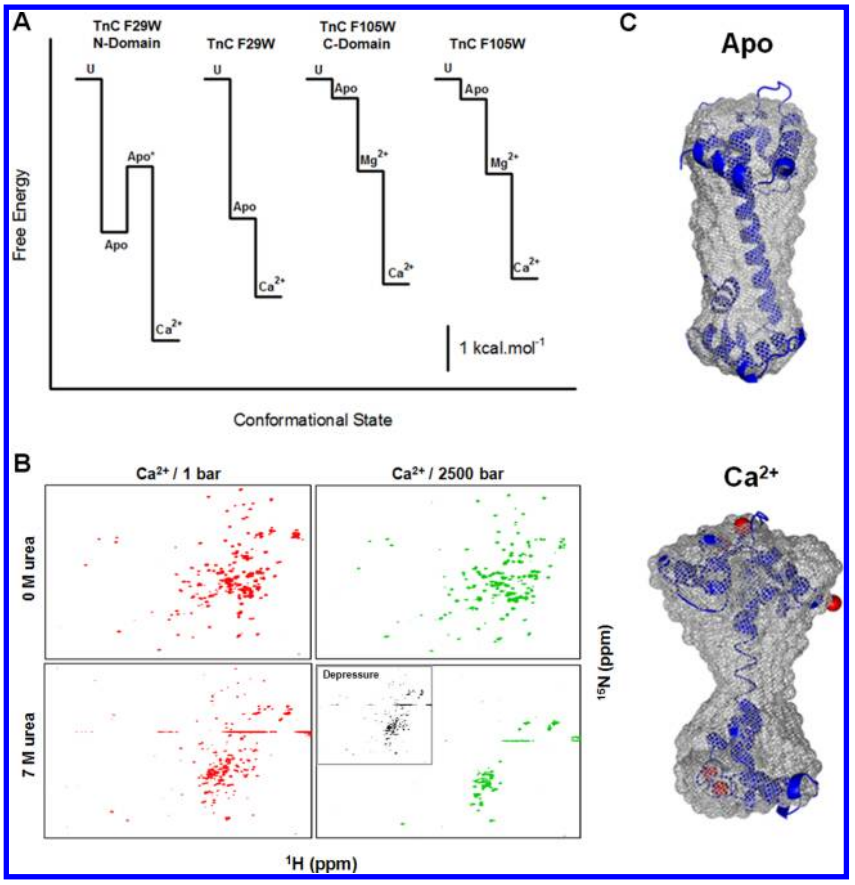

Figure 8. (A) Free-energy change for folding of the entire TnC subunit and individual N- and C-domains. Apo* corresponds to the free-energy change related to the conformational change that precedes Ca^{2+} binding. (B) High-pressure HSQC spectra for the Ca^{2+}-bound F29W TnC and (C) molecular reconstructions from SAXS data for the apo and Ca^{2+}-bound F29W TnC subunit. Reprinted with permission from ref 136. Copyright 2013 American Chemical Society.

striking result of these analyses was that different volume changes (measured from NMR) were observed for different residues, but the residues in the hydrophobic core exhibited values that were very similar to those measured by Trp emission.[39]

Furthermore, the thermodynamic parameters ΔG°_{atm} and ΔV, which govern the folding of the N and C domains in all of their possible physiological states, were determined (Figure 8A).[135,136] The obtained volume and free-energy diagrams revealed a landscape of different conformations, from the less structured (more hydrated) denatured apo form to the highly structured (less hydrated) Ca^{2+}-bound form (Figure 8B). The large change in the folding free energy of the C domain coupled with Ca^{2+}-binding explains the much higher Ca^{2+} affinity of sites III and IV. Furthermore, comparison of the volume and free-energy changes related to the folding of the isolated domains with those observed for intact TnC revealed that, whereas the C domain has only a small effect on the structure of the N domain, the stability of the N domain in the Ca^{2+}-bound state is significantly decreased when Ca^{2+} is bound to sites III and IV of the C domain. The cross-talk between the C and N domains appears to be mediated by the central helix, which shows a smaller volume and, most likely, a greater rigidity and stability upon Ca^{2+}-binding to the EF-hand sites, as revealed by low-resolution small-angle X-ray scattering (Figure 8C).[136]

The interaction of another EF-hand protein, calmodulin, with a physiological model partner, a peptide corresponding to the calmodulin-binding domain of smooth muscle myosin light chain kinase (smMLCKp), was studied by measuring the dependence of amide hydrogen exchange as a function of pressure.[34] The amino acid residues in the core of the bound helical domain were similarly affected by pressure with an apparent volume of dissociation of −74 mL/mol. These values correspond to the breakage of 2−4 ion pairs and the consequent water electrostriction.[34]

Recently, Smeller and co-workers[137] published an interesting paper that presents a pressure−temperature phase diagram for cod parvalbumin (Gadm1) and its coupling to Ca^{2+} binding. Gadm1 is associated with fish allergies. These authors explored the $P-T$ phase diagram using FT-IR. The phase diagram was found to be quite complex, including partially unfolded and molten globule states. Ca^{2+} ions were observed to be essential for the formation of the native structure. Complete pressure unfolding could only be achieved at high pressure (1.14 GPa) and increased temperature (40 °C). A strong correlation was found between Ca^{2+} binding and protein conformation. The completely unfolded molecule, from which Ca^{2+} was released, could not refold.

Another spectacular example of coupling between pressure stability and ligand binding was described in a study by Marchal et al.[138] on odorant-binding proteins (OBPs). OBPs belong to a class of proteins in the lipocalin family, which includes proteins that participate in the transport of small hydrophobic molecules in the nasal mucosae of vertebrates. The authors detected a peculiar dependence on pressure. While the substrate-bound

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protein remains in its native configuration at up to 330 MPa (at which it dissociates from its substrate), the substrate-free protein dissociates into monomers at 200 MPa. On the other hand, the monomeric substrate-free form of the protein unfolds at 120 MPa. Whereas pressure unfolding was observed to be reversible, dimerization and substrate binding were not. The authors proposed that refolding requires relatively large changes in structure and hydration, in accordance with the conformational drift hypothesis previously proposed by Weber.[117] The occurrence of the drifted and hydrated state would explain the relatively low stability of the monomeric form of this protein.

Knowledge on the effects of hydrostatic pressure on protein−ligand interactions has been extended to biotechnological applications. Laugharn and co-workers have developed several technological approaches to the pressure-assisted binding and dissociation of biomolecular complexes.[139−142] A particularly interesting application is the use of pressure to dissociate immune complexes with the aim of increasing the sensitivity and specificity of clinical assays.[140]

4. AMYLOID AGGREGATES: NANOSTRUCTURES THAT ARE HIGHLY SENSITIVE TO PRESSURE

In his Nobel lecture given in 1972, Christian Anfinsen summarized the main dogma of protein folding by stating that "The three-dimensional structure of a native protein in its normal physiological milieu is the one in which the Gibbs free energy of the whole system is lowest; that is, the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment".[143] At that time, the existence of "protein misfolding", amyloid aggregates, and related diseases had not been addressed in the protein folding literature. Today, protein misfolding and aggregation are known to be of great importance not only because of their implications for disease but also due to their relevance to physiological function.[63,144−146] Thus, understanding the thermodynamic and kinetic basis of protein aggregation is crucial, especially if we wish to develop therapeutic strategies to combat these devastating diseases. Hydrostatic pressure is a powerful tool for assessing protein aggregation. One of the first studies along these lines was performed by our research group on the amyloidogenic protein transthyretin (TTR), which is involved in senile systemic amyloidosis and familial amyloidotic polyneuropathy.[42] We characterized the pressure-induced dissociation of TTR tetramers. After one cycle of compression−decompression at 1 °C, the monomers reassociated into tetramers with an altered conformation, designated T(4)*. The T(4)* tetramers were less stable under pressure and aggregated rapidly at 37 °C. The "preaggregated" state of T(4)* exhibited all of the properties of a conformationally drifted state with looser subunit interactions. Disease-related TTR variants were shown to exhibit stabilities and properties similar to those of T4*.[147]

Following this work, many studies were published involving the use of high pressure to explore the role of cavities and hydration in the formation of amyloid aggregates.[41,44,71,126,148−152] The general findings obtained in several of these studies were that amyloid fibrillar aggregates are usually sensitive to pressure and that their pressure sensitivity is correlated with the disruption of hydrophobic cavities. This was expected because forces similar to those that operate in the native state, especially the formation of water-excluded cavities, maintain amyloid and misfolded conformations. Figure 9 shows how pressure acts to dissociate amyloid oligomers and fibrils. However, not all amyloid aggregates exhibit larger specific volumes than are found in the proteins in their native states.

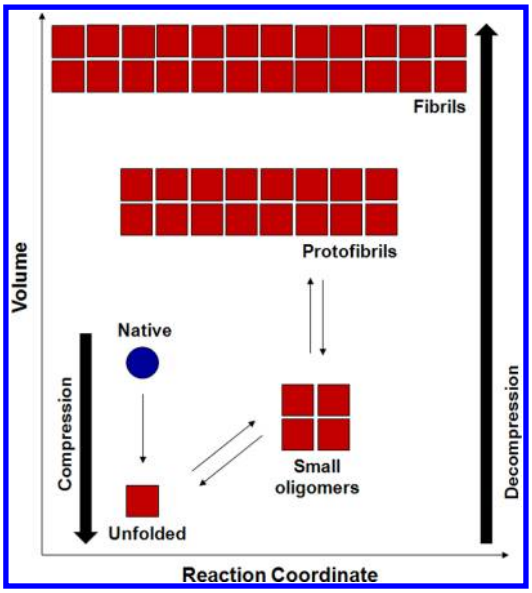

Figure 9. General volume diagram for protein folding and aggregation. Similar to native proteins, aggregates have packing defects that make them sensitive to reversible pressure dissociation. Adapted with permission from ref 41. Copyright 2004 American Chemical Society.

In general, early aggregated species and protofilaments are more sensitive to pressure than consolidated fibrils.[41,63] For example, the amyloid fibrils of β2-microglobulin (β2-m), which are involved in dialysis-related amyloidosis, are not tightly packed, instead presenting a larger number of cavities than the denatured protein, making them highly sensitive to pressure.[153] However, mature amyloid-like fibrils formed from a fragment of the β2-m protein are resistant to pressure, indicating a smaller partial specific volume, most likely due to a greater contribution from hydrogen bonds.[153] Similar results were observed when comparing whole fibrils of transthyretin (wild-type and mutant forms) with TTR peptides;[42,154] whereas the former could be dissociated by pressure, the latter were quite resistant.

Additionally, the application of hydrostatic pressure has been evaluated as an economical method for the recovery of native proteins from inclusion bodies (IB) expressed in heterologous cells.[155−160] The aggregates found in inclusion bodies are usually nonamyloid, but in many cases, they can be dissociated by pressure. This methodology is currently being evaluated by pharmaceutical and biotechnological companies (see patents in refs 156 and160).

4.1. Pressure Studies in Amyloid and Neurodegenerative Diseases

The cytotoxic species that are responsible for amyloid neurodegeneration are thought to be fibrils and oligomeric intermediates.[161] Many studies have investigated protein aggregation/fibril formation as well as the disassembly of these aggregates and attempted to characterize their structure and stability. These approaches are important for understanding amyloid aggregation as well as prion conversion (see the following section) and for designing drugs that might prevent or delay the aggregation process. As described above for transthyretin, pressure can promote the formation of intermediates that are prone to aggregation (Figure 9).[42,64,147]

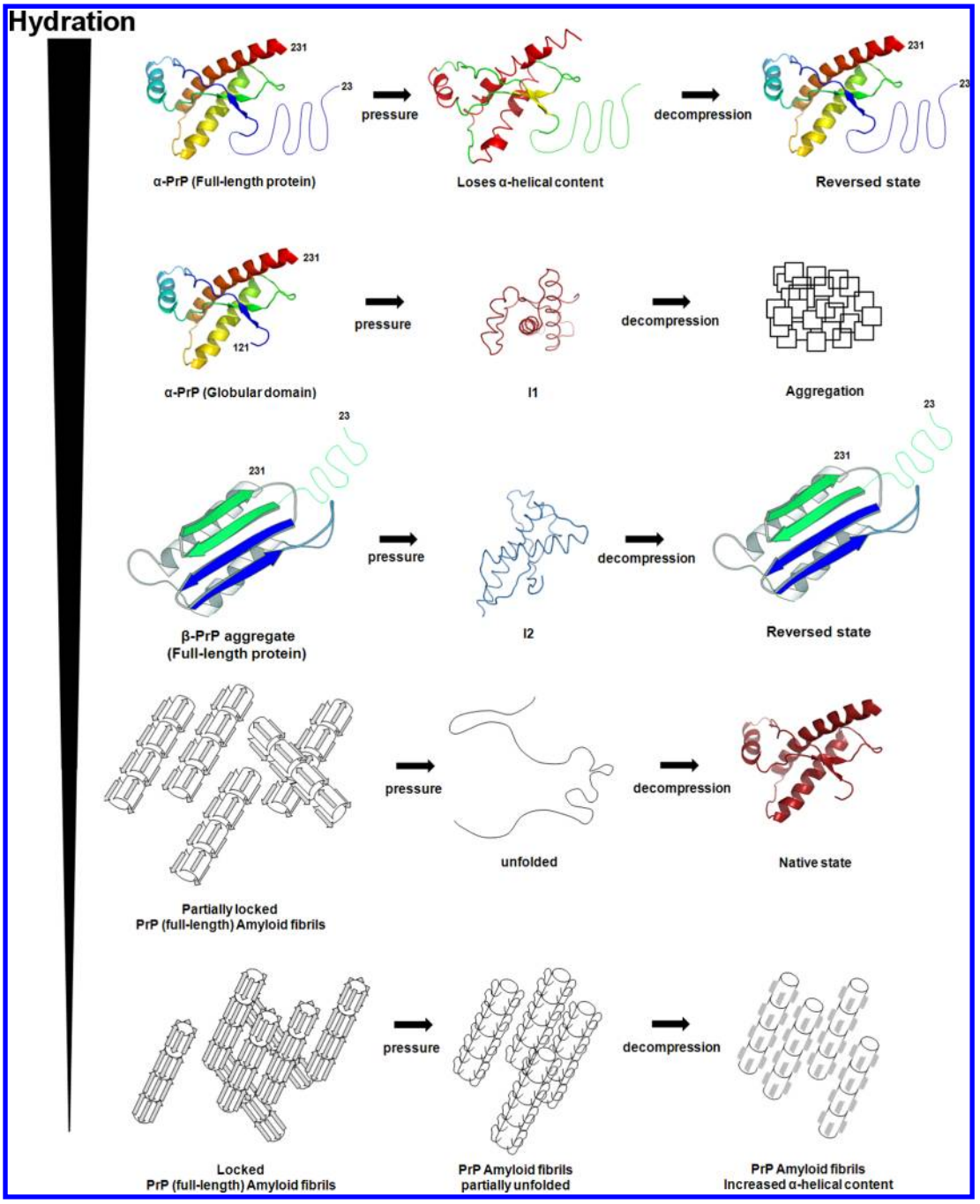

Figure 10. High-pressure effects on monomer PrP and its aggregates. Pressure leads to hydration of cavities generating PrP less folded intermediates. Its effects are reversible after decompression over full-length α-PrP and full-length β-PrP aggregates.[170] Irreversible aggregation occurs when pressure is applied to α-PrP globular domain[168,169] or amino-terminal deletions PrP constructs.[171] These results reveal that the N-terminal domain increases protein hydration, conferring more stability. The effect over PrP fibrils is also irreversible, dissociating partially locked fibrils, leading to monomer resolubilization, and affecting the secondary content of locked fibrils. PDB code: 1QLX.

Recently, high pressure was used to explore a potential therapy for amyloidogenic diseases via trapping the monomer of a nonamyloidogenic variant (T119M) of transthyretin.[162] In this study, pressure produced long-lived monomers of T119M. When the pressure-populated monomers of T119 M were mixed with aggressive mutants of TTR, nonamyloidogenic heterotetramers were generated.[162]

Studies using pressure as a tool have also been conducted on islet amyloid polypeptide (IAPP) by Winter's group.[163] Aggregation of IAPP can be causative for Type II diabetes mellitus because it results in the deposition of amyloid in the extracellular matrix of pancreatic beta cells, leading to islet cell death. These authors employed high hydrostatic pressure, FT-IR, and AFM to study IAPP fibrillation. They found that preformed IAPP fibrils exhibited strong polymorphism and formed heterogeneous structures. As was observed for TTR and β2-m,[42,153] most of the fibrils were very sensitive to high hydrostatic pressure, demonstrating the participation of hydrophobic interactions and cavities in the packing of the structures. However, fragments 1−19 and 1−29 of IAPP were found to be resistant to pressure treatment, suggesting that they were more densely packed.[163] On the other hand, evidence was obtained

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that hydrophobic interactions and poor packing of amino acids in the 30−37 amino acid region of the polypeptide are responsible for the high-pressure sensitivity of full-length IAPP.

Alzheimer's (AD) and Parkinson's disease (PD) are the first and second most common neurodegenerative brain disorders, respectively. Both diseases affect a significant fraction of the population over 65 years of age. The symptoms of Parkinson's disease include muscular rigidity, resting tremor, bradykinesia, and impaired postural reflexes, which are caused primarily by the loss of dopaminergic neurons and depletion of dopamine in the *substantia nigra pars compacta*. Development of α-synuclein (α-syn) aggregates is believed to be the major cause of Parkinson's disease. The formation of α-synuclein fibrils occurs quite slowly, usually over a period of days. When synuclein fibrils are subjected to hydrostatic pressure (below 3.0 kbar), they dissociate into soluble proteins showing the same intrinsically disordered structure that the protein possessed prior to forming fibrils.[64] Pressure-dissociated α-syn undergoes fibrillogenesis with typical slow kinetics, demonstrating the reversible character of the pressure effects.[64,164] Strikingly, we found that fibrils formed by the α-syn variants A30P and A53T, which are associated with early Parkinson's disease, are more susceptible to pressure than wild-type α-syn.[64] On the basis of these results, we deduced that fibrils of α-syn formed from these variants would be more easily dissolved into small oligomers by the cellular machinery. This finding is relevant in light of the belief that the pathogenic species of α-syn are its small aggregates, rather than the mature fibrils.[165]

More recently, we employed an approach involving pressure to investigate how dopamine (DA) differentially modulates the stability of protofibrils (PF) and fibrils (F) composed of wild-type α-syn or the α-syn variants A30P and A53T.[164] In the absence of DA, all of the α-syn mutant PFs exhibited identical levels of stability. However, in the presence of DA, the PFs formed from α-syn variants were much more stable than the wild-type α-syn PFs. This result suggests that the variant α-syn PFs persist longer, which could explain why these mutations are so aggressive. When added to mesencephalic and cortical neurons in culture, A30P protofibrils (PFA30P) and A30P DA-protofibrils (DAPFA30P) decreased the number and length of the neurites present and increased the number of apoptotic cells. Surprisingly, high hydrostatic pressure abolished the toxic effects of PFA30P and DAPFA30P. Atomic force microscopy revealed that pressure fragments the PFs into smaller aggregates.[164] These findings imply that strategies aimed at disrupting and/or clearing these aggregates may be promising for improving the clinical course of Parkinson's disease.

Alzheimer's disease (AD) is associated with the aggregation of amyloid beta peptide (Aβ) and tau protein. Aβ is a peptide of 39−43 amino acids that is derived from a larger, type I transmembrane protein termed amyloid precursor protein (APP). Aβ is generated from APP by the beta- and gamma-secretase proteases. Aβ, especially its larger form containing 42−43 amino acids, is highly apolar and undergoes aggregation rapidly when dissolved in an aqueous environment. Aβ-1−40 and Aβ-1−42 are the most common peptides found in amyloid plaques. Aβ amyloid fibrils are much more resistant to pressure than α-synuclein fibrils (unpublished results),[64] similar to the fibrils derived from short fragments of TTR, β2-m, and IAPP.[42,154,163]

A recent report has evaluated the changes in the conformation of Aβ monomers and the distribution between states 1 (more folded and packed) and 2 (partially unfolded).[166] State 1 is the one with higher affinity to aggregating seeds and was increased by temperature and decreased by pressure. Pressure likely causes the dissociation of Aβ oligomers and fibrils, similar to what has been found with α-synuclein. In fact, the authors detected some dissociation of Aβ fibrils by detecting increases in signal intensities in the ^{1}H NMR spectra.[166]

4.2. Effects of Pressure on Prion Protein Misfolding and Aggregation

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that are associated with the misfolding and aggregation of prion protein (PrP). PrP can be found as a cell surface protein that exhibits an α-helix-rich structure. This isoform, termed cellular PrP (PrP^{C}), can be misfolded into a β-sheet-rich form, prion scrapie (PrP^{Sc}). The mechanisms underlying this conversion have been extensively investigated, but they are not completely understood.[167]

Several studies utilizing pressure have been performed with the goal of characterizing PrP intermediates and elucidating the PrP aggregation pathway (Figure 10). Depending on the protein concentration and pH involved, pressures above 400 MPa have been shown to induce aggregation of the Syrian hamster PrP globular domain to generate a proteinase K (PK)-resistant form with an increased beta content.[43,168,169] The pressure-induced aggregation of the globular domain was found to be time-dependent: the formation of soluble oligomers was followed by the appearance of fibrils, and aggregation was irreversible after decompression.[43,169]

Our group showed that pressures above 400 MPa lead to a type of denaturation of full-length mouse PrP that is totally reversible, without the use of temperature or other denaturants. The α-helical structure of the protein decreases, while the content of turns increases, with no significant changes in the random coil or β-sheet conformation being observed.[170] The amino-terminal region of PrP has been shown to be important for conversion and toxicity. When we investigated PrP constructs with amino-terminal deletions under high pressure, we observed a reduction of pressure stability with a nonreversible effect and aggregation after decompression (Figure 10).[171] These differences in pressure susceptibility likely reflect the distinct levels of hydration and packing of the full-length protein and its globular domain, with the amino-terminal domain resulting in increased stability and hydration of the protein.

As described in previous sections, high pressure dissociates many types of protein aggregates. We investigated the effect of pressure on mouse PrP aggregates obtained via thermal treatment.[170] The temperature-induced aggregates showed an increase in intermolecular antiparallel β-sheet content and a decrease in α-helical and random coil structures. Pressure reversibly dissociated the temperature-induced aggregates, leading to a decrease in β-sheet structures; however, the denatured species were different from those formed via α-PrP denaturation, revealing different folding routes (Figure 10).[170] A similar combination of temperature and pressure led to decreased PrP^{Sc} resistance to proteinase treatment.[172,174] β-PrP also undergoes pressure denaturation at pressures below 400 MPa, indicating that it exhibits a greater number of solvent-excluded cavities when compared to α-PrP.[170] The increased sensitivity of β-PrP to pressure was shown to be time-dependent; the longer was the exposure to temperature, the greater was the pressure resistance.[170] Pressure perturbation

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calorimetry experiments (PPC) showed that β-PrP also exhibits a smaller surface-accessible area, indicating that the hydration level is very important for PrP aggregation.[170] Analyses of molecular dynamics have also been employed to verify the role of hydration in the stability of prions.[175,176]

Decreased hydration and occlusion of the hydrophobic surfaces of proteins can also be achieved by binding to ligands such as nucleic acids (NA),[177−179] 4,4-dianilino-1,1-binaphthyl-5,5-sulfonate (bis-ANS),[180] or glycosaminoglycans.[181] The "protein-only hypothesis" postulates that PrP^{Sc} is the infectious agent responsible for converting PrP^{C} to PrP^{Sc}, resulting in self-propagation. There is a great deal of supporting data for this model, although the purely proteinaceous nature of PrP has been argued.[179] PrP^{C} and its recombinant forms have been shown to undergo conversion in cell-free assays. However, the requirement for partially purified PrP^{Sc} as seed and the low infectivity of the converted products suggest that other cofactors might act to enhance this conversion.[179] NA and GAGs were shown to bind preferentially to the amino-terminal domain of PrP, leading to protein aggregation.[177−179,181] Studies involving pressure suggest that the amino-terminal region leads to hydration of the protein, increasing its stability and the energy barrier for conversion (Figure 10). The interaction of the amino-terminal region with polyanions is important for releasing the structured water around it, decreasing this barrier and facilitating conversion.[179,182]

Mouse PrP fibrils that formed under partially denaturing solvent conditions[183] were shown to be partially and irreversibly dissociated under a pressure of 600 MPa at 25 °C, with 30% of PrP being resolubilized. The fibrils exhibited a loss of their β-sheet structure and a concomitant increase in their α-helical structure. Furthermore, following pressure treatment, they formed aggregates with a different tertiary/quaternary structure due to the loss of amyloid features. The remaining aggregates were also resistant to a new pressure cycle. These experiments suggest that fibrils formed from full-length PrP under denaturing conditions may present two different types of packing: one involving cavities that are highly sensitive to pressure, whose disruption leads to fibril dissolution, and another that is not dissociated by pressure, although its structure is affected.[184] The growth of fibrils is believed to follow a dock-lock mechanism in which a monomer first docks to the fibril, then goes through structural rearrangements that cause it to bind with a higher affinity to the fibril (lock process) and become part of the fibril structure.[185−187] A simulation study showed that monomers interacting with fibrils exhibit conformational fluctuations and are not stable.[187] From this point of view, high pressure could destabilize the locking process, leading to monomer resolubilization, and stabilize hydrogen-bond interactions that are important for stacking.

High pressure has also been applied to scrapie brain homogenates. High pressure reduces the proteinase resistance and infectivity of PrP^{Sc} when applied at temperatures of approximately 60 °C, with a reduction of infectivity to 6−7 log infectious units/g being observed.[188] On the other hand, purified PrP^{Sc} is resistant to this treatment.[174,188] It is believed that centrifugation steps performed during purification lead to the formation of resistant highly aggregated species with fewer packing defects, as has been observed for late temperature-induced aggregates[170] and fibrils following pressure treatment.[184] However, an alternative explanation is that these two scrapie conformations can coexist.

The amyloid fibrils formed in vivo[189] and in vitro show polymorphism, and pressure treatment studies have revealed that they exhibit different water contents, chain packing, and quaternary structures. In the case of prion diseases, differences of this type could account for strain phenomena.[190] The fibrillation reaction is strongly affected by pH, protein concentrations, temperature, agitation, and other environmental conditions. The structures observed in vivo and in vitro may arise from different pathways, demonstrating the complexity of this scenario. The importance of these pathways is not predictable because not all of these structures are infectious.[191] Generally, these pathways involve a first step that is thermodynamically unfavorable and involves nucleus formation and structural changes. A second, thermodynamically favorable step involves the addition of monomers to the nucleus, resulting in polymerization.[192] Monomers can also be added directly to existing fibrils.[193] Heterogeneous nuclei can be formed under different initial conditions but also from a unique starting condition, producing great experimental variability.[191]

TSEs are infectious diseases that do not elicit an immune response, and there is currently no treatment for them.[194] Prion diseases can be transmitted to humans through blood transfusions,[195] contaminated surgical materials, and ingestion of contaminated meat.[196] Ingestion of contaminated meat from cattle with bovine spongiform encephalopathy (BSE) is believed to cause variant Creutzfeldt−Jakob disease (vCJD) in humans.[197] Although the number of vCJD cases has not increased in recent years and prophylactic actions have been implemented with respect to cattle, the number of cases of chronic wasting disease (CWD) in elk and deer has been increasing in the U.S., and one case of BSE has been reported.[198] The potential for zoonotic potentiation of CWD is still uncertain.[199] Thus, the concern about prion contamination and the fact that the infectious agent is highly resistant to many conventional inactivation procedures make it necessary to develop inactivation methods for both reusable and consumed materials.[200] Contaminated wastes can be incinerated at high temperatures (>700 °C). Reusable materials have been successfully decontaminated using sodium hypochlorite or sodium hydroxide solutions, followed by autoclaving at 134 °C.[200] In the decontamination of food, alterations such as loss of vitamins and unsaturated fatty acids, changes in taste and color, and formation of undesirable compounds should be avoided. In this context, heat sterilization is not a good strategy. High pressure has been proposed as an alternative because it does not affect covalent bonds and therefore does not greatly alter the composition of food.[201]

Many authors have evaluated the effects of high pressure on TSE-contaminated samples from tissue or processed meat. The combination of high pressure (500−1200 MPa) and temperature (milder temperatures that do not affect food quality, of up to 137 °C) was shown to decrease infectivity, but the obtained efficiency was dependent on the conditions applied and the material treated.[172−174,188,201] The resultant inactivation of prion biological activity was shown to be irreversible, and although it was not complete, the remaining infective units would be very few and most likely would be insufficient for oral infection.[202]

Pressure has also been shown to reduce the toxicity of amyloid fibrils formed from recombinant proteins,[184] confirming that the observed decrease in β-sheets and increase in α-helical structures is correlated with a loss of infectivity.[203]

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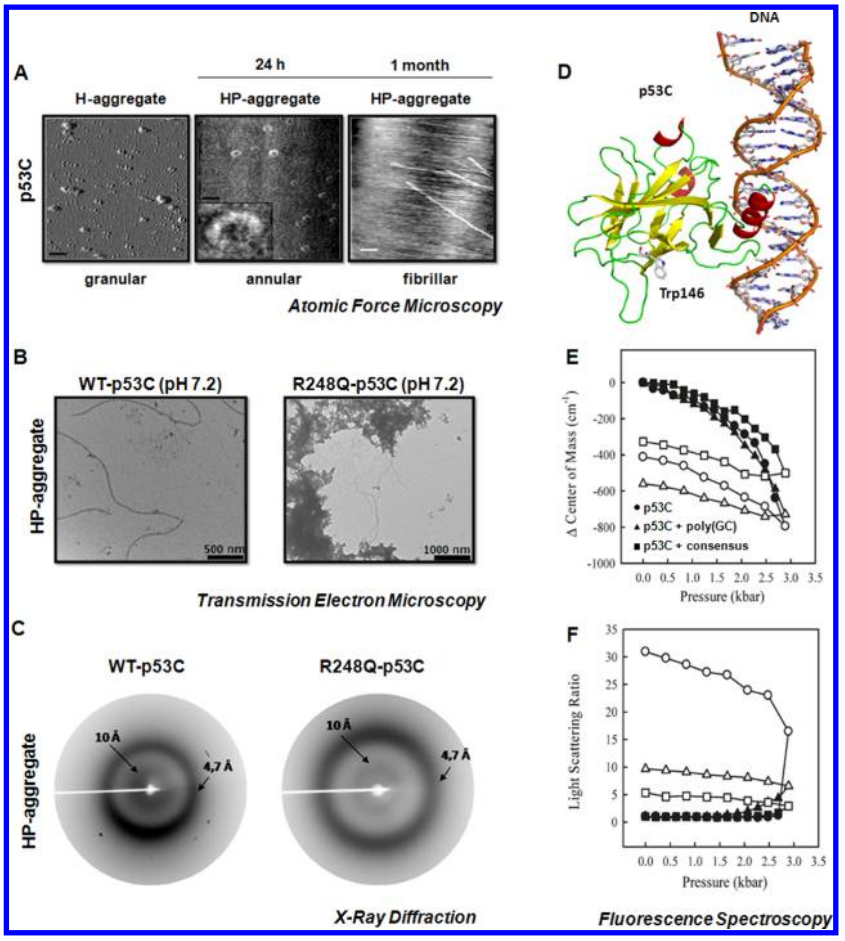

Figure 11. High-pressure studies on p53 DNA binding domain (p53C). (A) p53C aggregates assessed by atomic force microscopy. Granular aggregates induced by heating (left, H-aggregate). Annular (middle) and fibrillar (right) aggregates induced by high pressure (HP). (B) HP aggregates from wild-type and R248Q mutant p53C assessed by transmission electron microscopy. (C) X-ray diffraction spectra from wild-type and R248Q mutant p53C. (D) Structural architecture for p53C bound to a double-stranded DNA (PDB code: 1TUP). (E) Center of spectral mass and (F) light scattering for DNA-binding contributions to p53C behavior under pressure, followed by fluorescence emission from Trp146. Part (A) reprinted with permission from ref 126. Copyright 2003 American Chemical Society. Parts (B) and (C) reprinted with permission from ref 127. Copyright 2012 American Society for Biochemistry and Molecular Biology. Parts (D)−(F) reprinted with permission from ref 217. Copyright 2009 American Chemical Society.

Interestingly, following pressure treatment, the residual infectivity was mainly attributed to the soluble fraction.[184] The nature of the toxic species associated with amyloid diseases is still not well understood. Fibrils have been shown to be toxic, but many authors currently consider oligomers to be the main toxic species.[204] These findings raise important questions. For example, do fibers prepared via concentration constitute a source of toxic species, and can pressure treatment disrupt toxic species and decrease their release due to the induction of increased fiber packaging? Because the observed pressure effect was shown to be dependent on PrP conformation and heterogeneity is always found within and between samples, additional studies should be conducted to ensure safe application of this technology in decontamination procedures. In any case, the overall results are very promising. Figure 10 provides a summary of the studies addressing prion proteins.

Yeast prions exhibit a behavior different from that of mammalian prions.[205] Amyloid fibrils of the Sup35 protein of *Saccharomyces cerevisiae* are highly resistant to pressure. It was observed that high pressure induces a new structural organization of these fibrils that results in a higher affinity for the amyloid dye thioflavin-T.

5. PRESSURE TREATMENT STUDIES ON PROTEINS INVOLVED IN CANCER: MISFOLDING AND PRION-LIKE BEHAVIOR OF P53

The tumor suppressor protein p53, which is considered the guardian of the genome, is a tetrameric nuclear phosphoprotein that plays a vital role in mammalian cells. When cells experience stress, p53 activates a signaling cascade, partly through its role as a transcription factor that results in cell cycle arrest or apoptosis.[206] Mutations in p53 are strongly associated with increased susceptibility to cancer and are associated with worse prognosis.[206] In approximately 60% of human cancers, p53 function is lost, making it a tempting target for cancer therapies. The p53 monomer contains 393 amino acid residues and consists of an N-terminal transactivation domain, followed by a proline-rich region, a central DNA-binding domain (p53C), a tetramerization domain, and the extreme C terminus.[207] The central region, p53C, which is also referred to as the core domain, is composed of residues 94−312 and is responsible for DNA recognition.[207] Among all of the known point mutations in p53 that are related to cancer, 97% are found in the p53C region of the protein. Most mutations in p53C lead to changes in its structure and/or DNA binding. The misfolding of mutant

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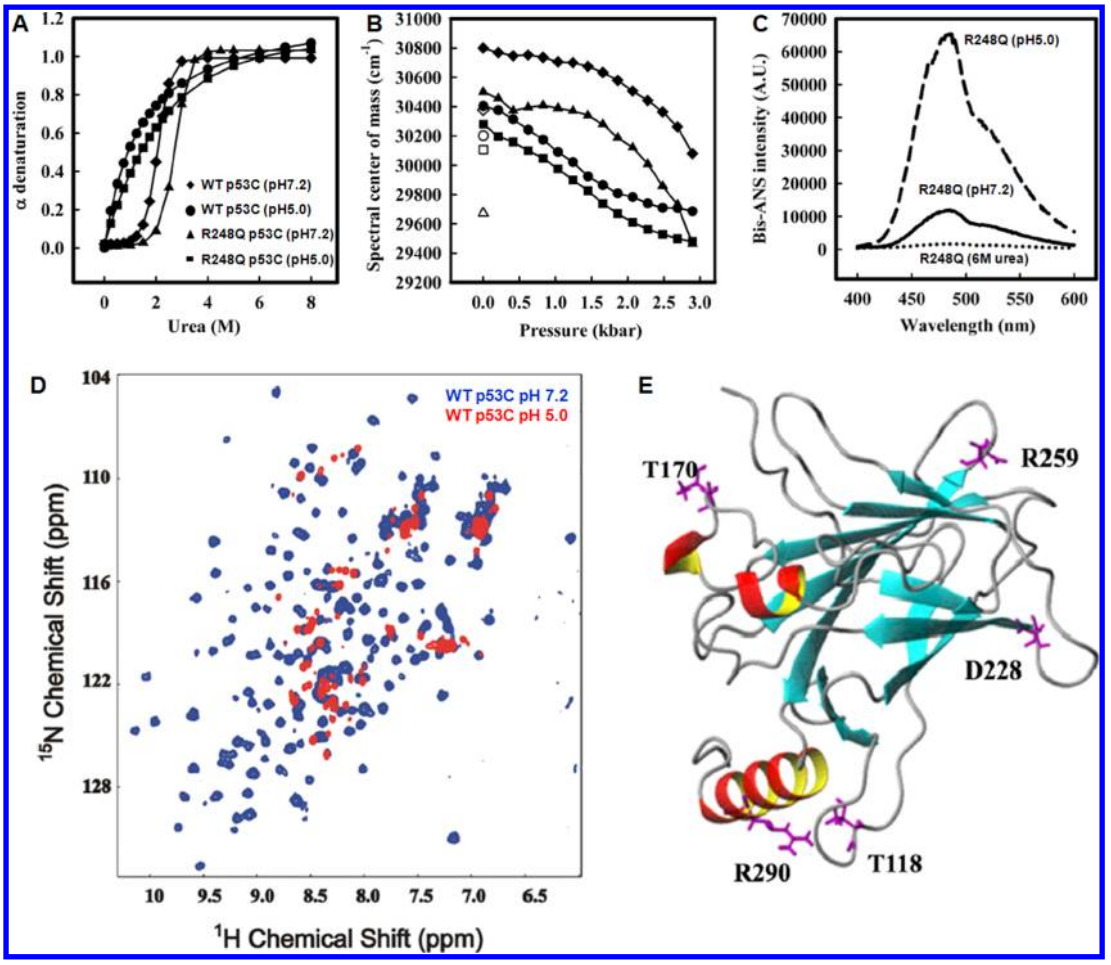

Figure 12. p53C may assume a molten-globule state at low pH. Wild-type and R248Q p53C induced unfolding by urea (A) and high pressure (B). (C) Acidic pH exposes hydrophobic pockets for R248Q p53C mutant as probed by bis-ANS fluorescence. (D) HSQC spectra for wild-type p53C at neutral and acidic pH. (E) Residues that underwent significant chemical shift perturbation at acidic pH are highlighted. See ref 219 for more details. Reprinted with permission from ref 219. Copyright 2010 American Society for Biochemistry and Molecular Biology.

p53 translated from a single mutant allele is associated with the phenomenon of negative dominance, in which wild-type p53 protein translated from the remaining wild-type p53 allele is somewhat affected.[206,208] As we describe more fully below, this finding suggested to us that p53 mutants might act as prions or prionoids.[126,127]

Our group showed that the core domain of p53 forms β-sheet-rich fibrillar aggregates following hydrostatic pressure treatment (Figure 11A).[126,209] On the basis of this result, we hypothesized that p53 aggregation might participate in tumor pathogenesis.[63,126,127] The response of p53C to pressure was different from that found with other aggregating proteins that usually are dissociated by pressure,[58] which can be explained by differences in volume changes between native and aggregated state. Corroborating our finding, other p53 regions have been reported to undergo aggregation under physiological conditions.[210−212] An intermediate oligomer of p53C was also observed during equilibrium and kinetic folding/unfolding transitions.[213] Thus, fibrillogenesis of p53 might contribute to its loss of function and act as seed for the accumulation of conformationally altered proteins in cancerous cells resulting in a negative-dominant effect as well as a gain of function.[63,126,127]

We recently evaluated the amyloid aggregation of wild-type (WT) p53 and the hot-spot mutant R248Q in detail under physiological conditions and determined whether the mutant could seed the aggregation of the wild-type form in a prion-like mode.[127] The central p53C domains of wild-type protein and the R248Q mutant aggregated into a mixture of oligomers and fibrils, and R248Q displayed a much greater tendency to aggregate than WT p53 (Figure 11B). We also characterized the aggregation of full-length p53 into amyloid-like species. The aggregation of p53 was quite heterogeneous and generated a mixture of amyloid oligomers and fibrils. We found that the application of pressure allowed the production of a higher proportion of fibrils. The X-ray diffraction pattern of the fibrillar aggregates induced by hydrostatic pressure was consistent with the characteristic conformation of cross-β-sheet amyloid fibers, with reflections of 4.7 and 10 Å being observed (Figure 11C).[127]

In testing for prion-like behavior, we found that using R248Q p53C amyloid oligomers and fibrils as seed could accelerate the aggregation of WT p53C.[127] Further support for a prion-like effect of mutant p53 was obtained from the examination of breast cancer biopsy samples.[127,214] We found that the R248Q mutant colocalized with amyloid-like species in these samples. In another experiment, a tumor cell line containing mutant p53 displayed massive aggregation of p53 in the nucleus. We conclude that aggregation of p53 into a mixture of oligomers and fibrils sequesters the native protein into an inactive conformation that is typical of a prionoid. The prion-like

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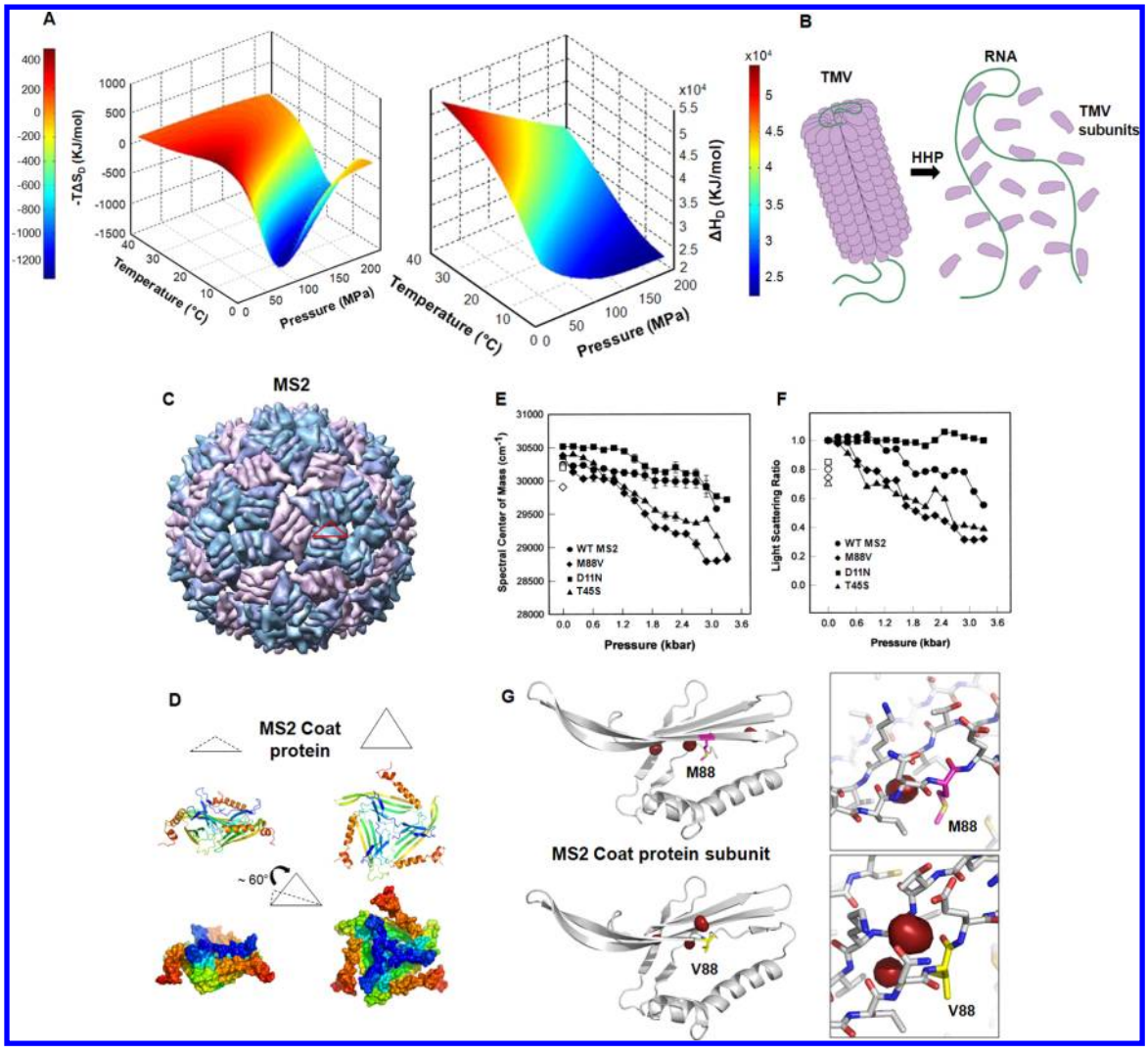

Figure 13. High-pressure studies on viruses particles. (A) Surface plots of the $p-T$ dependence of changes in entropy (left) and enthalpy (right) during Tobaccos' mosaic virus (TMV) dissociation. (B) Schematic showing TMV dissociation upon high pressure. (C) Biological assembly of bacteriophage MS2 coat protein. (D) Structural architecture of MS2 coat protein (PDB code: 2MS2). (E) Fluorescence spectral center of mass and (F) light scattering evaluating pressure stability of MS2 coat protein and mutants. (G) M88V variant leads to cavity increase in MS2 coat protein (PDB code: 2MS2). Cavities are shown as red surface. Part (A) reprinted with permission from ref 225. Copyright 2012 American Chemical Society. Parts (E) and (F): Used with permission from ref 229. Copyright 2004 Wiley.

behavior of oncogenic p53 mutants provides an explanation for their negative dominance effect and indicates that they may serve as a potential target for cancer therapy. In a recent study,[215] amyloid oligomers of p53 were shown to be present in basal cell carcinomas (BCC). In another study,[216] mutant p53 was observed to coaggregate with two of its paralogues, p63 and p73.

Additionally, we found that interaction with a cognate DNA sequence stabilizes p53 against the effects of pressure and prevents aggregation of the protein into an amyloid-like structure (Figure 11D–F).[217] Sequence-specific DNA also stabilized full-length p53. The effects of cognate DNA could be simulated by high concentrations of osmolytes, implying that the observed stabilization is caused by water exclusion. We propose that aptameric nucleic acids can be employed as a therapeutic approach for preventing the occurrence of misfolded species of p53 and treating malignant tumors.[217] The search for molecules that may inhibit aggregation has also been conducted by Fersht and co-workers.[218]

Pressure treatment of p53 allowed us to characterize a molten-globule conformation of the protein observed at acidic pH.[219] The molten-globule conformation showed decreased stability in response to pressure and lacked cooperativity under urea denaturation (Figure 12A and B). At pH 7.2, WT p53C was more resistant to pressure than the mutant, whereas at pH 5.0, the molten-globule conformations of both WT and R248Q (pH 5.0) were much less stable than the native conformations and showed much smaller volume changes (Figure 12A–C).[219] The smaller volume confirms that the protein is loosely packed, with many fewer water-excluded cavities being observed. However, pressure denaturation of the molten-globule conformation was considerably more reversible than that of native p53C (neutral pH). Another interesting finding was that at pH 5.0, the observed difference in stability was much smaller and the reversibility much greater (Figure 12A and B). HSQC NMR spectroscopy confirmed that the protein exhibits a typical molten-globule structure at acidic pH (Figure 12D and E).[219] Human breast cells (MCF-7) transfected with p53-GFP in culture also showed localization of p53 to acidic vesicles,

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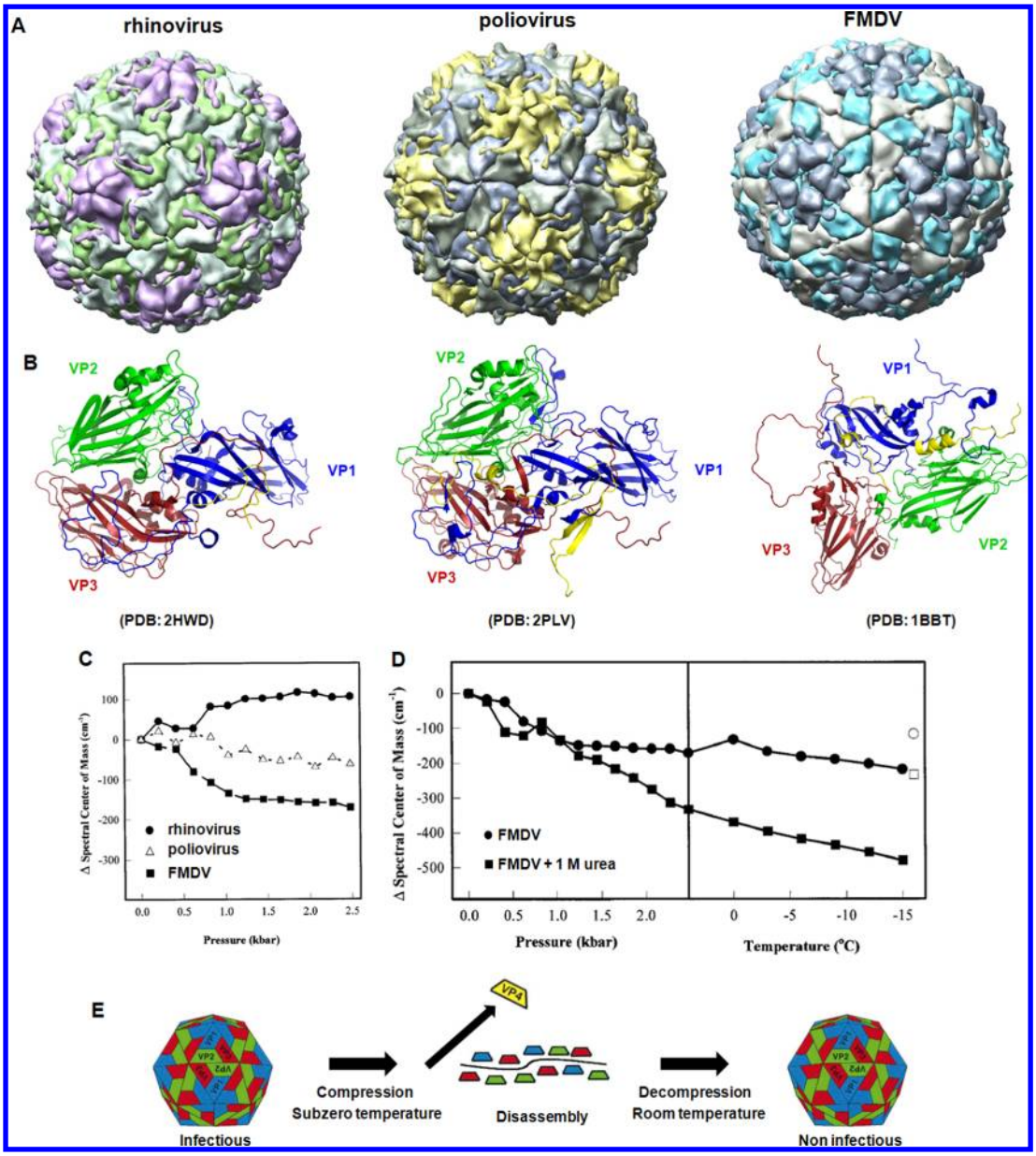

Figure 14. (A) Biological assemblies for rhino (left), polio (middle), and FMD (right) viruses. (B) Structural architecture for the trimeric unit (VP1, VP2, and VP3) from the corresponding particles as shown in (A). (C) Pressure stability of picornaviruses monitored by the center of spectral mass from Trp fluorescence emission. (D) Pressure and cold stability for FMDV monitored as in (C). (E) Proposed model for picornaviruses disassembly by pressure and low temperature. Parts (B)−(E) reprinted with permission from ref 226. Copyright 1999 Elsevier.

suggesting that the low-pH conformation is present in the cells. Additionally, low-pH stress tends to favor high levels of p53 in the cells. More recently, Park et al.[220] reported that the molten-globule conformation of p53 is the client protein of the Hsp90 chaperone protein. Pressure-assisted cold denaturation of p53C also reveals the complex folding landscape of this protein.[209] When wild-type p53C was treated with the combination of high pressure and subzero temperature, the protein denatured without aggregation. However, when the conditions were reversed to room temperature and atmospheric pressure, the protein adopted an alternative conformation similar to that exhibited by the hot-spot oncogenic mutant R248Q.[209]

6. EFFECTS OF PRESSURE ON VIRUSES

6.1. Virus Particles as Compressible Nanostructures

Viruses are excellent examples of nanomachines. A virus particle is composed of a membrane-enveloped or non-enveloped protein shell and nucleic acid. To package an infectious viral genome, integral multiples of 60 subunits are required to form the icosahedral protein shell, resulting in nonidentical contacts between subunits.[221] It is clear that assembly involves stringent packing constraints, which makes virus particles ideal macromolecular assembly models for study under high pressure. As described in previous sections, high pressure is very effective in dissociating large assemblies such as microtubules, amyloid aggregates, amorphous aggregates, and inclusion bodies.[10,15,23,41,42,113,155] Because viral structure is highly dependent on protein−protein interactions, hydrostatic

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pressure has been a valuable tool for assessing viral structure−function relationships.[10,15,122]

In general, monomeric or dimeric species of capsid coat proteins are much more sensitive to pressure than the assembled icosahedral particles.[10,103,123,222] In addition, empty capsids are usually much less stable than viral ribonucleoprotein particles.[10,15] Similar to amyloids, the assembly of virus particles involves the formation of water-excluded cavities. Viruses with a helical structure are also destabilized by high pressure.[223−225] For example, dissociation of tobacco mosaic virus (TMV) occurs when subjected to hydrostatic pressure, as first reported by Lauffer and Dow.[223] Several types of virus particles also undergo cold denaturation under pressure, indicating that assembly can be driven by entropy.[35,36,103,104,224−226] In a thermodynamic analysis of TMV dissociation, Bispo and co-workers showed that although enthalpy predominated during dissociation, this characteristic was not necessarily uniform because it depended on the experimental conditions to which the protein was exposed, with possible switching from an enthalpy-driven to an entropy-driven process being observed (Figure 13A and B).[225]

In a number of icosahedral viruses, ribonucleoprotein intermediates have been detected when their particles are subjected to pressure.[10,15,35,123,124,226−228] When the pressure is released, the ribonucleoprotein intermediate acts as a scaffold for the regeneration of the particle.[10,15,227,228] In the absence of RNA, the subunits tend to drift toward a disorganized structure and do not renature when the perturbation is withdrawn. A common finding in pressure-mediated analyses of viruses is a progressive decrease in the folding structure when moving from assembled capsids to ribonucleoprotein intermediates (in the case of RNA viruses), free dissociated units (dimers or monomers), and, finally, denatured monomers.[10,15,227,228]

Bacteriophage MS2 consists of 180 copies of a coat protein arranged in a T = 3 quasi-equivalent surface lattice surrounding the ssRNA genome (Figure 13C and D). It has provided a convenient model for pressure-treatment studies of protein−protein and protein−nucleic acid interactions. Utilizing this model, we have studied how the packing and stability of virus capsids are affected by single amino acid substitutions in the coat protein.[229] M88V and T45S mutant particles were found to be much less stable than their wild-type forms and completely dissociated at 3.0 kbar of pressure (Figure 13E and F). The lower stability of M88V particles could be correlated with an increase in the size of the cavity of the hydrophobic core (Figure 13G).[229] The infectious titer of the virus particles was recovered after high-pressure treatment. Thus, following pressure-induced dissociation of the virus, information allowing correct reassembly was preserved. In contrast to M88V and T45S, the D11N mutant virus particle was more stable than the wild-type virus, despite also possessing a temperature-sensitive growth phenotype.

The stability of native bacteriophage MS2 was also compared to that of virus-like particles (VLPs) containing nonviral RNAs as well as an assembly defective coat protein mutant (dlFG) and its single-chain variant (sc-dlFG).[222] The VLPs were observed to be more stable than the virus particles, indicating the possible presence of additional contacts between coat protein subunits and heterologous RNA. Although the dlFG mutant dimerizes correctly, it fails to assemble into capsid particles because of the absence of the FG loop (15 aa residues), which is crucial for interdimer interactions along the viral 5-fold and quasi-6-fold axes. The dlFG mutant was less stable than both the VLPs and wild-type virus particles, most likely due to the absence of the interdimer protein−protein interactions that normally occur in the capsid. In contrast, the single-chain dimer, sc-dlFG, was stabilized against pressure. The presence of a 34-base-pair poly(GC) DNA sequence also resulted in pressure stabilization. These studies provide information regarding the mechanisms that can ensure that protein−protein interactions in the capsid lattice are sufficiently stable and specific to ensure assembly, while at the same time allowing the structural changes necessary for nucleic acid release during infection.

In another model system, the DNA bacteriophage p22, we found that the monomeric capsid protein was highly sensitive to pressure.[103] In contrast, the assembled T = 7 procapsid was highly resistant to pressure dissociation and denaturation. P22 procapsid shells dissociated only at high pressures and low temperatures, demonstrating that they are stabilized by entropy.[103] Several single amino acid substitution mutants were utilized in these analyses. W48Q mutant shells were found to be easily dissociated by pressure at room temperature, showing little dependence on reduced temperature, suggesting a smaller entropic contribution. In contrast, lower stabilities of the G232D and T294I mutants were associated with defective protein cavities.[35,36]

6.2. Pressure Effects on Insect and Mammalian Viruses

Extensive pressure manipulation studies have been performed on viruses of the family *Picornaviridae*, which is a group that is of great economic and medical relevance.[230] These viruses display a common capsid structure consisting of 60 copies of four different proteins, termed VP1−VP4 (Figure 14A and B). One remarkable feature of picornaviruses is that despite the structural homology of their capsid proteins, they exhibit a wide range of stabilities in response to pressure and cold denaturation.[226] Both poliovirus and rhinovirus are stable under high pressure at room temperature: pressures of up to 240 MPa do not promote viral disassembly or inactivation in these viruses (Figure 14C). Within the same pressure range, foot-and-mouth disease virus (FMDV) particles are greatly affected by pressure, showing a loss of infectivity of more than 4 log units. The dissociation of polio- and rhinoviruses is observed only under high pressure at low temperatures in the presence of low concentrations of urea (1−2 M) (Figure 14D). Pressure and low temperature data reveal clear differences in stability among these three picornaviruses, with FMDV being the most sensitive, poliovirus the most resistant, and rhinovirus displaying intermediate stability.[226]

The changes observed in picornaviruses following pressure and low temperature treatment are illustrated in Figure 14E. The most important finding of these analyses is that after a pressure cycle, the virus particles reassociate into a non-infectious form, referred to as a P-particle. Pressure-inactivated picornaviruses may resemble the A-particles formed by polio- and rhinoviruses when these agents interact with their host cells. A-particles lack the internal capsid protein VP4.[231] The release of VP4 could explain the inactivation of the virus particles. The A-particle is substantially less infectious than the natural virion and has been considered an intermediate in uncoating. The A-state is similar to the "fusion intermediate state" observed in enveloped viruses.[232] Upon interaction with host cells, the conformations of the viral coat proteins and envelope glycoproteins change. This can potentially lead to the production of noninfectious particles, or it may result in the

exposure of previously occult epitopes that could be important for vaccine development. Some of the irreversible conformational changes observed in viral particles after exposure to high pressure resemble the changes that occur in vivo. These changes are discussed below for most of the viruses we have studied.

Our group recently showed that human rhinovirus type 14 (HRV14) releases VP4 after pressure treatment, as observed via MALDI-TOF mass spectrometry.[233] This observation can explain the pressure-induced inactivation of this virus. Additionally, an antiviral drug, WIN 52084, blocks the pressure-induced release of VP4, a potential indication of the antiviral mechanism of action of this drug.

We have also used high pressure to evaluate the function of maturation cleavage in flock house virus (FHV), a nonenveloped icosahedral insect virus, by comparing WT and cleavage-defective mutant (D75N) FHV virus-like particles (VLPs).[234] Mature FHV particles were observed to be less stable under pressure than cleavage-defective particles, possibly due to the metastability elicited upon particle maturation. We also have evidence that the gamma subunit is released from the viral capsid after a cycle of compression and decompression, similar to the pressure-induced release of picornavirus VP4 (Figure 14E). In both cases, pressure induces the formation of a noninfectious particle, which apparently represents a fusion-active state of these nonenveloped viruses. We also investigated the stability of the authentic FHV particles. VLPs package cellular RNA, and native particles contain viral RNA. Our results, which demonstrate that native particles are more stable under physical and chemical treatments than VLPs, emphasize the specificity of the interaction between the capsid protein and viral RNA.[235]

6.3. Effects of Hydrostatic Pressure on Membrane-Enveloped Viruses – Induction of the Fusogenic State

Enveloped animal viruses generally fuse with cellular membranes to deliver their genomes into host cells.[232] We have found that high pressure inactivates enveloped viruses, including influenza, Sindbis, and VSV, by trapping their particles in a fusion-like intermediate state.[236−238] In the case of influenza and alphaviruses, we showed that hydrostatic pressure triggers a conformational change in the viral glycoprotein at neutral pH that is very similar to the change triggered by the low pH within endosomes.[237] Hydrostatic pressure converts the conformation of the metastable, native state, which has a larger volume, into the fusogenic state, which is characterized by a smaller volume. As discussed in section 7.1, the application of pressure to populate fusion-active states can be exploited in the development of new antiviral vaccines and drugs.

The pressure-induced formation of noninfective particles has been demonstrated in many viruses, including rotaviruses,[239] infectious bursal disease virus (IBDV),[240] vesicular stomatitis virus (VSV),[236,238] simian immunodeficiency virus,[241] HIV,[242] influenza virus,[237,243] lambda phage,[244] alphaviruses,[37,237] and picornaviruses.[226,245] In IBDV, high pressure results in the elimination of infectivity, while the virions retain their original immunogenic properties and could elicit high titers of virus-neutralizing antibodies.[240] In the case of VSV, a membrane-enveloped virus, application of 250 MPa pressure for a sufficient amount of time abolishes infectivity.[236,238] Electron microscopy of the compressed samples shows no detectable dissociation, but the subunits appear to be displaced from their normal positions by the pressure, as indicated by bulges under the viral membrane.

In membrane-enveloped viruses, pressure treatment causes the membrane glycoproteins to undergo a conformational change that is similar to the receptor-activated conformational change.[237,238] In influenza virus, this change is usually referred to as the "spring-loaded" model, indicating a mechanism in which the fusion peptide region is inserted into the target membrane in an early step of the fusion process.[232] Low pH elicits the spring-loaded mechanism in influenza virus. We observed that subjecting influenza virus to pressure exposes hydrophobic domains.[237] Pressure also produces an increase in the fusion activity of the virus at neutral pH.[237] These data indicate a change in the labile native state of the envelope complex to a more stable one, mimicking the fusion-active conformation.

7. BIOTECHNOLOGICAL APPLICATIONS OF HIGH HYDROSTATIC PRESSURE

In parallel to the advancements in the basic fields of biochemistry, biophysics, and chemical biology, a stunning progression has occurred in the application of high pressure in biotechnology. We detail below the applications in the fields of microbiology for the sterilization of microorganism and viruses, vaccine development, and food processing. We have already discussed, in section 4, the use of high pressure to refold proteins from aggregates, especially inclusion bodies,[155−160] as well as its applications in ligand binding (section 3.6, refs 139−142).

Pressure has also been proposed for the isolation and purification of biomolecules from mixtures, particularly the pressure-assisted isolation and purification of nucleic acid molecules.[246] In another application, the same group has developed a method using pressure to control nucleic acid hybridization.[247]

The combination of high pressure and subzero temperature has also been explored to treat biological samples with the aim of isolating specific biomolecules.[248]

7.1. Pressure Sterilization and Pressure Inactivation of Viruses and Other Microorganisms – A Pascalized Vaccine

Viral inactivation using hydrostatic pressure has been utilized aiming two potential applications: vaccine development and virus sterilization. Immunization is by far the most effective way to prevent infectious diseases in animals and humans. There are three types of immunization approach against virus diseases: using live (attenuated) particles; using killed (inactivated) whole-virion particles; and using subunit vaccines.

Hydrostatic pressure has been suggested as an approach for viral inactivation and vaccine development.[10,232,241−244] Because high pressure does not introduce exogenous substances into a vaccine and is often selective in its action on macromolecular structures, its use frequently results in highly immunogenic preparations.[236,240,245,249] It is also practical for large-scale immunization.

Antibodies raised against pressurized virus particles have been found to be as effective as those raised against intact virus, as measured by their neutralization titers in plaque reduction assays.[236,239,245,250] Pressure induces the formation of inactive rotavirus,[239] which is an important viral agent that causes a form of gastroenteritis that is responsible for millions of cases of fatal diarrhea in children in developing countries. The new

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conformation of the pressurized rotavirus particle does not result in a loss of immunogenicity. Pressure alters the receptor-binding protein VP4 by triggering changes similar to those that occur when the virus interacts with target cells.[239]

More recently, we studied the pressure-induced inactivation of the yellow fever virus and the implications of this process for the development of an inactivated yellow fever vaccine.[250] The successful yellow fever (YF) vaccine consists of live attenuated 17D-204 or 17DD viruses. Despite this vaccine's excellent record of efficacy and safety, serious adverse events have been recorded and have influenced the ability to achieve extensive vaccination in endemic areas. Therefore, alternative strategies, including the use of inactivated whole virus, should be considered. Following this approach, we employed hydrostatic pressure as a method for viral inactivation and vaccine development. The application of 310 MPa pressure for 3 h at 4 °C abolished YF infectivity and eliminated the ability of the virus to cause disease in mice. Although the pressure-inactivated virus did not elicit high neutralizing antibody titers, the immunized mice exhibited complete protection against an otherwise lethal challenge with 17DD virus.[250] These data provide a basis for the possible further development of a pressure-inactivated vaccine against YF.[250]

Influenza virus poses a serious global health threat, particularly in light of newly emerging strains such as avian virus H5N1. Recently, we used H3N8 avian influenza virus that had been inactivated by hydrostatic pressure as a vaccine.[243] Our goal was to assess the immunogenic and protective capacity of the pressurized virus in a Balb/c mouse model. After vaccination, the mice were challenged and monitored for virus-specific antibodies, clinical symptoms, and death. Following immunization, there was an increase in IgG1 and IgG2a levels in the serum of the mice and in IgA levels in nasal lavage specimens. A viral neutralization assay showed that neutralizing antibodies were produced. After challenge, the control group, which was immunized with saline, showed all of the examined clinical signs of disease (weight loss, ruffled fur, lethargy, and huddling), whereas animals that were vaccinated did not develop any clinical signs. The results indicated that the animals presented a satisfactory humoral response after vaccination and were protected against viral challenge.[243]

In a recent study, we evaluated whether fully pressure-inactivated human influenza A virus would confer protection to mice against infection.[251] Our study showed that full viral inactivation by pressure was obtained with an overall preservation of viral structure and fusogenic activity. When the virus was inoculated in mice, a strong protection against infection was correlated with an immune response consisting of serum immunoglobulin IgG1, IgG2a, and serum and mucosal IgA.[251]

High hydrostatic pressure (HHP) has also been used to inactivate leptospires,[252] which are the causative agents of several important zoonotic and human diseases. Pressure treatment abolished the leptospires' infectivity. However, when the pressure-treated leptospires were injected into rabbits, they were highly immunogenic. This preparation has the potential to assist in formulating a vaccine.

High hydrostatic pressure may be more advantageous over other methods to prepare antiviral vaccines. Attenuated live viruses can revert after a period of time and may cause the disease that they are intended to prevent or worsen the real disease. Immunization with isolated subunits is also associated with several problems. A reliable physical method of preparing killed vaccines, such as high pressure, should not have the same drawbacks. The reason pressurized viruses maintain their immunogenic potential most likely resides in the fact that the structural changes that occur under pressure treatment are very subtle. As discussed above, pressure treatment appears to mimic the changes that are produced when viruses bind to their cellular receptors. The substantial evidence that high pressure traps viruses in "fusion intermediate states" that are not infectious but are highly immunogenic is promising for vaccine development.[228,237,250] For example, particles of pressure-inactivated VSV are able to attach to the membrane and are partially internalized, but not by endocytosis.[253] As a result, the other steps in the infection cycle are compromised.

Those viruses that are stable when pressure is applied at room temperature can be inactivated when the temperature is decreased under pressure.[226] Studies examining the effects of pressure are important in determining its potential application not only in the production of antiviral vaccines but also in new methods for sterilizing biological products such as blood, plasma, and their derivatives[244,254] as well for food processing, as discussed below (section 7.3). Another recent application is the use of high pressure (in combination with other conditions) to sterilize medical and pharmaceuticals materials against *Mycobacterium abscessus*, an important pathogen acquired in hospitals.[255]

7.2. High Pressure as a New Method for Vaccine Preservation

In a recent study, we applied HHP with another aim. Three attenuated poliovirus serotypes were compared with regard to pressure and thermal resistance. Interestingly, we found that HHP does not inactivate any of the three serotypes studied.[256,257] Rather, HHP treatment was found to stabilize poliovirus by increasing the thermal resistance of the virus at 37 °C.[256] The trivalent oral polio vaccine (OPV) is a live attenuated viral vaccine that is currently used in several countries for the prevention of poliomyelitis. OPV is heat labile, and thus it must be stored frozen and used immediately after thawing to ensure effective immunization. Most live vaccines are temperature sensitive, and the live attenuated polio vaccine is among the least stable of all common childhood vaccines. Following our proposal of high hydrostatic pressure treatment as an alternative for increasing the thermostability of the three attenuated poliovirus serotypes, HHP is now being considered as a possible stabilizing method, which would circumvent the problems associated with the requirement for refrigeration during storage and transport of the vaccine prior to use.[256,257] In addition to being both extremely secure and inexpensive, this technology can be applied alone or in combination with other methods to guarantee effective stabilization of commercial poliovirus vaccines.[256,257]

7.3. High-Pressure Processed Food

HHP is a nonthermal process that has the ability to inactivate numerous microorganisms, including pathogens, as well as endogenous enzymes, preserving the sensorial characteristics of food products and prolonging their shelf life.[258] HHP is currently being used in the food industry as an alternative to other food processing technologies, especially thermal processing.[253,257]

The first HHP-treated product that appeared on the market was a high-acid jam produced in Japan in the 1990s. A wide range of HHP-treated food products have now been

commercialized, including juices and other fruit and seafood products.[244,253,258]

Viruses are responsible for approximately 70% of foodborne illnesses worldwide.[259] Although innumerable enteric viruses can be transmitted orally, the two principal foodborne threats are currently human norovirus (HuNoV) and hepatitis A virus (HAV). The challenge regarding the inactivation of these viruses arises from the fact that their viral particles are very stable; they are resistant to low pH, detergents, and organic solvents.[260] HHP shows enormous potential as a method to inactivate these foodborne viruses.

HuNoVs cause the majority of foodborne illnesses in the U.S. and are responsible for 11% and 25% of deaths and hospitalizations, respectively, due to such illnesses.[261] The primary symptoms of HuNoV infection are diarrhea and vomiting. The most important source of infectious HuNoV is fecal contamination of food or water. Direct assessment of human norovirus viability requires the use of human volunteers. Initial work to assess the feasibility of inactivating HuNoV was therefore performed using the surrogate viruses feline calicivirus (FCV) and murine norovirus (MNV). Given that HuNoV is the most common foodborne etiologic agent and that both MNV and FCV were found to be pressure sensitive,[260,262,263] direct testing of pressure-induced inactivation was highly desirable. Consequently, a study in human volunteers was performed using pressure-treated HuNoV-contaminated oysters.[264] In this study, the authors concluded that pressures of at least 400 MPa or higher were required to make human norovirus-contaminated shellfish safe for human consumption.[263]

A key concern is that fresh food products often carry a high risk of norovirus contamination because they undergo no processing, as there is currently no effective method to eliminate the virus from fresh products. Lou and colleagues investigated the efficiency of high-pressure processing (HPP) for inactivating murine norovirus (MNV-1), one of the substitutes for human norovirus, in aqueous medium and fresh products.[259] They demonstrated that MNV-1 was effectively inactivated by HPP.

To determine the possibility of MNV inactivation within shellfish, live oysters were contaminated in a large flow-through oyster tank that permitted simulated natural bioaccumulation of MNV to levels of approximately 6 $\log_{10}$ per oyster. Treatment for 5 min at 400 MPa and 5 °C was sufficient to inactivate 4 $\log_{10}$ MNVs.[263] Subsequent experiments showed that high-pressure inactivation of MNV could be confirmed to an equal extent by in vivo infection of mice and in vitro cell culture.[265] Inactivation of MNV present in clams was recently demonstrated by Arcangeli and co-workers.[266] Furthermore, Kovač et al.[267] recently reported a 2.63 $\log_{10}$ reduction of active MNV in strawberry puree after 5 min of treatment at 300 MPa and complete inactivation of MNV after a 5 min treatment at 400 MPa.

With the advent of a specific vaccine, hepatitis A virus (HAV) is becoming increasingly rare in the developed world,[268] although it remains endemic in the developing world. HAV exhibits age-associated virulence. The complete inactivation of a 7 $\log_{10}$ HAV stock was observed at 460 MPa.[262] Grove et al.[269] subsequently evaluated HAV and reported reductions of >1 $\log_{10}$, >2 $\log_{10}$, and >3.5 $\log_{10}$ TCID50 after 10 min of treatment with pressures of 300, 400, and 500 MPa, respectively. Belonging to the family *Picornaviridae*, HAV seems to be as susceptible to pressure as foot-and-mouth disease virus.[226,245] Subsequent studies have sought to characterize the potential to inactivate foodborne HAV viruses in foods such as oysters, green onions, and strawberry puree.[260]

In conclusion, HHP is able to inactivate both HuNoV and HAV.[260] However, given the complexities of food matrixes and the variable responses of viruses to HHP, validation of the HP conditions within the food or food matrix being produced is required. High-pressure processing is a valuable tool that represents a promising intervention for inactivating and eliminating foodborne viruses from food products with minimal or no impact on food quality.

8. CONCLUSIONS AND PERSPECTIVES

The increasing use of high hydrostatic pressure in chemical biology has marked this approach as one of the most promising new tools available for addressing basic problems such as protein folding and recognition as well as for the development of new applications in biotechnology and medicine. The application of pressure has allowed us to characterize the high degree of plasticity of proteins, their folding landscape, their interaction with ligands, and how proteins assemble into supramolecular structures. In addition to equilibrium approaches, detailed studies of the kinetics of the pressure-induced unfolding and folding of proteins, together with the development of several theoretical approaches, have increased our understanding of the effects of pressure on protein conformation. The use of structural tools with atomic resolution, such as X-ray diffraction and NMR,[30,87] promises to bring new insight into how energy and volume constraints are minimized in the protein folding landscape. Furthermore, the isolation of folding intermediates, which is crucial for understanding protein misfolding and aggregation, has been made possible through pressure-treatment methodology. The ability to populate an amyloidogenic intermediate protein conformation, without proceeding to aggregation, is a unique property of pressure treatment that has provided the possibility of characterizing the structural properties of amyloidogenic forms of proteins. Most protein misfolding diseases have only palliative treatments, and the use of pressure technology to study errors in protein folding and aggregation may reveal new structural targets for drug development.

The reduced stability of virus particles at high pressure and low temperature suggests the potential application of pressure techniques to inactivate viruses as well as prions. We have found that a number of viruses can be inactivated by pressure at room temperature. The substantial evidence that high pressure traps viruses in "fusion intermediate states" that are not infectious but are highly immunogenic is very promising for vaccine development. The pressure-induced population of fusion-active states can be utilized in the development of new antiviral vaccines and drugs. These studies of the effects of pressure on viruses are important not only for indicating the usefulness of pressure as a potential tool for the production of antiviral vaccines but also because they suggest novel ways to sterilize biological products, such as blood and plasma derivatives. In addition, the new approach of using high pressure to stabilize human vaccines provides new opportunities and possibilities for disease prevention. In addition, high-pressure processing (HPP) is an increasingly popular nonthermal food processing technology. In recent years, several groups have demonstrated the potential of HPP to inactivate different foodborne viruses, representing a great advance for the food industry.

Currently, the use of pressure technology is flourishing. This period is reminiscent of the novel *Flatland* from 19th century writer Edwin Abbott Abbott[270] about a visit from an inhabitant of a three-dimensional world to a world of two dimensions. Pressure technology provides a new dimension with which to illuminate the dynamics and structure of biological molecules.

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Notes

The authors declare no competing financial interest.

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Andrea Cheble de Oliveira was born in Rio de Janeiro, Brazil. She received her Ph.D. degree in Biological Chemistry from the Federal University of Rio de Janeiro (UFRJ) in 1998 under the supervision of Professor Jerson L. Silva. She works at the Gregorio Weber Laboratory of Thermodynamics of Proteins and Viruses at the UFRJ. In 2001, she obtained a position as Associate Professor at the Medical Biochemistry Institute at the same university, and from 2004 to 2006 she joined the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana−Champaign as a research assistant. Dr. Oliveira's research interests are focused on the area of the structural biology of viruses, virus−cell interaction, and protein−protein, protein−lipid, and protein−nucleic acid interactions. Her current projects involve the use of fluorescence spectroscopy, hydrostatic pressure, and two-photon microscopy, including single particle tracking and fluorescence correlation spectroscopy (FCS), for the study of viral particle inactivation, assembly, and infection of cells.

Tuane Vieira graduated in Biology from the Federal University of Rio de Janeiro (UFRJ) in 2004. She received her Master degree in 2005 and Ph.D degree in 2009, both in Biological Chemistry at UFRJ. From 2000 to 2005 her research was on the area of glycobiology, and from 2005 to now she has been devoting her research to structure biology, focusing on biological systems of medical importance. Nowadays she is a postdoctoral fellow in the laboratory of Professor Jerson Silva at UFRJ working on prion biology, investigating PrP−polysaccharide interactions and its importance for prion conversion, at the molecular level and systems biology level.

Guilherme A. P. de Oliveira was born in 1985 in Rio de Janeiro, Brazil, and graduated in Biomedicine at the Federal University of Rio de Janeiro in 2008. He received his M.S. degree in 2009 and his Ph.D. in 2013, both in Biological Chemistry at the Medical Biochemistry Institute, same University, under the supervision of Prof. Jerson Lima Silva. Currently, he is a post-doc at the same Institute. His research is devoted to applying the synergism among different structural approaches that include fluorescence spectroscopy, circular dichroism, high-pressure nuclear magnetic resonance, and small-angle X-ray scattering using synchrotron radiation to understand biologic systems involved in cancer and muscle contraction.

Marisa Carvalho Suarez graduated in Dentistry, Federal University of Rio de Janeiro (1992), M.Sc. (1995), and Ph.D. (2001) in Biological Chemistry from the Federal University of Rio de Janeiro (UFRJ), under the supervision of Professor Jerson Lima Silva (M.Sc. degree) and Professor Débora Foguel (Ph.D. degree). From 2006–2008, she worked as professor at the West State University (UEZO). She is currently Associate Professor of the Medical Biochemistry Institute – Polo Xerém at UFRJ and also works in the laboratory of Professor Débora Foguel. She has experience in the area of Biochemistry and studies the process of aggregation of proteins such as transthyretin (TTR) and bovine serum albumin.

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