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New Insights into the Mechanisms of Protein Misfolding and Aggregation in Amyloidogenic Diseases Derived from Pressure Studies†

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ABSTRACT: Hydrostatic pressure is a robust tool for studying the thermodynamics of protein folding and protein interactions, as well as the dynamics and structure of folding intermediates. One of the main innovations obtained from using high pressure is the stabilization of folding intermediates such as molten-globule conformations, thus providing a unique opportunity for characterizing their structure and dynamics. Equally important is the prospect of understanding protein misfolding diseases by using pressure to populate partially folded intermediates at the junction between productive and off-pathway folding, which may give rise to misfolded proteins, aggregates, and amyloids. High hydrostatic pressure (HHP) has also been used to dissociate nonamyloid aggregates and inclusion bodies. In many proteins, the competition between correct folding and misfolding can lead to formation of insoluble aggregates, an important problem for the biotechnology industry and for human pathologies such as amyloidosis, Alzheimer's, Parkinson's, prion's, and tumor diseases. The diversity of diseases that result from protein misfolding has made this theme an important research focus for pharmaceutical and biotechnology companies. The use of high-pressure promises to contribute to the identification of the mechanisms behind these defects and creation of therapies against these diseases.

Although protein folding is a highly efficient process, sometimes it can go awry. Protein misfolding has been implicated in a large number of diseases, which are now grouped under the name of protein folding disorders (PFDs)[1] (*1*, *2*). In principle, protein folding diseases can be divided into two groups. In the group of diseases known as amyloidoses, large quantities of wrongly folded proteins undergo aggregation, destroying brain cells and other tissues. Such disorders include Alzheimer's disease, Parkinson's disease, transmissible spongiform encephalopathies, familial amyloid polyneuropathy, Huntington's disease, and type II diabetes, among other well-known diseases (*1*, *2*). In the

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[1] Abbreviations: HHP, high hydrostatic pressure; PFD, protein folding disorders; IB, inclusion bodies; TTR, transthyretin; p53C, core domain of tumor suppressor protein p53; α-syn, α-synuclein.

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other group, a small genetic error, usually affecting a single amino acid residue, leads to a misfolded conformation, which either affects its function or makes it extremely susceptible to cellular proteases. These latter diseases include cystic fibrosis, inherited emphysema, and several types of cancer. In some of these diseases, both aggregation and a lack of function may contribute to its pathogenesis. For more than 50% of human cancers, mutations in the p53 protein usually affect DNA binding or the protein stability (*3*). In the case of prions, the outstanding additional property is that they are infectious; they cause transmissible spongiform encephalopathies, such as the mad cow disease and its human counterpart, new-variant Creutzfeldt-Jakob disease (*2*, *4*).

A novel approach to the *in vitro* studies of protein misfolding is the use of high hydrostatic pressure at equilibrium or in kinetic experiments (*5*−*10*). High pressure perturbs the three-dimensional organization of the protein structure based on the volume changes associated with the differential solvation of the multiple conformational states of the energy landscape of proteins (*11*−*13*). This perturbation is rather "gentle" and generally leads to the population of intermediates that are not significantly accessed under more drastic conditions. The use of several pressure approaches in the past few years has allowed biochemists and biophysicists to dissect the role of packing and cavities in the generation of aggregates and amyloids. These studies have shed light on the understanding of the protein folding disorders. A wide spectrum of analyses has been performed on protein misfolding and aggregation, including studies on the amyloidogenic protein transthyretin (*5*, *6*, *14*), prions (*15*−*17*), ataxin (*7*), α-synuclein (*10*), the tumor suppressor protein p53 (*9*, *18*), myoglobin (*19*, *20*), tailspike protein (*21*, *22*), recombinant human IFN-γ (*23*), rhodanese (*24*), and insulin (*25*). For all these cases, pressure reveals details of the folding process for the native protein as well for the assembly of aggregates. The general trend is that amyloid aggregates are sensitive to pressure and that the susceptibility to pressure depends on the susceptibility to infiltration of water. The diagram in Figure 1 shows how misfolded protein and aggregates are formed from intermediates and how pressure may affect the balance between the different conformations. Because pressure shifts the equilibrium toward the species with smaller volumes, it has the potential to dissociate aggregates. It can be clearly deduced that similar forces present in the native state maintain amyloid and misfolded conformations, especially the formation of water-excluded cavities.

Pressure Affects the Distribution of Cavities and Packing in Proteins

The folded structure of a protein is highly dependent on the solvation and distribution of water-excluded cavities. Both features can be explored by using hydrostatic pressure (*13*). The pressure derivative of the Gibbs free energy change between two states is equal to their difference in volume. Therefore, the application of pressure can shift the equilibrium to protein conformers ranging from slightly altered structures to partially or completely unfolded conformations (*26*). These states are more solvated than the native state, and thus, osmolytes tend to counteract the effects of pressure (*27*). The specificity and stability of each individual structure reside in the packing characteristics of each protein (*13*, *28*).

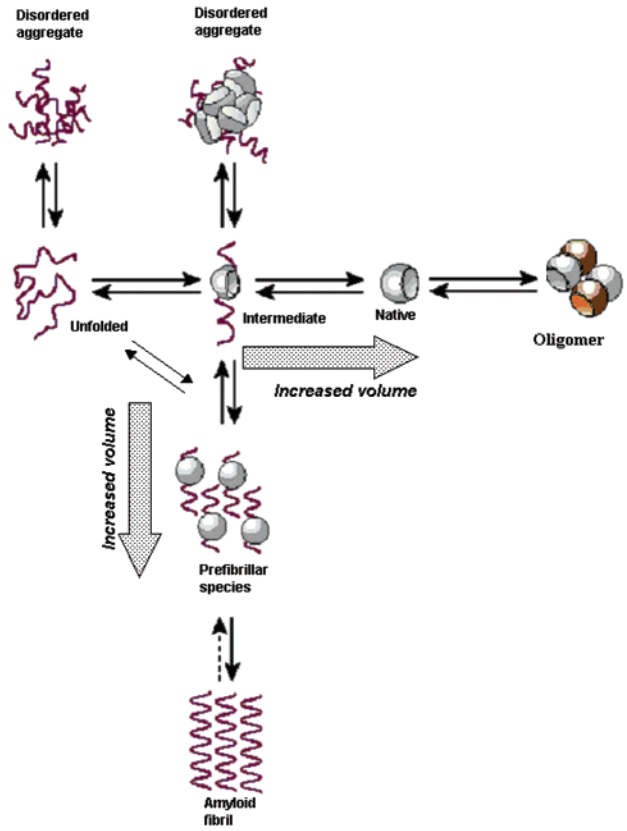

FIGURE 1: Protein folding and misfolding pathways. The diagram shows how amyloid fibrils and other aggregates can be formed from totally or partially unfolded intermediates. As pointed out by Dobson (*49*), other assemblies such as oligomers, containing natively folded molecules, are usually in the most favorable species. As indicated by the large arrows, the native states and aggregates (especially amyloid fibrils) share the property of having water-excluded cavities, having larger volumes, which makes their structures highly susceptible to pressure. Modified from ref *49*.

The decrease in the volume of proteins upon partial or complete disruption of the native structure can be explained by hydration of exposed nonpolar amino acid residues, electrostriction of exposed charges, and the loss of free volume arising from packing defects in the completely folded structure. Cold denaturation of proteins can also be assisted by high pressure because it lowers the freezing point of water (*29*, *30*). Both experimental (*27*) and theoretical (*31*, *32*) approaches have provided a convincing explanation for why pressure induces the disruption of the hydrophobic core, leading to partially or fully unfolded states that are more hydrated and with smaller volumes than the native state (Figure 1). Usually the unfolded and intermediate states experience no significant changes in volume (*13*). Several studies on the effects of pressure on protein structure have utilized high-resolution multidimensional NMR (*33*, *34*) and X-ray crystallography (*35*). High-pressure NMR studies have revealed that considerable secondary structure remains in the pressure-denatured states (*30*, *33*, *36*, *37*). These studies have also shown that the intermediate states have considerably smaller volumes than the native states (Figure 1).

According to Figure 1, both folded and aggregated states would exhibit larger specific volumes because of the formation of water-excluded cavities. Therefore, pressure would dissociate aggregates very easily, as will be exemplified in several cases described below. Early aggregates and protofibrils

may be more easily dissociated by pressure than the mature fibrils. Although fibrils may be less hydrated (with more cavities) than early aggregates, they are more stable which may require much higher pressures. On the other hand, pressure may promote the formation of intermediates that are prone to aggregation.

Recovery of Folded Protein from Amorphous Aggregates and Inclusion Bodies (IB)

The use of high hydrostatic pressure (HHP) as a tool for dissociating large protein aggregates such as inclusion bodies (IB) is a recent development (*21*, *22*). An earlier effort to apply hydrostatic pressure to recover folded protein from a heterologous expression system was described by the group of Hager (*38*). They reconstituted recombinant chloroperoxidase from the denatured apochloroperoxidase by a high-pressure treatment (207 MPa) at −12 °C. The pressure-assisted preparation of holochloroperoxidase exhibited a much higher content of native-like secondary structure than the nonpressurized preparations.

In the past two decades, hydrostatic pressure has been widely utilized to dissociate highly structured protein assemblages such as virus particles (*39*). The observed effects vary from small perturbations in the conformation of the envelope proteins to complete disassembly of the virus capsid (*40*−*43*). In all these studies, highly specific protein−protein and protein−nucleic acid interactions are the targets for the high-pressure perturbation. The same interactions that hold a protein in its native state are also responsible for the assembly of oligomeric proteins, virus particles, and large aggregates. In collaboration with A. S. and C. R. Robinson, we reasoned that pressure could be used as a new and cheap methodology to improve the yield of recovery of native proteins from IB (*21*). Using tailspike protein from P22, we observed that when previously formed aggregates of this protein were subjected to a pressure of 2.4 kbar for 90 min in the absence of any cosolvent, there was more than 50% dissociation of the aggregates by HHP. Strikingly, the rescued protein was the trimer with native properties since it was able to attach to P22 particles and render them infectious (*21*). The tailspike trimer by itself is resistant to pressure, which contributed to the success of recovery of the native protein. More recently, Lefebvre and Robinson (*22*) extended the studies on the tailspike system. They compared the simultaneous on- and off-pathway behavior following dilution of freshly denatured P22 tailspike protein and clearly showed that, under high pressure, a structured intermediate is formed in a rapid manner from a pressure-sensitive aggregate population.

Gorovits and Horowitz (*24*) previously demonstrated that HHP could increase the yield of protein folding by inhibiting nonspecific interactions responsible for the aggregation of rhodanese. Aggregates of rhodanese induced by 3.9 M urea could be dissociated by pressure (2 kbar). The authors found that when the refolding was performed at 2 kbar, the yield of the folded, active enzyme was ∼25%, a value significantly higher than the value of 5% observed when the refolding was performed at atmospheric pressure. More recently, this group extended their studies with rhodanese (*44*) and found that the gain in activity was maximal (56% in the presence of 4 M glycerol) only when fresh aggregates (formed 5−15 min after the onset of the refolding) were compressed. Activity recovered from aggregates that spent 65 min under refolding conditions was much lower (32%). This suggests the aggregates can change with time, giving rise to functional proteins that cannot be recovered by HHP.

St. John and co-workers (*45*) also used pressure to dissociate large aggregates. They combined pressure in the range of 1−2 kbar with low, nondenaturing concentrations of guanidine hydrochloride (GdmHCl) to dissociate aggregates of lysozyme and of recombinant human growth hormone (rhGH) as well as IB of β-lactamase. Compared with the samples of lysozyme that remained at atmospheric pressure, the fraction of soluble protein in the samples subjected to HHP was up to 100% greater, with a 70% recovery of catalytic activity. This result is very promising since it suggests that the four disulfide bridges present in lysozyme were remade, rendering the protein active.

The successful dissociation of IB and large aggregates by HHP is a valuable result for biotechnology purposes (*46*−*48*). In this context, we note that HP equipment has been adapted for industrial purposes for the preparation of pressure-processed foods and for the experimental production of pressure-inactivated viruses for vaccines (*39*, *43*).

Pressure Studies on Proteins Involved in Amyloidogenic Diseases

Protein aggregation is a puzzle in several human diseases (*1*, *2*, *49*−*51*). Several soluble cellular proteins undergo fibrillogenenesis under special conditions, and the accumulation of fibrils in specific organs and tissues can ultimately lead to death. We have studied the folding and aggregation of transthyretin (TTR). Wild-type TTR is responsible for senile systemic amyloidosis (SSA), a disease that affects 25% of people who are more than 80 years old, and is characterized by heavy amyloid deposits in the heart (*50*, *52*). On the other hand, more than 80 point mutants of TTR have been described, most of them involved in familial amyloidotic polyneuropathy (FAP). In general, FAP patients present the first symptoms by the second or third decade with peripheral neuropathy, cardiomyopathy, carpal tunnel syndrome, and vitreous opacities (*52*). The hallmark of these diseases is the accumulation of insoluble protein fibrils made up of TTR. This protein is a tetramer consisting of 127 amino acid β-sheet subunits that circulates in plasma and is known to bind retinol binding protein and thyroxine (*30*, *52*, *53*).

TTR fibril formation is thought to depend on tetramer dissociation (*50*, *54*, *55*). Pathological mutations have been shown to decrease tetramer stability, and fibril formation can be inhibited by molecules that bind to and stabilize the TTR tetramer (*55*). Treatment of TTR by high pressure leads to partially unfolded monomers, and when the pressure is released, one obtains altered tetramers with weaker subunit interactions and with amyloidogenic properties (*5*, *6*). These altered monomers and tetramers were studied using fluorescent dyes at equilibrium. At 3.0 kbar, the tryptophan emission of TTR exhibited a large red shift, indicating substantial denaturation, but the protein could still bind bis-ANS, suggesting persistence of some tertiary contacts. The most surprising result from these studies was the observation of fibril formation immediately after pressure release under conditions that are close to physiological conditions (Figure

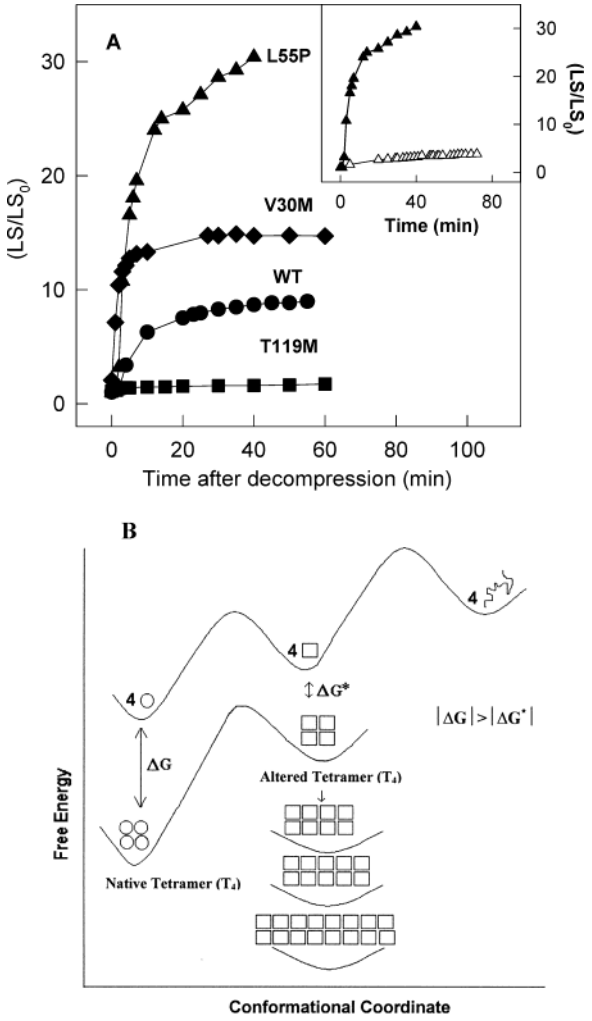

FIGURE 2: Amyloid fibrils formed after decompression and their energetics. (A) Measurement of light scattering after decompression (from 3 kbar) of wild-type transthyretin (●) and the different variants, T119M, V30M, and L55P. In the inset, L55P was incubated at pH 5.6 and atmospheric pressure and LS was recorded (△). For comparison, the curve obtained for L55P after the release of pressure is presented (▲). (B) Free energy diagram for dissociation, denaturation, and aggregation. The native tetramer and the monomer are represented with circles and the altered tetramer, the monomer, and aggregates with squares, and the denatured, unfolded monomer is represented with a crooked line. Modified from refs *5* and *6*.

2A). The oligomeric state of the protein that gives rise to these fibrils is a "loose" tetramer in equilibrium with a small fraction of monomers, as visualized by gel filtration chromatography (*5*).

Several studies had shown before that the tetramers must dissociate and be denatured into a partially native monomer (M*) before fibril formation (*54*, *55*). The pressure data indicated that there is an altered tetramer, called T_4* (preaggregated state), in equilibrium with monomeric species, that is also able to aggregate into fibrils (Figure 2B). The T_4* state appears to be physiologically relevant either because it seeds aggregation or because it acts as a reservoir of altered monomers that aggregate readily (Figure 2B). Recently, we have expanded our studies with TTR by comparing the thermodynamic stability of wild-type (wt) TTR with that of the aggressive amyloidogenic mutants (L55P and V30M) and the nonamyloidogenic mutant T119M (*6*). Our data show that there is an inverse correlation between the extent of amyloidogenesis and thermodynamic stability, where L55P is the least stable variant and has the highest propensity for fibrillogenesis (*6*).

Another advantage of the use of HHP is in drug screening. Since aggregation of TTR occurs within a few minutes of decompression (∼30 min), in a day it is possible to scan several compounds with potential for inhibiting fibril formation (*8*). In the case of TTR, this represents an enormous improvement, since under optimal conditions at atmospheric pressure, TTR aggregation takes 72 h to be completed (*8*).

Recently, by using HHP, Webb and co-workers (*23*) studied the dissociation/unfolding and aggregation of homodimeric IFN-γ in the presence of different concentrations of sucrose. They also observed that HHP increased the aggregation rate through an increased level of solvation of the protein, which exposes more surface area, thus shifting the equilibrium to the transition state, which has a smaller volume than the native state. The addition of sucrose (from 0.125 to 1 M) inhibited aggregation under pressure by shifting the equilibrium in favor of the dimeric, native species.

Smeller and co-workers (*19*) also observed a strong tendency of horse heart metmyoglobin to aggregate after a cycle of compression and decompression at high temperatures (45 and 60 °C). They showed that there were two types of pressure-induced aggregates: one that could be dissociated by moderate pressures (<3 kbar) and one that was pressure-insensitive. The pressure-induced aggregation of proteins may seem to be counterintuitive, because pressure often dissociates multimeric proteins. Thus, for each protein, one must investigate whether pressure populates a "sticky" folding intermediate with a smaller partial molar volume. If so, aggregation is the preferential route.

Pressure Studies on the Misfolded Conformation of Tumor Suppressor Protein p53

p53 is a tumor suppressor protein, ubiquitously expressed in vertebrate cells, that integrates general stress responses that can lead to one of several alternatives: cell cycle arrest, apoptosis, or adaptive responses (*56*). It is the core domain, p53C (≈24 kDa), which accounts for DNA binding. Mutations in the p53 gene are found in more than 50% of human cancers, and p53C is the region where 90% of these mutations have been detected (*3*, *57*). Mutations lead to inactivation of p53's wild-type function as a tumor suppressor. R248Q, the mutation found most frequently in human cancers, is an interesting example since it can be classified as both a contact and a structural mutant (*57*, *58*). An aggravating factor in the impairment of p53 caused by many mutants is the negative dominance phenomenon (*59*). Most p53 mutants (translated from a single mutant allele) are able to drive wild-type p53 (translated from the remaining wild-type p53 allele) into a mutant conformation, in a way that resembles the action of the prion protein (*59*, *60*).

Several carcinomas exhibit an abnormal accumulation of wild-type tumor suppressor protein p53 either in the cytoplasm or in the nucleus of the cell (*61*). We decided to investigate whether wt and mutant forms of p53 would be susceptible to formation of amyloid-like aggregates. We found that the wild-type p53 core domain (p53C) can form

fibrillar aggregates after a mild high-pressure treatment (*9*). Aggregates could be obtained by a cycle of compression and decompression at 37 °C, and their fibrillar character was demonstrated by electron and atomic force microscopies, by binding of thioflavin T, and by circular dichroism. In contrast, drastic thermal denaturation led to the formation of granular-shaped aggregates. Annular aggregates similar to those found in the early aggregation stages of several amyloidogenic proteins were also observed by atomic force microscopy immediately after pressure treatment. All the different aggregates of p53C were very toxic to cells (*9*). The hot-spot mutant R248Q underwent similar aggregation behavior when perturbed by pressure or high temperature. We proposed that the fibrillogenesis of p53 may contribute to the loss of function of p53 and to the seeding of the accumulation of the conformationally altered protein in malignant cells.

In a subsequent study, we aimed to isolate a soluble altered form of p53, under a condition that impeded aggregation (*18*). The altered conformation of p53 is a good target for rational cancer therapy. The development of drugs or peptides that re-establish and stabilize the active conformation of the tumor suppressor protein is the main goal in this field (*57*, *62*). Accordingly, it is essential to understand the molecular basis that drives p53 folding and stabilizes its native conformation to understand why some mutations, or even some alternative conformations of the wild-type protein, lead to loss of protein function, and ultimately to tumors. To explore the folding landscape of the wild-type p53 core domain (p53C) under nonaggregating conditions, we used high hydrostatic pressure (HHP) and sub-zero temperatures (*18*). Aggregation of the protein always accompanied pressure denaturation at 25 and 37 °C. On the other hand, when HP experiments were performed at 4 °C, the extent of denaturation and aggregation was significantly less pronounced (*18*). Lowering the temperature below zero caused cold denaturation of p53C without aggregation. Reversal of conditions yielded a nonaggregated, alternative conformation of the protein. Nuclear magnetic resonance (^{1}H−^{15}N NMR) data showed that the alternative p53C conformation resembled that of the hot-spot oncogenic mutant R248Q (Figure 3). This alternative state was as susceptible to denaturation and aggregation as the mutant R248Q when subjected to HP at 25 °C. Together, these data demonstrate that wild-type p53C adopts an alternative conformation with a mutant-like stability, consistent with the dominant-negative effect caused by many mutants (*59*, *60*). This alternative conformation is likely related to inactive forms that appear *in vivo*, usually driven by interaction with mutant proteins. The inactive conformation of p53C can be a valuable target in the search for ways to interfere with protein misfolding and hence to prevent tumor development (*18*).

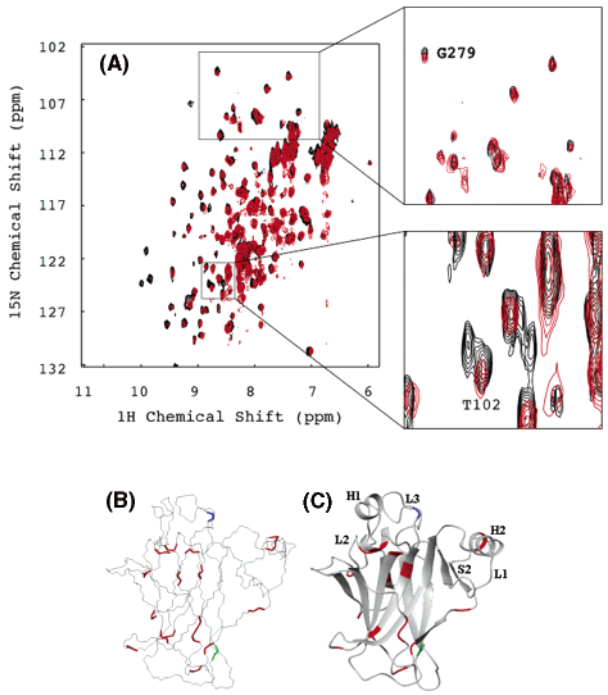

FIGURE 3: Conversion of the wild-type p53C into a conformation that mimics a hot-spot mutant. (A) NMR spectra of the wild-type p53 core domain: (left) superposition of ^{1}H−^{15}N HSQC spectra of native p53C (black) and pressurized cold-denatured p53C (red) in 10% D_2O at 293 K and (right) expanded regions of the two-dimensional spectra showing significant ^{15}N chemical shifts (G279 in the top panel and T102 in the bottom). (B and C) Residues with changes in amide chemical shifts of >0.15 ppm are colored red in the backbone (B) and ribbon (C) representations. W146 is green, and R248 is blue. The DNA-binding region is localized at the top. The hydrophobic cluster is localized at the bottom. Helices H1 and H2, loops L1 and L2, and strand S2 are labeled in the ribbon structure (C). Adapted from ref *18*.

Metastability and Conformational Drift as Crucial Factors for Amyloidogenesis

Hysteresis occurs during the association−refolding processes of several proteins (for review, see refs *11* and *63*; *64*−*66*) and is a clear indication that complete equilibrium has not been reached. Hysteresis was found for both the high-pressure unfolding (*5*, *6*) and the guanidine denaturation (*64*) of TTR. G. Weber proposed an elegant hypothesis to explain the hysteresis based on the slow interconversion between the species, termed "conformational drift" (*67*). His proposal accounts for the rapid dissociation of an oligomeric protein coupled to a slow isomerization process (a first-order reaction) that takes place after dissociation (*67*). The partial loss of affinity between subunits results from this progressive conformational change upon dissociation. Reciprocally, conformational adjustments restoring the original properties of the oligomer occur upon reassociation (*11*, *67*, *68*).

In several of the studies described above, where pressure can populate amyloidogenic conformations of proteins, the observed changes are intimately related to the hysteresis behavior found in the pressure perturbation curves. For TTR, we showed that massive aggregation of wild-type TTR can be triggered at relatively mild pH values provided that the subunit stability has been affected by populating an altered oligomer (T_4*) (*5*, *6*) (Figure 2A). The stability of T_4* is much lower than that of native TTR, as represented in the free energy diagram of Figure 2B. A similar situation occurs with p53C when pressure denaturation is performed at low temperatures (*18*). For TTR, the pressure data do not show whether aggregation proceeds from T_4* or from the monomeric species generated from the microscopic dissociation of T_4*, in part because a small fraction of monomers persists after a return to atmospheric pressure. Because aggregation is inhibited by high pressure (*5*, *8*), the properties of the monomeric species could be characterized. The altered

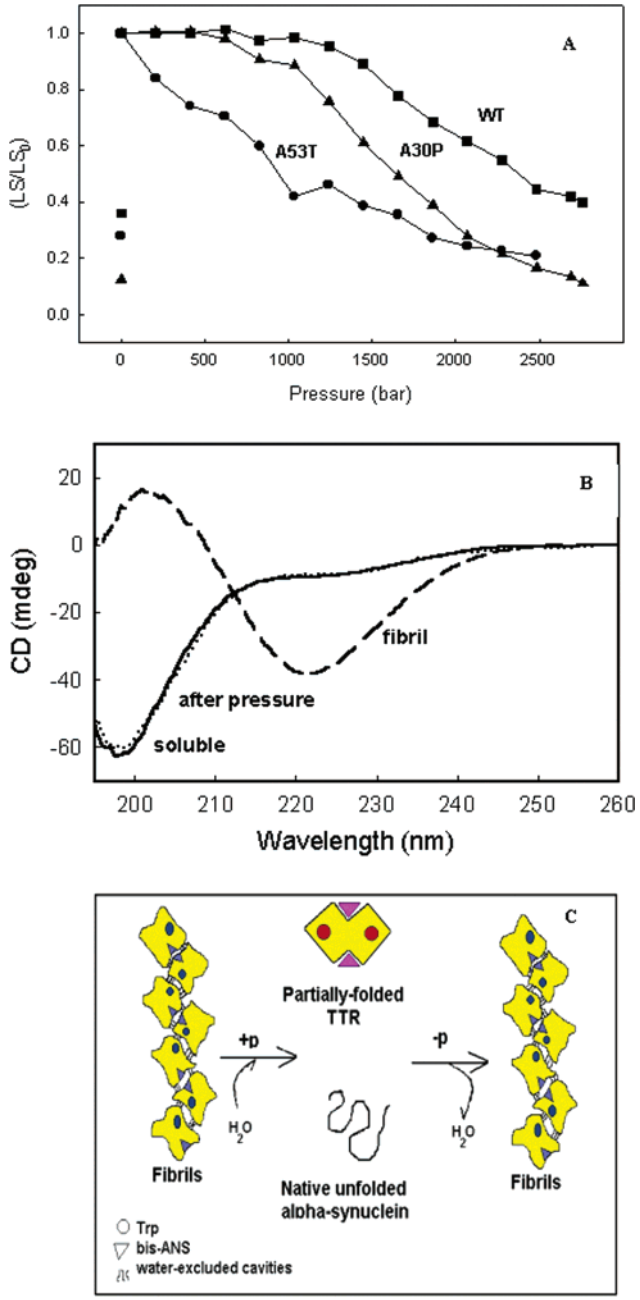

FIGURE 4: Dissociation of amyloid fibrils of α-synuclein. (A) Pressure titration of α-syn fibrils from wt and variant proteins. Fibrils of wt (■), A53T (●), and A30P (▲) were subjected to pressure, and light scattering was recorded. Isolated symbols at the left correspond to the values after decompression. (B) Circular dichroism (CD) spectra of the soluble wt α-syn (—), fibrils (– – –), and the species rescued from HHP treatment (···). (C) Schematic representation of the effects of HHP on the fibrils of wt TTR and α-syn. The core of the amyloid fibrils is not perfectly packed, creating cavities that render the fibrils susceptible to HHP. Modified from ref *8*.

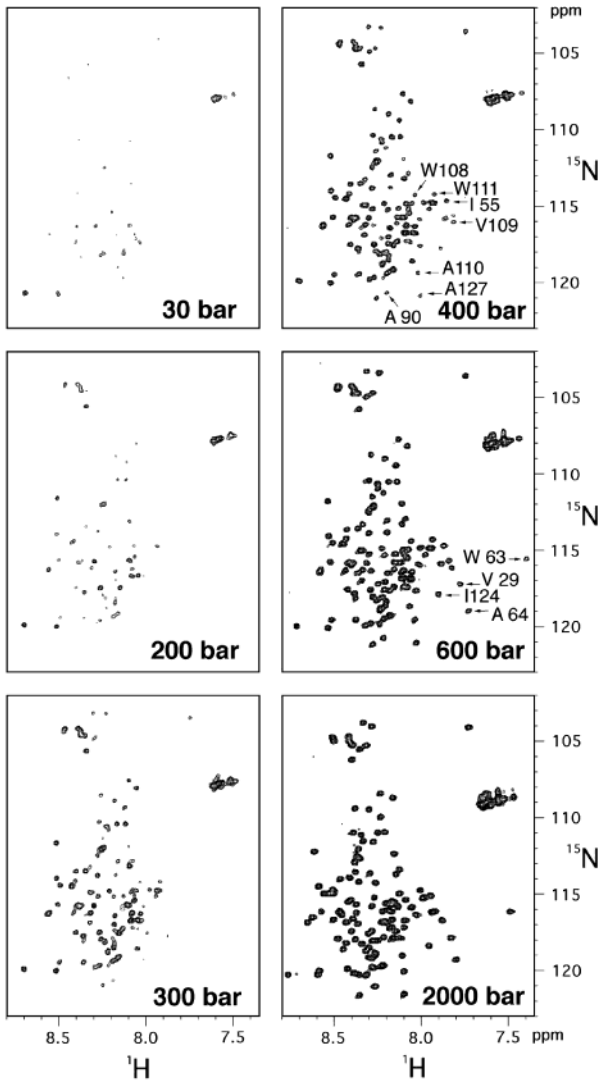

FIGURE 5: Reversible pressure dissociation of intrinsically denatured lysozyme. $^{15}N-^{1}H$ HSQC spectra of the uniformly ^{15}N-labeled 0SS variant at different pressures. Spectra at 30, 400, and 2000 bar were taken during an increasing pressure cycle, and those at 600, 300, and 200 bar were taken during a decreasing pressure cycle (from ref *10*).

conformation of TTR (T_4*) produced by the compression−decompression cycle was typical of a "conformationally drifted" state. The free energy diagram in Figure 2B illustrates the dissociation and isomerization to an altered conformation and reassociation to form a loose tetramer T_4* (preaggregated state). A substantial activation barrier between the T_4 and T_4* specieswould exist. The much lower stability of T_4* may be explained by a higher state of hydration (*6*). Amyloidogenic mutants could resemble the more hydrated T_4* state, with a lower affinity between subunits and a stronger propensity to aggregate. Either the amyloidogenic state obtained under pressure or the altered tetramer might provide a target for development of a drug against amyloid disease.

It is clear that for complex protein motifs, especially those involved in the misfolding and amyloidogenic diseases, kinetics are at least as important as the equilibrium constraints, and possibly even more important. Accordingly, the properties of a specific protein assemblage may differ markedly depending on the previous history of the sample and may rely on protein concentration, the presence of specific ligands, temperature, and pressure. In the cell, chaperones and other folding-assistant factors would interfere to keep the right protein conformation. However, their limited efficiency may be causally related to the fast turnover of proteins in the cell, in contrast to the usually high stability of isolated proteins in solution.

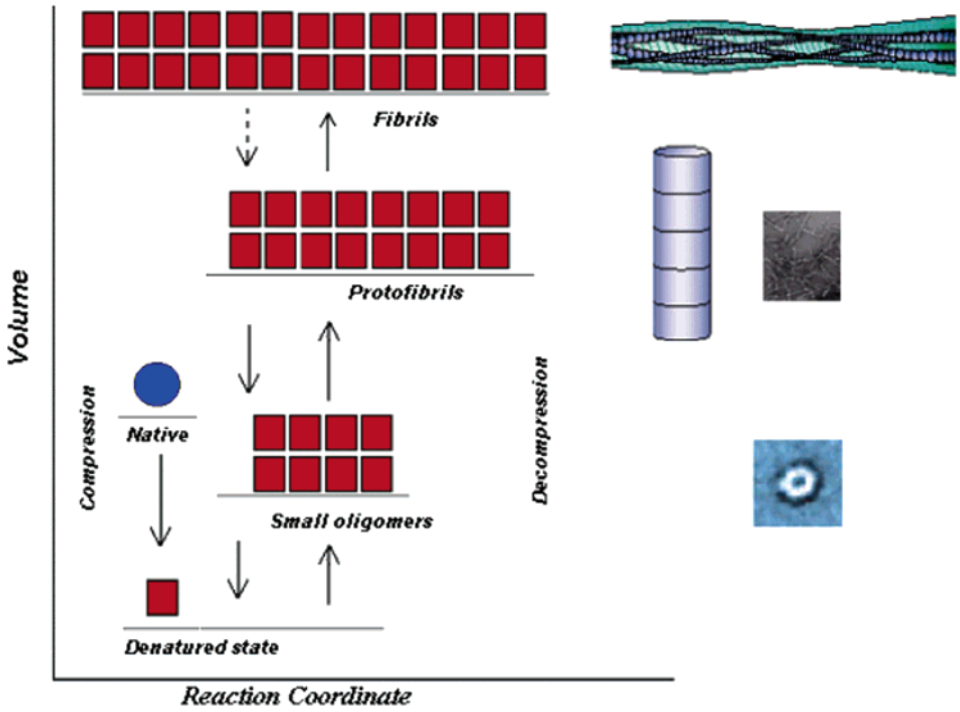

FIGURE 6: General volume diagram for protein folding and aggregation. Thus, aggregates, similar to native proteins, have packing defects making them sensitive to reversible pressure dissociation. The pictures in the right side of the diagram represent the early aggregates, prefibrillar and fibrillar structures.

Reversibility of Fibrillogenesis and the Volume Diagram for Protein Aggregation

The development of drugs that are able to dissociate preformed fibrils is an objective of several research and pharmaceutical laboratories (*69*, *70*). These drugs would be candidates for the treatment of the amyloidogenic diseases. To design the ideal drug, the interactions that hold a fibril together need to be dissected. This problem has been tackled in our laboratory and others in studies using hydrostatic pressure to dissociate fibrillar aggregates of TTR and α-synuclein (*8*) and *β*-sheet aggregates of recombinant PrP protein (*17*). Our data show that all these fibrils are very sensitive to HHP, undergoing dissociation immediately when subjected to high pressures. In the case of TTR, its fibrils were dissociated by pressure (3 kbar) into a partially folded monomer. When the pressure was released, aggregation occurred and resulted in the appearance of new fibrils. Aggregates of rhodanese were also reversibly dissociated by pressure (*24*, *44*).

In the case of α-synuclein (α-syn) involved in Parkinson's disease, fibrillogenesis is very slow, taking several days. Parkinson's disease is the second most common neurodegenerative disorder in humans. It is associated with resting tremor, postural rigidity, and progressive degeneration of dopaminergic neurons in the *substantia nigra* pars compacta (*71*). When the synuclein fibrils are subjected to HHP, they dissociate into a soluble protein that undergoes fibrillogenesis again very slowly (*8*) (Figure 4). Fibrils formed by wt α-syn are less susceptible to pressure denaturation than the variants that are linked to one of the forms of Parkinson's disease, A30P and A53T (Figure 4A). This implies that fibrils of α-syn formed from the variants would be more easily dissolved into small oligomers by the cellular machinery. This result is physiologically important in light of the growing body of evidence which shows that the pathogenic species are the small aggregates rather than the mature fibrils (*2*, *49*).

The high susceptibility of α-syn fibrils to HHP (Figure 4) suggests that hydrophobic interactions may play a crucial role in fibril maintenance. In conclusion, these results point to HHP as a promising tool for dissociating aggregates and IB of proteins that are important for humans and animals. Since HHP gently perturbs the structure of proteins, it allows important intermediate states that lie on the folding pathway of several proteins to be populated and characterized. In the case of amyloidogenic proteins, these intermediates are the main targets for the development of drugs that can block fibrillogenesis.

In a recent article, Niraula et al. (*10*) showed in very elegant experiments the reversible dissociation of the multimeric precursor of amyloid fibrils of the disulfide-deficient mutant of hen lysozyme. Among other approaches, they used $^{15}N-^{1}H$ HSQC spectra to quantify the dissociation of the *β*-sheet-rich aggregate (Figure 5). As the pressure is increased, the cross-peaks start to appear at positions corresponding to the typical unfolded and hydrated protein. Their study also demonstrated that amyloid fibrils can form from an intrinsically denatured protein (*10*), similar to what happens with synuclein (*8*). The *β*-sheet aggregates of the prion protein are also reversibly dissociated by pressure (*16*, *17*). The *β*-rPrP aggregate is much more sensitive to pressure than the cellular α-helical isoform (α-rPrP) (*17*). The greater susceptibility of *β*-rPrP to pressure may reflect its less hydrated structure. Pressure perturbation calorimetry has corroborated the view that the accessible surface area of α-rPrP is much greater than that of *β*-rPrP, which explains the lower degree of hydration of *β*-rPrP. The free energy

and volume diagrams indicate the existence of different folded conformations as well as different denatured states of PrP, which may explain the elusive character of conversion of prion into a pathogenic form (*17*). These studies support recent findings that other molecules such as nucleic acids may participate in the prion conversion reaction by changing the solvent accessible surface and the distribution of cavities (*72*−*74*).

A general volume diagram for protein folding and aggregation is presented in Figure 6. In this diagram, the capacity for forming an amyloidogenic intermediate without proceeding to aggregation is a unique property of pressure, which opens the prospect of characterizing the structure of the amyloidogenic form. In the case of transthyretin, a less stable tetramer is formed after decompression and aggregation can be prevented by keeping the sample at 4 °C (*5*, *6*). The pressure studies on this protein led directly to the idea of a "preaggregated", oligomeric state with loose subunit interactions. There is substantial evidence that the formation of intermediate assembled states may contribute significantly to the neurodegeneration in amyloidogenic diseases, such as Parkinson's (*2*). The mature fibrils are less hydrated, and thus have larger volumes (with more water-excluded cavities). Their susceptibility to pressure will depend on the total free energy, explaining why there are fibrils that are highly susceptible to pressure such as synuclein (*8*) and early aggregates of prion (*17*) and some fibrils that are highly resistant to pressure such as mature prion fibrils (*17*, *75*). This later case is represented by the dashed arrow in Figure 6. Overall, the pressure results on the amyloidogenic proteins transthyretin (*5*, *6*), ataxin (*7*), α-synuclein (*8*), tumor suppressor p53 protein (*9*, *18*), and prion protein (*14*, *17*, *75*) suggest that stable intermediates can be achieved by utilizing appropriate pressure and temperature conditions. The isolation of these intermediates provides targets for the development of lead compounds capable of blocking protein misfolding and aggregation, potential drugs against the protein folding disorders.

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REFERENCES

1. Horwich, A. (2002) Protein aggregation in disease: a role for folding intermediates forming specific multimeric interactions, *J. Clin. Invest. 110*, 1221−1232.
2. Caughey, B., and Lansbury, P. T. (2003) Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders, *Annu. Rev. Neurosci. 26*, 267−298.
3. Hernandez-Boussard, T., Rodriguez-Tome, P., Montesano, R., and Hainaut, P. (1999) IARC p53 mutation database: a relational database to compile and analyze p53 mutations in human tumors and cell lines, *Hum. Mutat. 14*, 1−8.
4. Prusiner, S. B. (1998) Prions, *Proc. Natl. Acad. Sci. U.S.A. 95*, 13363−13383.
5. Ferrão-Gonzales, A. D., Souto, S. O., Silva, J. L., and Foguel, D. (2000) The preaggregated state of an amyloidogenic protein: hydrostatic pressure converts native transthyretin into the amyloidogenic state, *Proc. Natl. Acad. Sci. U.S.A. 97*, 6445−6450.
6. Ferrão-Gonzales, A. D., Palmieri, L., Valory, M., Silva, J. L., Lashuel, H., Kelly, J. W., and Foguel, D. (2003) Hydration and packing are crucial to amyloidogenesis as revealed by pressure studies on transthyretin variants that either protect or worsen amyloid disease, *J. Mol. Biol. 328*, 963−974.
7. Marchal, S., Shehi, E., Harricane, M. C., Fusi, P., Heitz, F., Tortora, P., and Lange, R. (2003) Structural instability and fibrillar aggregation of non-expanded human ataxin-3 revealed under high pressure and temperature, *J. Biol. Chem. 278*, 31554−31563.
8. Foguel, D., Suarez, M. C., Ferrao-Gonzales, A. D., Porto, T. C., Palmieri, L., Einsiedler, C. M., Andrade, L. R., Lashuel, H. A., Lansbury, P. T., Kelly, J. W., and Silva, J. L. (2003) Dissociation of amyloid fibrils of α-synuclein and transthyretin by pressure reveals their reversible nature and the formation of water-excluded cavities, *Proc. Natl. Acad. Sci. U.S.A. 100*, 9831−9836.
9. Ishimaru, D., Andrade, L. R., Teixeira, L. S., Quesado, P. A., Maiolino, L. M., Lopez, P. M., Cordeiro, Y., Costa, L. T., Heckl, W. M., Weissmuller, G., Foguel, D., and Silva, J. L. (2003) Fibrillar aggregates of the tumor suppressor p53C core domain, *Biochemistry 42*, 9022−9027.
10. Niraula, T. N., Konno, T., Li, H., Yamada, H., Akasaka, K., and Tachibana, H. (2004) Pressure-dissociable reversible assembly of intrinsically denatured lysozyme is a precursor for amyloid fibrils, *Proc. Natl. Acad. Sci. U.S.A. 23*, 4089−4093.
11. Silva, J. L., and Weber, G. (1993) Pressure stability of proteins, *Annu. Rev. Phys. Chem. 44*, 89−113.
12. Mozhaev, V. V., Heremans, K., Frank, J., Masson, P., and Balny, C. (1996) High-pressure effects on protein structure and function, *Proteins 24*, 81−91.
13. Silva, J. L., Foguel, D., and Royer, C. A. (2001) Pressure provides new insights into protein folding, dynamics and structure, *Trends Biochem. Sci. 26*, 612−618.
14. Niraula, T. N., Haraoka, K., Ando, Y., Li, H., Yamada, H., and Akasaka, K. (2002) Decreased thermodynamic stability as a crucial factor for familial amyloidotic polyneuropathy, *J. Mol. Biol. 320*, 333−342.
15. Kuwata, K., Li, H., Yamada, H., Legname, G., Prusiner, S. B., Akasaka, K., and James, T. L. (2002) Locally disordered conformer of the hamster prion protein: a crucial intermediate to PrPSc? *Biochemistry 41*, 12277−12283.
16. Torrent, J., Alvarez-Martinez, M. T., Heitz, F., Liautard, J. P., Balny, C., and Lange, R. (2003) Alternative prion structural changes revealed by high pressure, *Biochemistry 42*, 1318−1325.
17. Cordeiro, Y., Kraineva, J., Ravindra, R., Lima, L. M. T. R., Gomes, M. P. B., Foguel, D., Winter, R., and Silva, J. L. (2004) Hydration and packing effects on prion folding and β-sheet conversion: High-pressure spectroscopy and pressure perturbation calorimetry studies, *J. Biol. Chem. 279*, 32354−32359.
18. Ishimaru, D., Maia, L. F., Maiolino, L. M., Quesado, P. A., Lopez, P. C., Almeida, F. C., Valente, A. P., and Silva, J. L. (2003) Conversion of wild-type p53C core domain into a conformation that mimics a hot-spot mutant, *J. Mol. Biol. 333*, 443−451.
19. Smeller, L., Rubens, P., and Heremans, K. (1999) Pressure effect on the temperature-induced unfolding and tendency to aggregate of myoglobin, *Biochemistry 38*, 3816−3820.
20. Meersman, F., Smeller, L., and Heremans, K. (2002) Comparative Fourier transform infrared spectroscopy study of cold-, pressure-, and heat-induced unfolding and aggregation of myoglobin, *Biophys. J. 82*, 2635−2644.
21. Foguel, D., Robinson, C. R., Sousa, P. C., Jr., Silva, J. L., and Robinson, A. S. (1999) Hydrostatic pressure rescues native protein from aggregates, *Biotechnol. Bioeng. 63*, 552−558.
22. Lefebvre, B. G., and Robinson, A. S. (2003) Pressure treatment of tailspike aggregates rapidly produces on-pathway folding intermediates, *Biotechnol. Bioeng. 82*, 595−604.
23. Webb, J. N., Webb, S. D., Cleland, J. L., Carpenter, J. F., and Randolph, T. W. (2001) Partial molar volume, surface area, and hydration changes for equilibrium unfolding and formation of aggregation transition state: high-pressure and cosolute studies on recombinant human IFN-γ, *Proc. Natl. Acad. Sci. U.S.A. 98*, 7259−7264.
24. Gorovits, B. M., and Horowitz, P. M. (1998) High hydrostatic pressure can reverse aggregation of protein folding intermediates and facilitate acquisition of native structure, *Biochemistry 37*, 6132−6135.
25. Dzwolak, W., Ravindra, R., Lendermann, J., and Winter, R. (2003) Aggregation of bovine insulin probed by DSC/PPC calorimetry and FTIR spectroscopy, *Biochemistry 42*, 11347−11355.
26. Royer, C. A. (2002) Revisiting volume changes in pressure-induced protein unfolding, *Biochim. Biophys. Acta 1595*, 201−209.

27. Oliveira, A. C., Gaspar, L. P., Da Poian, A. T., and Silva, J. L. (1994) Arc repressor will not denature under pressure in the absence of water, *J. Mol. Biol. 240*, 184−187.
28. Chalikian, T. V. (2003) Volumetric properties of proteins, *Annu. Rev. Biophys. Biomol. Struct. 32*, 207−235.
29. Foguel, D., and Silva, J. L. (1994) Cold denaturation of a repressor-operator complex: the role of entropy in protein-DNA recognition, *Proc. Natl. Acad. Sci. U.S.A. 91*, 8244−8247.
30. Nash, D. P., and Jonas, J. (1997) Structure of pressure-assisted cold denatured lysozyme and comparison with lysozyme folding intermediates, *Biochemistry 36*, 14375−14383.
31. Hummer, G., Garde, S., Garcia, A. E., Paulaitis, M. E., and Pratt, L. R. (1998) The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins, *Proc. Natl. Acad. Sci. U.S.A. 95*, 1552−1555.
32. Hillson, N., Onuchic, J. N., and Garcia, A. E. (1999) Pressure-induced protein-folding/unfolding kinetics, *Proc. Natl. Acad. Sci. U.S.A. 96*, 14848−14853.
33. Peng, X. D., Jonas, J., and Silva, J. L. (1993) Molten-globule conformation of Arc repressor monomers determined by high-pressure ^{1}H NMR spectroscopy, *Proc. Natl. Acad. Sci. U.S.A. 90*, 1776−1780.
34. Akasaka, K. (2003) Highly fluctuating protein structures revealed by variable-pressure nuclear magnetic resonance, *Biochemistry 42*, 10875−10885.
35. Fourme, R., Ascone, I., Kahn, R., Mezouar, M., Bouvier, P., Girard, E., Lin, T., and Johnson, J. E. (2002) Opening the high-pressure domain beyond 2 kbar to protein and virus crystallography: technical advance, *Structure 10*, 1409−1414.
36. Inoue, K., Yamada, H., Akasaka, K., Herrmann, C., Kremer, W., Maurer, T., Doker, R., and Kalbitzer, H. R. (2000) Pressure-induced local unfolding of the Ras binding domain of RalGDS, *Nat. Struct. Biol. 7*, 547−550.
37. Lima, L. M. T. R., Foguel, D., and Silva, J. L. (2000) DNA tightens the dimeric DNA-binding domain of human papillomavirus E2 protein without changes in volume, *Proc. Natl. Acad. Sci. U.S.A. 97*, 14289−14294.
38. Zong, Q., Osmulski, P. A., and Hager, L. P. (1995) High-pressure-assisted reconstitution of recombinant chloroperoxidase, *Biochemistry 34*, 12420−12425.
39. Silva, J. L., Oliveira, A. C., Gomes, A. M. O., Lima, L. M., Mohana-Borges, R., Pacheco, A. B., and Foguel, D. (2002) Pressure induces folding intermediates that are crucial for protein-DNA recognition and virus assembly, *Biochim. Biophys. Acta 1595*, 250−265.
40. Silva, J. L., Luan, P., Glaser, M., Voss, E. W., and Weber, G. (1992) Effects of hydrostatic pressure on a membrane-enveloped virus: high immunogenicity of the pressure-inactivated virus, *J. Virol. 66*, 2111−2117.
41. Oliveira, A. C., Ishimaru, D., Gonçalves, R. B., Smith, T. J., Mason, P., Sa-Carvalho, D., and Silva, J. L. (1999) Low temperature and pressure stability of picornaviruses: implications for virus uncoating, *Biophys. J. 76*, 1270−1279.
42. Tian, S. M., Ruan, K. C., Qian, J. F., Shao, G. Q., and Balny, C. (2000) Effects of hydrostatic pressure on the structure and biological activity of infectious bursal disease virus, *Eur. J. Biochem. 267*, 4486−4494.
43. Gaspar, L. P., Silva, A. C., Gomes, A. M. O., Ano Bom, A. P. D., Schwarcz, W. D., Mestecky, J., Novak, M. J., Foguel, D., and Silva, J. L. (2002) Hydrostatic pressure induces the fusion-active state of enveloped viruses, *J. Biol. Chem. 277*, 8433−8439.
44. Panda, M., Gorovits, B. M., and Horowitz, P. M. (2000) Productive and nonproductive intermediates in the folding of denatured rhodanese, *J. Biol. Chem. 275*, 63−70.
45. St John, R. J., Carpenter, J. F., and Randolph, T. W. (1999) High-pressure refolding of recombinant human growth hormone from insoluble aggregates, *Proc. Natl. Acad. Sci. U.S.A. 96*, 13029−13033.
46. King, J., Haase-Pettingell, C., Robinson, A. S., Speed, M., and Mitraki, A. (1996) Thermolabile folding intermediates: inclusion body precursors and chaperonin substrates, *FASEB J. 10*, 57−66.
47. Clark, E. D. (2001) Protein refolding for industrial processes, *Curr. Opin. Biotechnol. 12*, 202−207.
48. Carrio, M. M., and Villaverde, A. (2002) Construction and deconstruction of bacterial inclusion bodies, *J. Biotechnol. 96*, 3−12.
49. Dobson, C. M. (2003) Protein folding and misfolding, *Nature 426*, 884−890.
50. Kelly, J. W. (1998) The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways, *Curr. Opin. Struct. Biol. 8*, 101−106.
51. Soto, C. (2001) Protein misfolding and disease; protein refolding and therapy. *FEBS Lett. 498*, 204−207.
52. Damas, A. M., and Saraiva, M. J. (2000) TTR amyloidosis-structural features leading to protein aggregation and their implications on therapeutic strategies, *J. Struct. Biol. 130*, 290−299.
53. Blake, C. C., Geisow, M. J., Oatley, S. J., Rerat, B., and Rerat, C. (1978) Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 Å, *J. Mol. Biol. 121*, 339−356.
54. Lai, Z., Colon, W., and Kelly, J. W. (1996) The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. *Biochemistry 35*, 6470−6482.
55. Lashuel, H. A., Lai, Z., and Kelly, J. W. (1998) Characterization of the transthyretin acid denaturation pathways by analytical ultracentrifugation: implications for wild-type, V30M, and L55P amyloid fibril formation, *Biochemistry 37*, 17851−17864.
56. Prives, C., and Hall, P. A. (1999) The p53 pathway, *J. Pathol. 187*, 112−126.
57. Bullock, A. N., and Fersht, A. R. (2001) Rescuing the function of mutant p53C, *Nat. Rev. Cancer 1*, 68−76.
58. Wong, K. B., DeDecker, B. S., Freund, S. M., Proctor, M. R., Bycroft, M., and Fersht, A. R. (1999) Hot-spot mutants of p53 core domain evince characteristic local structural changes, *Proc. Natl. Acad. Sci. U.S.A. 96*, 8438−8442.
59. Chenè, P. (1998) *In vitro* analysis of the dominant negative effect of p53 mutants, *J. Mol. Biol. 281*, 205−209.
60. Milner, J., and Medcalf, E. A. (1991) Cotranslation of activated mutant p53 with wild-type drives the wild-type p53 protein into the mutant conformation, *Cell 65*, 765−774.
61. Ostermeyer, A. G., Runko, E., Winkfield, B., Ahn, B., and Moll, U. M. (1996) Cytoplasmically sequestered wild-type p53 protein in neuroblastoma is relocated to the nucleus by a C-terminal peptide, *Proc. Natl. Acad. Sci. U.S.A. 93*, 15190−15194.
62. Foster, B. A., Coffey, H. A., Morin, M. J., and Rastinejad, F. (1999) Pharmacological rescue of mutant p53 conformation and function, *Science 286*, 2507−2510.
63. Weber, G. (1992) *Protein Interactions*, Chapman and Hall, New York.
64. Lai, Z., McCulloch, J., Lashuel, H. A., and Kelly, J. W. (1997) Guanidine hydrochloride-induced denaturation and refolding of transthyretin exhibits a marked hysteresis: equilibria with high kinetic barriers, *Biochemistry 36*, 10230−10239.
65. Sinclair, J. F., Ziegler, M. M., and Baldwin, T. O. (1994) Kinetic partitioning during protein folding yields multiple native states, *Nat. Struct. Biol. 1*, 320−326.
66. Schuler, B., Rachel, R., and Seckler, R. (1999) Formation of fibrous aggregates from a non-native intermediate: the isolated P22 tailspike β-helix domain, *J. Biol. Chem. 274*, 18589−18596.
67. Weber, G. (1986) Phenomenological description of the association of protein subunits subjected to conformational drift. Effects of dilution and of hydrostatic pressure, *Biochemistry 25*, 3626−3231.
68. Silva, J. L., Miles, E. W., and Weber, G. (1986) Pressure dissociation and conformational drift of the β dimer of tryptophan synthase, *Biochemistry 25*, 5780−5786.
69. Sacchettini, J. C., and Kelly, J. W. (2002) Therapeutic strategies for human amyloid diseases, *Nat. Rev. Drug Discovery 1*, 267−275.
70. Cohen, F. E., and Kelly, J. W. (2003) Therapeutic approaches to protein-misfolding diseases, *Nature 426*, 905−909.
71. Spillantini, M. G., Schmidt, M. L., Lee, V. M. Y., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) α-Synuclein in Lewy bodies, *Nature 388*, 839−840.
72. Cordeiro, Y., Machado, F., Juliano, L., Juliano, M. A., Brentani, R. R., Foguel, D., and Silva, J. L. (2001) DNA converts cellular prion protein into the β-sheet conformation and inhibits prion peptide aggregation, *J. Biol. Chem. 276*, 49400−49409.
73. Deleault, N. R., Lucassen, R. W., and Supattapone, S. (2003) RNA molecules stimulate prion protein conversion, *Nature 425*, 717−720.
74. Cordeiro, Y., Lima, L. M. T. R., Gomes, M. P., Foguel, D., and Silva, J. L. (2004) Modulation of prion protein oligomerization, aggregation, and β-sheet conversion by 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate (bis-ANS), *J. Biol. Chem. 279*, 5346−5352.

75. Torrent, J., Alvarez-Martinez, M. T., Harricane, M. C., Heitz, F., Liautard, J. P., Balny, C., and Lange, R. (2004) High-pressure induces scrapie-like prion protein misfolding and amyloid fibril formation, *Biochemistry 43*, 7162−7170.

76. Martins, S. M., Chapeaurouge, A., and Ferreira, S. T. (2003) Folding intermediates of the prion protein stabilized by hydrostatic pressure and low temperature, *J. Biol. Chem. 278*, 50449−50455.
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