# Ligand Binding and Hydration in Protein Misfolding: Insights from Studies of Prion and p53 Tumor Suppressor Proteins[†]

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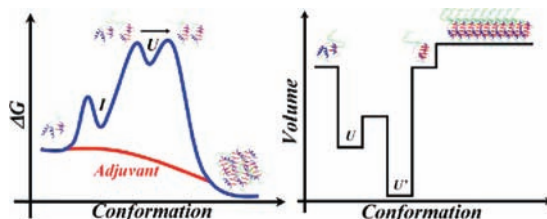
Protein misfolding has been implicated in a large number of diseases termed protein-folding disorders (PFDs), which include Alzheimer's disease, Parkinson's disease, transmissible spongiform encephalopathies, familial amyloid polyneuropathy, Huntington's disease, and type II diabetes. In these diseases, large quantities of incorrectly folded proteins undergo aggregation, destroying brain cells and other tissues.

The interplay between ligand binding and hydration is an important component of the formation of misfolded protein species. Hydration drives various biological processes, including protein folding, ligand binding, macromolecular assembly, enzyme kinetics, and signal transduction. The changes in hydration and packing, both when proteins fold correctly or when folding goes wrong, leading to PFDs, are examined through several biochemical, biophysical, and structural approaches. Although in many cases the binding of a ligand such as a nucleic acid helps to prevent misfolding and aggregation, there are several examples in which ligands induce misfolding and assembly into amyloids. This occurs simply because the formation of structured aggregates (such as protofibrillar and fibrillar amyloids) involves decreases in hydration, formation of a hydrogen-bond network in the secondary structure, and burying of nonpolar amino acid residues, processes that also occur in the normal folding landscape. In this Account, we describe the present knowledge of the folding and misfolding of different proteins, with a detailed emphasis on mammalian prion protein (PrP) and tumoral suppressor protein p53; we also explore how ligand binding and hydration together influence the fate of the proteins.

Anfinsen's paradigm that the structure of a protein is determined by its amino acid sequence is to some extent contradicted by the observation that there are two isoforms of the prion protein with the same sequence: the cellular and the misfolded isoform. The cellular isoform of PrP has a disordered N-terminal domain and a highly flexible, not-well-packed C-terminal domain, which might account for its significant hydration. When PrP binds to biological molecules, such as glycosaminoglycans and nucleic acids, the disordered segments appear to fold and become less hydrated. Formation of the PrP–nucleic acid complex seems to accelerate the conversion of the cellular form of the protein into the disease-causing isoform. For p53, binding to some ligands, including nucleic acids, would prevent misfolding of the protein. Recently, several groups have begun to analyze the folding–misfolding of the individual domains of p53, but several questions remain unanswered. We discuss the implications of these findings for understanding the productive and incorrect folding pathways of these proteins in normal physiological states and in human disease, such as prion disorders and cancer. These studies are shown to lay the groundwork for the development of new drugs.

1. Introduction

In the year 2009, we commemorate Charles Darwin's 200th birthday and 150 years since the publication of the seminal book *On the Origin of Species*.[1] Biology has evolved in fascinating and unexpected ways, and most of the general predictions of Darwin's theory of evolution have been tested at the molecular level. Chemists, biochemists, and physicists are trying to describe the conformation landscape of biomolecules through the use of their sophisticated tools, including quantum mechanics, kinetics, and thermodynamics. However, the understanding of apparently simple processes, such as protein folding, has so far eluded us. The applications of some of the laws of chemistry and physics do not always result in success, as was brilliantly pointed out by Schrödinger in *What is Life* (1944):[2] "... about the structure of living matter, we must be prepared to find it working in a manner that cannot be reduced to the ordinary laws of Physics"; and that is so "because the construction is different from anything we have yet tested in the physical laboratory".

Schrödinger's forecasts have become real, and most of our attempts to frame Biology according to a deterministic view have failed. This is particularly true in the processes that lead a biopolymer to evolve in space and time. Water is the ubiquitous background for all these processes, and although it tended initially to be overlooked, theoreticians and experimentalists have had to take it into account more and more.[3] From enzyme catalysis to cell signaling throughout the different compartments in the cell, water activity plays a crucial role. A protein will fold after successfully sampling the lowest free energies of the protein folding funnel according to the interactions among the different amino acid residues as well as to the differential interactions with water molecules (Figure 1).

The protein energy landscape gains considerable complexity with the inclusion of interactions with water.[3] For some proteins, the energy landscape becomes more complicated when the system drifts into an aggregation pathway, as exemplified in Figure 1. Protein misfolding and aggregation are involved in more than 30 human diseases.[4] Protein aggregation also proceeds with changes in hydration similar to folding and ligand binding.[5] Protein folding intermediates have been spotted as precursors to the misfolded and aggregated species. The choice of the folding intermediates that lead into a native or misfolded conformation will depend on how the different states are populated, based on their energies, energy barriers, and exposure of hydrophobic surfaces to the aqueous milieu (Figure 1). Homeostasis of cellular proteins is controlled by proteasomes, chaperones, and other folding
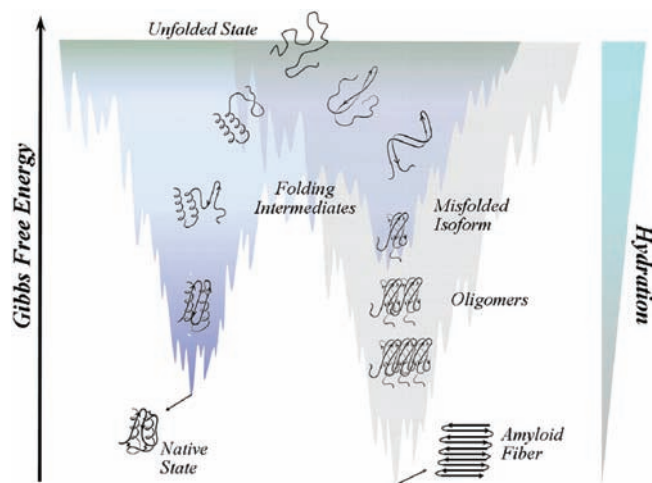

FIGURE 1. Free-energy landscape of protein folding versus misfolding. Unfolded proteins (represented in the top of the diagram) have high conformational entropy and are highly hydrated. As the proteins evolve in the funnel, the intermediate species become more structured and less hydrated. Some proteins face a bifurcation in the landscape, leading to metastable conformations, which depending on the conditions might stabilize misfolded and aggregated species.

assistants that play a crucial role in preventing the deleterious effects of misfolding.[6] Folding and misfolding/aggregation are equally driven by dehydration, and therefore, it is critical to evaluate the contribution of hydration to the formation of folded and misfolded species. Ligand binding leads to a decrease in solvent exposure very similar to that observed when the protein folds or aggregates.

Here, we review how ligand interaction affects protein folding and misfolding. The effects might be paradoxical; depending on concentration and the presence of partners, the outcome can be prevention of misfolding or its acceleration.

2. Hydration in Protein Folding, Misfolding, and Amyloid Assembly

The importance of hydration for the formation of amyloids has been deduced from structural studies[7] as well by molecular dynamics.[8] The use of computer simulations to study protein aggregation encounters problems due to the complexity of the system. Using explicit and implicit solvent simulations, a study with the amyloidogenic β-hairpin peptide (109−122) of the Syrian hamster prion protein allowed the authors to demonstrate that solvent exposure of hydrophobic surfaces is the driving force for the folding of the peptide.[8]

There are several *in vitro* studies that address the effects of water deprivation on the aggregation of proteins.[9,10] Using model cosolvents, Grudzielanek et al.[10] described how solvational perturbations lead into pronounced and different effects
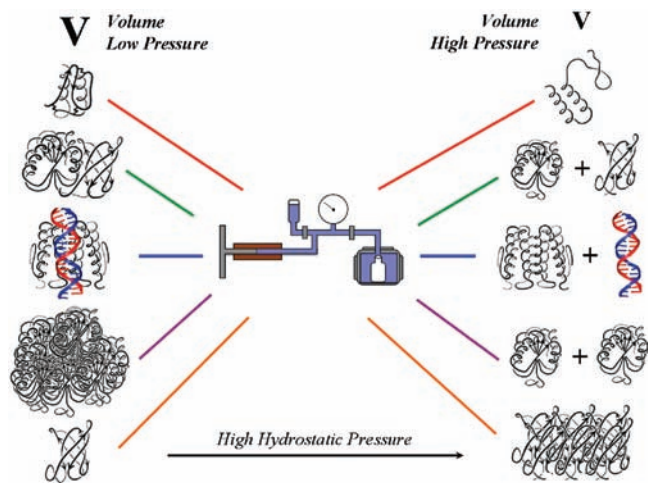
FIGURE 2. Hydrostatic pressure effects in different systems. Pressure acts on proteins by water infiltration and shifts the equilibrium to smaller volumes, and may induce protein denaturation, protein dissociation, dissociation of protein−nucleic acid complexes, disassembly of aggregates, including amyloids, and formation of preamyloidogenic intermediates.

on the unfolding, non-native assembly and fibril formation of insulin. Mukherjee and co-workers[9] utilized reverse micelles to show that the aggregation rates of two amyloid-forming peptides increase when hydration is decreased.

As described below, pressure and volumetric approaches make it possible to assess the effects of hydration and ligands on the folding and misfolding of proteins.

Hydration Effects on Protein Misfolding and Amyloid Aggregation as Studied by High Hydrostatic Pressure. Proteins undergo dissociation and unfolding by pressure mostly because the final states are more hydrated, have fewer nonhydrated cavities, and, therefore, occupy smaller volumes[5] (Figure 2). For a typical case of protein denaturation, pressure will shift the equilibrium of the reactants ($P_N + nH_2O$) into the products ($P_D(H_2O)_n$), where P_N and P_D are native and denatured proteins, respectively. The decrease in volume is due to hydration of newly exposed nonpolar and polar residues as well as to the loss of free volume arising from packing defects in the folded structure. High pressure has been used to assess the underlying mechanisms of protein misfolding and aggregation.[5,11−13] Some fibrillar aggregates are highly sensitive to pressure, and this sensitivity is related to the infiltration of water molecules into the protein interior during pressurization.[5,11]

The similar sensitivity to pressure of folded and misfolded proteins indicates comparable forces maintaining these states, especially because they have similar water-excluded cavities. Thus, both the folded and aggregated states will be less hydrated and have larger specific volumes (in cm^3/mol) than

the unfolded and dissociated states (Figure 2). Early aggregated species and protofilaments always have larger volumes and are thus sensitive to hydrostatic pressure.[12−16] Although fibrils might be less hydrated than early aggregates, they are more stable and have greater contributions from hydrogen bonds. Thus, much higher pressures would be required to dissociate these aggregates. A typical case is the amyloid fibrils of β2-microglobulin (β2-m), involved in dialysis-related amyloidosis, which are not tightly packed; instead, they present a larger number of cavities than denatured protein.[16] However, mature amyloid-like fibrils formed from a fragment of the β2-m protein have a smaller partial specific volume, probably because of a greater contribution from hydrogen bonds.[16] Similar results had been observed when comparing whole transthyretin with TTR peptides.[11,13]

Pressure can also promote the formation of intermediates that are prone to aggregation[11,13] (Figure 2). Pressure would have a reshuffling activity, producing intermediates that might evolve into misfolded/aggregated species under decompression. For transthyretin, involved in senile systemic amyloidosis and in familial amyloidotic polyneuropathy, a less stable tetramer is formed after decompression.[11,14] More recently, high pressure was used to explore a potential therapy against amyloidogenic diseases by trapping the monomer of a non-amyloidogenic variant (T119M) of transthyretin.[17] Pressure produced long-lived monomers of T119M. They were mixed with aggressive mutants of TTR to generate heterotetramers, which became nonamyloidogenic.[17]

3. Prion Protein: A Hydrated Promiscuous Protein

Transmissible spongiform encephalopathies (TSEs) are rare, fatal neurodegenerative diseases.[18] The most intriguing feature of TSEs is that they can be infectious, in addition to the hereditary and sporadic forms. All TSEs are related to a single type of infectious agent that contains isoforms of a constitutive protein known as the prion protein (PrP).[18] The cellular prion isoform (PrP^C) is rich in alpha helices and occurs naturally in cells of the host, whereas the misfolded form is a conformational variant of the first, rich in β-sheets, involved in transmission of the disease, called the prion scrapie (PrP^{Sc}).[18] Mature PrP^C is anchored to the outer cell membrane through a glycosyl phosphatidyl inositol anchor. PrP^C has a globular domain and a highly disordered amino-terminal domain.[18] The physiological function of PrP is still a matter of intense debate.
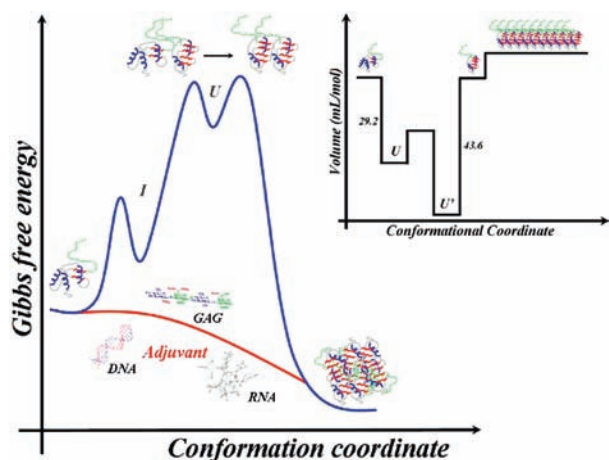
FIGURE 3. Energy and volume diagram of PrP misfolding. PrP^C (left) can misfold into an isoform rich in β-sheet structure capable of forming toxic and infectious aggregates (PrP^{Sc}) (right). The transition between the species is separated by a large energetic barrier. I and U represent intermediate and unfolded states, respectively. An adjuvant factor would lower the free-energy barrier, triggering formation of PrP^{Sc}. PrP^C has a larger solvent-accessible surface area than the misfolded/aggregated species, and the folding pathway also exhibits a kinetic barrier in the activation volume (inset, modified from ref 15). The pressure-denatured states of α-rPrP (PrP^C) and β-rPrP (PrP^{Sc}-like) are denoted as U and U', respectively.

Conversion of PrP^{Sc} into PrP^C Involves Changes in Hydration and Might Be Promoted by Ligands. The "protein-only hypothesis" postulates that the PrP is the main agent responsible for the outbreak of TSEs.[18] The discovery that PrP knockout mice are resistant to infection by prions[19] is the main groundwork for this hypothesis. The mechanisms that lead to conversion of PrP^C into PrP^{Sc} are still unknown, but there are several proposed models. Incubation of PrP^C with excess of PrP^{Sc} gives protease resistance,[20] suggesting that PrP^{Sc} catalyzes the conversion of PrP^C into newly formed PrP^{Sc}. However, it has been suggested by several groups that a still unknown cofactor might initiate or modulate the conversion of PrP^C to PrP^{Sc}.[21−23] This hypothetical molecule would lower the free-energy barrier that prevents conversion between PrP^C and PrP^{Sc}, triggering formation of PrP^{Sc} (Figure 3).

Biophysical studies have demonstrated that the transition between these states involves changes in hydration.[15,24,25] The free-energy and volume diagrams (Figure 3) show that the cellular isoform is in a metastable conformation, and surprisingly the differences involve larger changes in volume than in free energy. Studies employing high-pressure Fourier transform infrared (FTIR) and pressure perturbation calorimetry indicated that the cellular PrP isoform is more hydrated and has a larger solvent-accessible surface area than aggregated recombinant PrP (rPrP)[15,25] (Figure 3). Molecular dynamics

studies corroborated the role of hydration in the stability and amyloidogenicity of PrP.[24] Binding of a cofactor (such as nucleic acid or a glycosaminoglycan) would lead to a decrease in solvent-accessible surface area and a decrease in the level of hydration (Figure 3). Below, we focus on the ligand-binding properties of the prion protein and its implication in hydration changes in the latter leading to disease progression.

Binding of PrP to Nucleic Acids and Changes in Hydration. Nucleic acids are now believed to be important players[26] in prion biology. DNA or RNA molecules would participate in prion diseases as cofactors, helping to trigger the conversion of PrP^C into PrP^{Sc} (Figure 3). Nandi and Leclerc showed that recombinant murine prion protein (rPrP) polymerizes in a nucleic acid solution.[27] The first experimental evidence for a catalytic role of nucleic acids in PrP conversion was presented by some of us in 2001.[21] We showed that recombinant prion protein could bind DNA oligonucleotides with high affinity *in vitro*. Our main finding was that the PrP/nucleic acid complex could act as a catalyst, increasing the aggregation rate[21] (Figure 3). The structural data obtained from small-angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) measurements showed that rPrP interacts with DNA through both the globular and disordered domains.[28] The changes in NMR chemical shifts suggest a restructuring of the protein upon DNA binding and decrease of hydration.[28]

PrP can also bind RNA molecules.[29−32] Highly structured RNA molecules bind human rPrP with high affinity.[30] PrP can also form structures similar to retroviral proteins, therefore possessing RNA-binding chaperone characteristics, likely participating in nucleic-acid metabolism.[33] The interaction of PrP with RNA was shown to stimulate the conversion of PrP^C to PrP^{Res} (protease-resistant PrP) in hamster brain homogenates, and treatment of these homogenates with RNase inhibited the conversion.[22] Lately, synthetic RNAs were used to generate PrP^{Res} formation.[32] This result is consistent with our DNA-binding results suggesting that nucleic acids could be involved in prion conversion.[21] Intracerebral inoculation of a mixture of synthetic RNAs, purified PrP, and copurified lipids caused neurodegeneration in wild-type hamsters.[32]

Full-length rPrP interacts with RNA at the disordered and highly hydrated N-terminus, undergoing aggregation and losing most of its α-helical content.[31] NMR measurements with a synthetic RNA sequence showed that the soluble portion of PrP recovered most of its original fold, but with distinct changes in the NMR HSQC spectrum.[31] The aggregates derived from interaction of PrP with RNA extracted from neuroblastoma cells were highly cytotoxic.[31] In contrast, com-

plexes formed with synthetic RNAs were not toxic. The precise RNA structure needed for prion binding is still unknown, but the high flexibility of such molecules is certainly important for these interactions. It also seems crucial to the interaction changes in hydration of the protein itself that would decrease the solvent accessibility (Figure 3).

In the past decade, noncoding RNAs (ncRNA) have been shown to act in post-translational regulation. The finding that PrP interacts with nucleic acid with likely NA chaperone activities[33] raises the possibility that PrP might have some effects in the processing of ncRNA. In fact, recent studies showed that cytoplasmic PrP induced large ribonucleoprotein particles,[34] with potential function in post-transcriptional regulation. The participation of a nucleic acid in prion conversion would be a rare event because a seed of misfolded material would also be needed. Recombinant PrP can also translocate DNA to the intracellular space and promote DNA expression.[35] A putative nucleic acid chaperone function for PrP raises the question of how interaction with nucleic acids could contribute to the sporadic cases of prion diseases.

Binding of PrP to Glycosaminoglycans and Potential Therapeutic Approaches to Prion Diseases. Glycosaminoglycans (GAGs) have been implicated in many conformational diseases. Heparan sulfate was found in amyloid plaques in TSEs.[36] GAGs bind PrP^C both in its soluble form and at the cell surface,[37,38] and other studies showed that sulfated polysaccharides can inhibit the accumulation of PrP^{Res} in cells infected with scrapie.[38] Moreover, it was shown that sulfated GAGs could inhibit the polymerization of prion peptides into amyloid fibrils.[39] Thus, interaction of PrP (PrP^C and/or PrP^{Sc}) with endogenous GAGs might be needed for PrP^{Sc} propagation,[40] and exogenous GAGs might act as inhibitors, blocking the interaction of PrP with endogenous proteoglycans. In this respect, GAGs would act as some nucleic acids do[26] by reducing the access of the solvent to the protein surface.

A great variety of compounds have been tested in an effort to find agents that reverse or prevent the formation of PrP^{Sc}. Degenerate phosphorothioate oligonucleotides reduce PrP^{Sc} formation *in vivo*.[41] DNA thioaptamers bind with high affinity to different mammalian prion proteins[41,42] and have great potential as antiprion agents. GAGs are also considered as promising compounds for prion diseases.[39,43]

4. Misfolding of p53 and Cancer

When cells are subjected to stress, p53 works as a transcription factor, resulting in cell cycle arrest or apoptosis.[44] Failure of these responses leads into uncontrolled cell cycle.

p53 function is lost in more than 50% of human cancers, making it an appealing target for cancer therapies. p53 is a modular protein containing an N-terminal transactivation domain, followed by a proline-rich region, a central DNA-binding domain (p53C), a tetramerization domain, and a C terminus.[45] The central or core domain of p53 (p53C), comprising residues 94−312, is responsible for specific DNA interactions,[45] and 97% of the point mutations in p53 are in this domain.

An aggravating factor to the misfolding of p53 caused by single amino acid mutations is the negative dominance property: several p53 mutants (translated from a single mutant allele) are able to drive wild-type p53 protein (translated from the remaining wild-type p53 allele) into a mutant conformation,[46] in a way that resembles the action of the prion protein.

p53C is a relatively unstable protein undergoing easily chemical, thermal, and pressure denaturation.[47−50] Interestingly, p53C loses its DNA-binding activity spontaneously at 37 °C *in vitro* due to a kinetic partitioning between folding and misfolding pathways of the protein.[47] Recently, we found that the interaction with a cognate DNA sequence stabilizes p53 and prevents aggregation of the protein into an amyloid-like structure[51] (Figure 4). Sequence-specific DNA also stabilized full-length p53. The effects of cognate DNA could be simulated by high concentrations of osmolytes, implying that the stabilization is caused by water exclusion. We propose that aptameric nucleic acids can be used as therapeutic approaches to prevent misfolded species of p53 and treat cancer[51] (Figure 4).

The Intriguing Amyloid Potential of p53. Formation of amyloid-like aggregates has been described for the core,[49] for the tetramerization,[52,53] and for the transactivation[54] domains of p53. Rigacci and co-workers[54] elegantly demonstrated that the p53 N-terminal domain aggregates into amyloid assemblies that exhibit cytotoxicity. We found that the wild-type p53C can form fibrillar aggregates.[49] An intermediate oligomer of p53C was also observed during equilibrium and kinetic folding/unfolding transitions.[55] Annular and fibrillar aggregates of p53C were toxic to cells.[49] The hot-spot mutant R248Q also had a tendency to aggregate. Thus, the fibrillogenesis of p53 might contribute to its loss of function and seed the accumulation of conformationally altered protein in cancerous cells (Figure 4D).

Several carcinomas exhibit abnormal accumulation of wild-type or mutant tumor suppressor protein p53 either in the cytoplasm or in the nucleus of the cell.[56] Evidence that the three domains of p53 form amyloid-like aggregates is
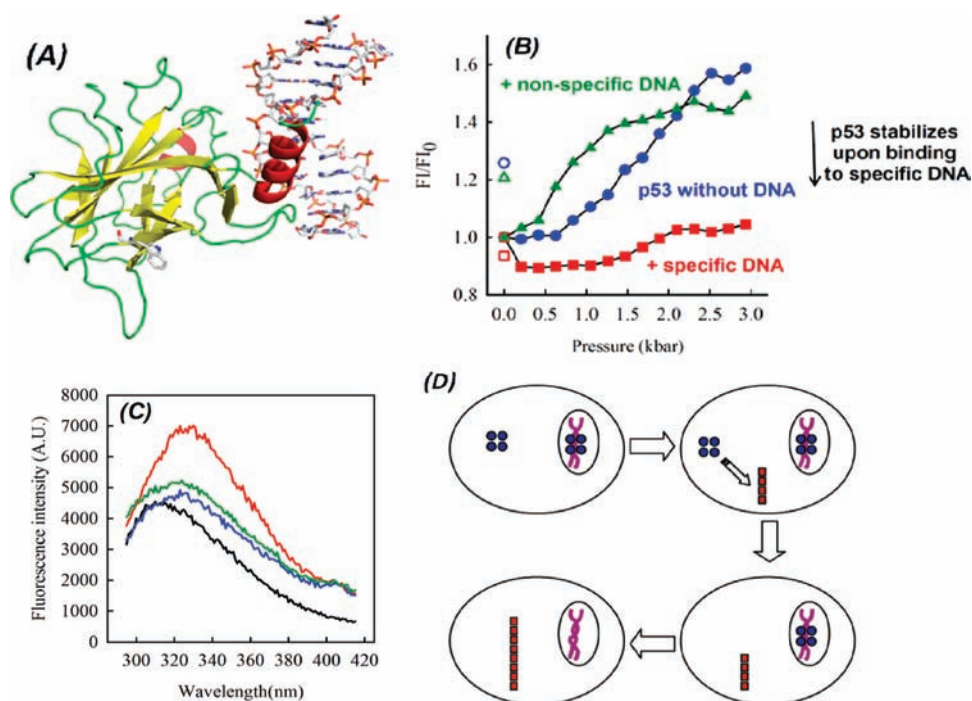
FIGURE 4. Stabilization of p53C upon sequence-specific DNA binding and recovery of misfolded aggregated species of p53C. (A) Structure of p53C bound to DNA (PDB entry 2ABY). (B) Full-length p53 is stabilized against pressure denaturation upon DNA binding as measured by fluorescence: p53 (blue circles), consensus-bound p53 (red squares), and poly(GC)-bound p53 (green triangles). Open symbols are values after return to atmospheric pressure. (C) Cognate DNA rescues the native conformation of p53C after misfolding and aggregation. Fluorescence of wild-type p53C at atmospheric pressure (solid black line); after the first cycle of pressurization in the absence of DNA (red line); after DNA addition at atmospheric pressure (blue line); and after the second pressure cycle in the presence of DNA (green line). (D) Proposed model for p53C aggregation. Conversion of native, active p53 (blue circles) into aggregates (red squares) in the cytoplasm (upper panels). Nuclear DNA is represented in purple. Adapted from refs 49 and 51.

quite striking, making it tempting to speculate that p53 amyloid formation might participate in the malignant process. Aggregation of p53 would act as a sink to sequester native protein into the inactive conformation, replicating the structural information, very much like a prion (Figure 4D). Hot-spot mutations of p53, related to malignant tumors, usually destabilize the folded conformation,[45,47] exposing hydrophobic surfaces to water, and we did find that they have a greater tendency to aggregate.[49] Because this aggregation is likely to include wild-type subunits, it could be the basis for the negative dominance of p53 mutants.[46] The search for molecules that preclude the formation of the misfolded conformation, which may ultimately lead to the prevention of tumor development, is a major goal in cancer research. The use of aptameric nucleic acids could be a good alternative to prevent aggregation and to rescue activity[51] (Figure 4). A more stable variant of p53 would shift the equilibrium toward the soluble and active form of the protein.

5. Nucleic-Acid Effects on Other Amyloidogenic Proteins

The effects of nucleic acids on the aggregation and misfolding properties are not restricted to prion protein[26] and p53.[51] For all the cases, the binding seems to be driven by decreases in the surface exposed to the aqueous environment. The replication initiator protein of *Pseudomonas* pPS10 plasmid (RepA) aggregates into amyloids.[57] DNA induced the aggregation of one of the domains of RepA into amyloids, which might have a role in the negative regulation of plasmid replication. However, DNA was not present in fibrils, similar to what we found for the interaction of PrP with DNA.[21]

In the case of α-synuclein, involved in Parkinson's disease, it has been found that DNA stimulates formation of fibrils.[58,59] There was a parallel between the effects of DNA-binding and osmolytes in inducing fibrillation.[59] These results are similar to that found with DNA-induced stabilization of p53C, which in turn resembles the stabilization

promoted by high concentrations of glycerol.[52] Formation of α-synuclein fibrils is driven by exclusion of molecules of water, as clearly shown by pressure studies of the fibrils.[11]

6. Overview and Future Perspectives

At the end of his book *Protein Interactions*, published in 1992,[60] Gregorio Weber wrote that "Future knowledge of the relation of protein function to structure and dynamics is much more likely to come from the comparative study of the proteins than from their study as isolated entities to which elementary physics and chemistry are applicable." To some extent, his words reinforce Schrodinger's statement that biomolecules are quite complex entities.[2] The great challenge in Biology for the next decades will be to discover how interactions among different biomolecules and with solvent occur in space and time in the cellular context. Even at one of the lowest hierarchical levels, such as the protein folding, understanding the frequent failure of polypeptides to reach the native state requires that we comprehend the interactions of a plethora of intermediate states with ligands and the solvent.[5] Here we exemplified this quite well with the prion protein, p53, and other amyloidogenic proteins.

For prions, they seem to have other accomplices (likely nucleic acids and GAGs) that chaperone their activity in converting the cellular form of the protein into the disease-causing isoform. There are, however, many questions that remain to be explored. The ability of the prion protein to bind nucleic acids may have broader implications for its native function than for disease. The great abundance of RNA in the cytosol that acts in a variety of cellular processes may hint at the physiological target of prion protein.

In the case of p53, it needs to interact with several other proteins to exert its functions, which contribute to p53's conformation and affinity for its target DNA. In the same manner, post-translational modifications likely interfere with protein folding. Another level of complexity for p53 folding involves p53 mutants that can alter the conformation of the wild-type protein either by forming heterotetramers or by aggregation, converting the wild-type monomer into an inactive form, as described for prions. The comprehension of p53 folding/misfolding may shed light on the mechanisms of p53 regulation and ultimately the cell's fate in tumorigenic processes.

BIOGRAPHICAL INFORMATION

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FOOTNOTES

[†]Dedicated to the memory of Professor Gregorio Weber.

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