Amyloid Dissociation

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Cold- and Pressure-Induced Dissociation of Protein Aggregates and Amyloid Fibrils**

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Protein folding is one of the most crucial steps during the life of a protein. If some malfunction occurs in achieving the native conformation, this will render the polypeptide totally inactive, or even worse, it can produce a misfolded molecule that can interfere with or block components of the cellular machinery to the point of causing cell malfunction or even death. In recent years, it has become evident that a wide range of human diseases are associated with aberrations in the folding process.[1,2] These diseases, which are also called "protein conformational diseases", include Alzheimer's disease (responsible protein: Aβ), Parkinson's disease (α-synuclein), prion protein related encephalopathies, and type II diabetes mellitus (islet amyloid polypeptide, IAPP).

Amyloid deposits exhibit similar, fibrillar submicroscopic structures. All amyloid is ordered in secondary structures, including a core cross β-sheet structure, in which continuous β sheets are formed with β strands running perpendicular to the fibril axis.[1] It has been suggested that the generic amyloid conformation, the cross β structure, may be a universal energetic minimum for aggregated proteins. Typically, amyloid fibrils consist of two to six unbranched protofilaments (2–5 nm wide) associated laterally or twisted together to form fibrils that are 4–13 nm wide. Once formed, the rigid structure of amyloids and the deep energy minima acquired make those structures extremely stable and hard to solubilize.[3]

The molecular mechanisms involved in the formation of these aggregate structures are still poorly understood, which is due to the fact that these insoluble structures are very large (in terms of molar mass) and generally cannot be crystallized. Although the amyloid structure is known to be toxic, there is considerable discussion as to its role in disease. It has also been suggested that the prefibrillar aggregates are more toxic than the amyloid fibrils themselves.[1,4]

In efforts to probe the stability and energetics of amyloid fibril, pressure and temperature perturbation as well as cosolvent dependence have been the focus of recent studies. Since the discovery of high-pressure-induced protein unfolding and denaturation by Nobel laureate P. W. Bridgman in 1914, it has been shown in numerous studies that hydrostatic pressure may lead to disruption of the intermolecular interactions maintaining the native protein structure, which is accompanied by a decrease in volume of the protein–water system and simultaneous unfolding.[5–9] Subsequently, high hydrostatic pressures (HHP) have also been shown to be effective for disaggregation and refolding of proteins from insoluble aggregates prepared in vitro.[10]

The appropriate way of expressing the thermodynamic stability of a protein is an energy landscape as a multidimensional function of temperature, pressure, and solution conditions. When the solution conditions (pH value, ionic strength, salt and cosolvent concentration) are kept constant, the stability of the protein is a function of only temperature and pressure. The Gibbs free energy difference $\Delta_u G(T,p)$ between the denatured (unfolded) and native state, relative to some reference point T_0,p_0 (e.g., the unfolding temperature at ambient pressure), can be approximated—assuming a second-order Taylor series of $\Delta_u G(T,p)$ expanded with respect to T and p around T_0,p_0—as given in Equation (1).[11]

$$\Delta_u G = \Delta_u G_0 + \frac{\Delta_u \kappa}{2}(p-p_0)^2 + \Delta_u \alpha (p-p_0)(T-T_0) - \Delta_u C_p \left[T\left(\ln \frac{T}{T_0} - 1 \right) + T_0 \right] + \Delta_u V_0 (p-p_0) - \Delta_u S_0 (T-T_0) \quad (1)$$

$\Delta_u\kappa$, $\Delta_u\alpha$, and $\Delta_u C_p$ refer to the changes in compressibility, expansibility, and heat capacity upon unfolding (u), respectively. In a simple approximation, the entropy and volume changes of unfolding, $\Delta_u S = \Delta_u H/T$ and $\Delta_u V$, respectively, may be decomposed into intrinsic contributions of the protein and those of the hydration shell. The entropy difference reflects mainly the increase in configurational entropy of the chain, $\Delta_u S_{conf}$, and the entropy change $\Delta_u S_{hydr}$ in the hydration shell owing to exposure of formerly buried residues ($\Delta_u S = \Delta_u S_{conf} + \Delta_u S_{hydr}$). The same reasoning applies to the volume change $\Delta_u V$ and to the enthalpy change $\Delta_u H$ ($\Delta_u H = \Delta_u H_{conf} + \Delta_u H_{hydr}$).

The transition line, where the protein unfolds upon a temperature or pressure change, is given by $\Delta_u G = 0$. The physically relevant solution of the curve in the p,T plane has an elliptical shape (Figure 1). Exposure of nonpolar groups upon unfolding tends to bind and immobilize water mole-

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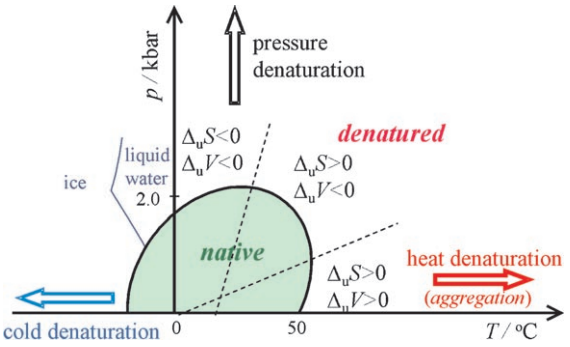

Figure 1. Schematic pressure/temperature stability diagram of a typical monomeric protein.[9] The different routes of unfolding of the native protein (green area) as well as the corresponding thermodynamic properties are depicted. Heat denaturation is often accompanied by irreversible aggregation. The water/ice phase-transition line is also given.

cules, thereby decreasing their entropy. This mechanism is strongly temperature-dependent. Its experimental signature is the positive heat capacity increment at constant pressure, $\Delta_u C_p > 0$ (as can be determined by calorimetry).

As also indicated in Figure 1, the slope of the p,T phase boundary between liquid water and ice I is negative. Hence, an increase in pressure extends the stability range of the liquid water phase to temperatures below 0 °C (e.g., −18 °C at ca. 2 kbar). The possibility to explore the temperature region below 0 °C at slightly elevated pressures can therefore be used to study the cold denaturation process of proteins. At low temperatures, a marked decrease of the hydration enthalpy term $\Delta_u H_{hydr}$ (increase of favorable, low-energy interactions between water and the protein interface) leads to the destabilization of the native structure.[12] Thus, with the enthalpy change of unfolding being a function of temperature, at a low enough temperature, the Gibbs free energy of unfolding $\Delta_u G$ becomes negative and the protein will unfold under the release of heat. Usually, the transition temperatures for the cold denaturation lie well below 0 °C, so that high pressure has to be applied to keep the water in the liquid state, or freezing of cold water has to be avoided using supercooling techniques and small sample volumes.[13] While the heat-induced denaturation, which is mainly driven by a large positive configurational entropy change ΔS_{conf}, is usually a highly cooperative process leading to a largely unfolded conformation, cold denaturation, which is fostered by the number and strength of interfacial H-bonds at low temperatures, is known to be a milder form of unfolding, leading only to a partial unfolding of the protein.[9]

According to the stability diagram shown in Figure 1, both pressure and cold can be used to destabilize proteins and hence have been used to destabilize protein aggregates and fibrils, which is facilitated by a combination of factors: HHP leads to a general weakening of hydrophobic interactions, while the presence of cavities within the folded proteins or in the interface of oligomers can favor the unfolding or dissociation of these structures. If water molecules fill the cavities when they become accessible upon unfolding or dissociation of the proteins, a volume decrease results, and hence the event is favored under high pressure. The dissociation of electrostatic interactions also leads to a marked reduction in the volume caused by electrostrictive effects of water molecules around the unpaired charged residues exposing charged groups. In a similar way, solvation of polar groups results in a decrease in volume of the water molecules.

The susceptibility of protein aggregates to pressure largely depends on the degree of structural order of the aggregate: fresh, amorphous aggregates are more sensitive to pressure and prone to refolding to the native state than mature amyloid fibrils. In the later case, the effectiveness of pressure-induced dissociation depends on the particular mode of polypeptide backbone and side-chain packing that allows reducing remaining void volumes. The pressure sensitivity of fresh aggregates and the insensitivity of most mature fibrils to HHP allow not only to differentiate between various stages of amyloid formation but also to obtain reliable thermodynamic data on the transformation process.[14–16]

As an example, we show pressure effects on insulin[15] and IAPP at various stages of the aggregation process (Figure 2). The capacity to bind the fluorophor thioflavin T (ThT) is used as a measure of fibril formation, which is indicated by an increase of fluorescence intensity upon binding to fibrillar species. Clearly, at the early stages of insulin aggregation, during which oligomeric species are formed in the nucleation and growth process, pressure leads to dissociation. These oligomers are stabilized mainly by electrostatic and hydrophobic interactions, yet they lack the precise packing of mature insulin fibrils. This renders them susceptible to pressure-induced dissociation. Unlike their precursors, the pressure insensitivity of mature insulin fibrils demonstrates that extensive hydrogen bonding and optimized side-chain packing are crucial for their stability. Figure 2b shows the effect of pressure-assisted cold denaturation conditions on IAPP fibrils grown at 25 °C for 6 days. No decrease in the ThT fluorescence intensity is observed under these conditions, indicating that, like insulin fibrils, mature IAPP fibrils grown at micromolar concentrations are resistant toward dissociation under these conditions. These results are corroborated by high-pressure NMR spectroscopy experiments. Only at much higher concentrations and higher pressures has partial dissociation of IAPP aggregate been observed.

Some amyloid fibrils (in particular protofibrils) might exhibit a larger partial specific volume than that of the composing proteins, because the assembly process, which involves various conformational transitions of native structures to non-native β-sheets, could create new water-excluded cavities and induce hydrophobic pockets.[16,17] In these cases, HHP has a potential to dissociate amyloid fibrils as well. For example, it has been shown that moderate pressures (1–3 kbar) can dissociate amyloid fibrils of transthyretin, β_2-microglobulin, and α-synuclein.[17,18] For α-synuclein, fibrils formed from mutants linked to Parkinson's disease (A53T and A30P) were more sensitive to high pressure than the wild-type fibrils, suggesting that those mutations affect the hydrophobic interactions and packing of amyloid fibrils. One of the most thrilling prospects for application of high pressure in protein aggregation research is the idea of destroying prion infectivity through pressure treatment.[19]

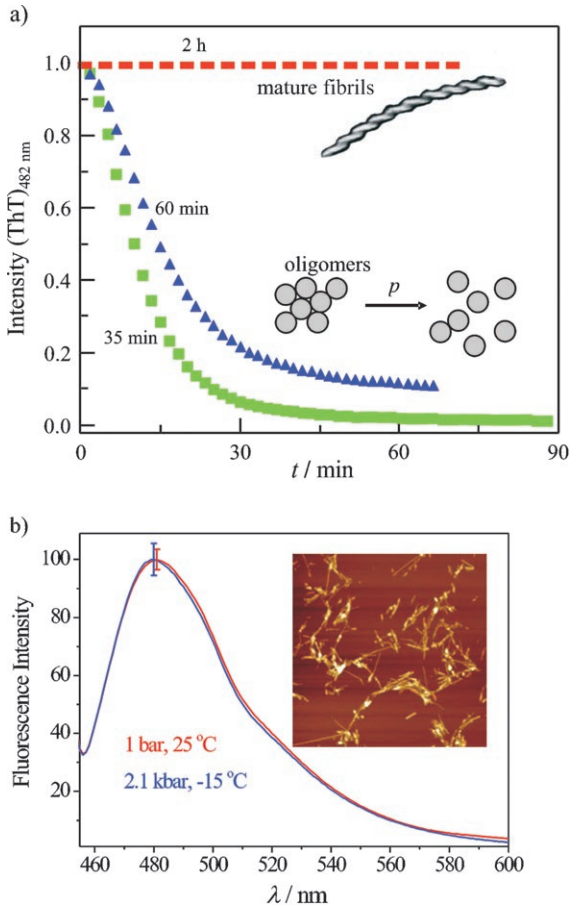

Figure 2. a) Characteristic features of the high-pressure treatment of 0.5 wt.% insulin in water (0.1 M NaCl, $T = 60\,^{\circ}C$) at different stages of the aggregation process. The decay of the normalized ThT intensity upon a pressure increase to 1 kbar at progressively longer incubation times is shown. At 35 and 60 min, the oligomeric states prevail, whereas at 2 h, the mature fibrillar state of insulin is reached, which exhibits pressure resistance. b) ThT fluorescence spectra of 10 μM IAPP mature fibrils grown for six days and kept at 1 bar, 25 °C (red) and under 2.1 kbar pressure at −15 °C for 72 h (blue).

In a very elegant way, Kim et al.[13] demonstrated recently that amyloid fibrils may be highly sensitive to low temperature as well. Amyloid fibrils of α-synuclein have been shown to rapidly dissociate in supercooled water at −15 °C. ^{15}N-labeled α-synuclein fibrils were injected into 1 mm glass capillaries, and the capillaries were placed in a 5 mm NMR tube. Subsequently, the NMR tube was incubated at −15 °C for one day in the supercooled state. After incubation, transmission electron microscopy (TEM) images, ThT fluorescence, and 2D HSQC NMR spectra were recorded, and the data showed only disordered aggregates and rare fibrillar structures. Similar to HHP, hydrophobic interactions decrease with decreasing temperature. The authors show that at −15 °C, the temperature dependence of hydrophobic and electrostatic interactions contribute to the cold dissociation of α-synuclein fibrils.

Hence, besides dissolution with aggressive chemical agents, low temperature or high pressure are also able to dissolve protein aggregates and fibrils. Moreover, temperature and pressure perturbation can yield a wealth of enlightening new information on the structure and stability of such aggregates as well as on the kinetics of their formation.

Figure 3 shows a schematic energy landscape for protein folding and aggregation phenomena.[9,20] At the high energy,

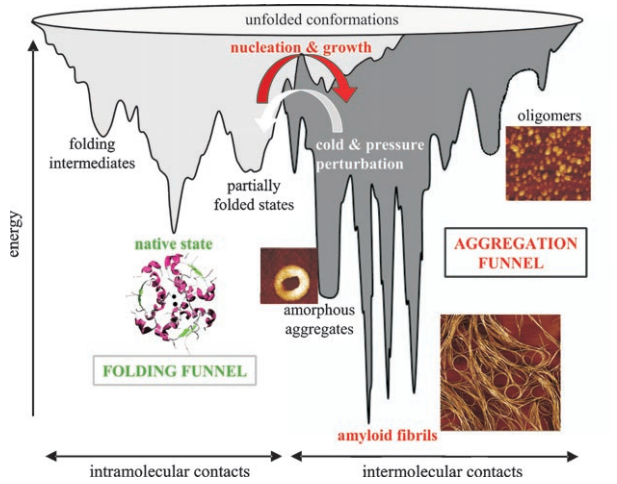

Figure 3. Schematic energy landscape for protein folding and aggregation/amyloid formation.[9,20] While the protein attains the native conformation at its global energy minimum under normal physiological conditions, the amyloidogenic stacking of many protein molecules may lead to lower energies beyond this level in the aggregation funnel. At low temperature and high pressure, dissociation of less densely packed protein aggregates and fibrils is possible.

high entropy surface, a multitude of unfolded conformations are present. "Funneling" on a rugged energy surface (or "landscape") occurs towards the natively folded state by rapid intramolecular contact formation. By a (generally slow) nucleation process and subsequent autocatalytic aggregation reaction of partially unfolded structures (induced by mutations, particular cosolvents, change in pH value, etc.), formation of aggregates and amyloid fibrils through intermolecular contacts may occur, and the system enters the aggregation funnel. Within the aggregation funnel, loosely packed oligomeric or amorphous aggregates as well as different amyloid fibrillar states (strains) with different packing properties and with deep energy minima may form, thus exhibiting conformational polymorphism. Dissociation of protein aggregates and, under favorable conditions, of amyloid fibrils is possible upon application of HHP or under cold denaturation conditions at temperatures well below 0 °C. We may assume that an amyloid structure without optimal packing will enable formation of various isoforms, suggesting the structural basis of multiple forms of amyloid fibrils in contrast to the unique native fold of functioning proteins.

In closing, the cold denaturation study by Kim et al.[13] as well as pressure perturbation approaches will invigorate further work on amyloidogenic proteins. These studies will provide further insight into the structural diversity of amyloid fibrils, which might be responsible for the most puzzling observation of prion diseases, that is, the presence and

propagation of different strain phenotypes even for one single protein.

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