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Protein Folding and Aggregation under Pressure

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INTRODUCTION

The protein folding problem

The folding problem, namely how a freshly synthesized polypeptide chain reaches its secondary, tertiary and sometimes quaternary structure belonging to the native state, has been studied widely in the last decades (Pain, 2000; Dobson, 2003; Gruebele, 2002, 2005; Royer, 2008). The field was initiated by Anfinsen who unfolded the bovine pancreatic ribonuclease and found that after dialyzing the denaturant out, the protein folded back and showed enzymatic activity. He concluded that the primary structure, which remains intact in the denatured state, unequivocally determines the native conformation of the protein (Anfinsen, 1973). This principle is known today as the Anfinsen dogma.

It is clear now that Anfinsen was fortunate with this experiment because his protein did not misfold, which is common nature for proteins, but his conclusion gave rise to a new area of research.

A theoretical approach by Levinthal led to a paradox. If one estimates the number of conformations, which are available for an average sized protein, using a simple approximation of 2 rotamers (rotational states) pro residue, the total number of possible conformations is an astronomic number (~10^{30} for a protein of 100 residues). Assuming that the protein needs only 10 ps to try one conformation, the time to sample the whole conformational space is much longer than the age of the universe (4 10^{11} years, while the

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universe is c.a. 10^{10} years old). It is clear that proteins do not fold by a random search in the conformational space. The above calculation, (known today as Levinthal's paradox) led to the idea of directed folding. Levinthal himself used such a calculation to support his idea of a directed folding, rather than believing in the random walk-type folding. According to this idea the possible choices in the folding path are restricted by the previous folding steps. This is visualized by the so called funnel model. (Fig. 1.) The funnel model was invented in order to visualize the nonrandom nature of the process (Bryngelson et al., 1995). The conformation of the protein backbone can be determined by two angles called dihedral angles (, □). This spans a 200 dimensional conformational space for a protein of 100 residues. For characterization of the complete structure one needs further coordinates, giving a conformational space with several hundred or thousand dimensions. Each point in this space represents a unique conformation with certain energy. Plotting the energy versus the coordinates of the conformational space one gets the so called energy landscape. In order to visualize the energy landscape one reduces on the plot the number of the conformational coordinates to two, resulting in a three-dimensional plot seen in Fig. 1. According to the funnel hypothesis this plot has one global minimum. The side of the funnel is a rugged surface with several local minima, representing intermediate states with different lifetime.

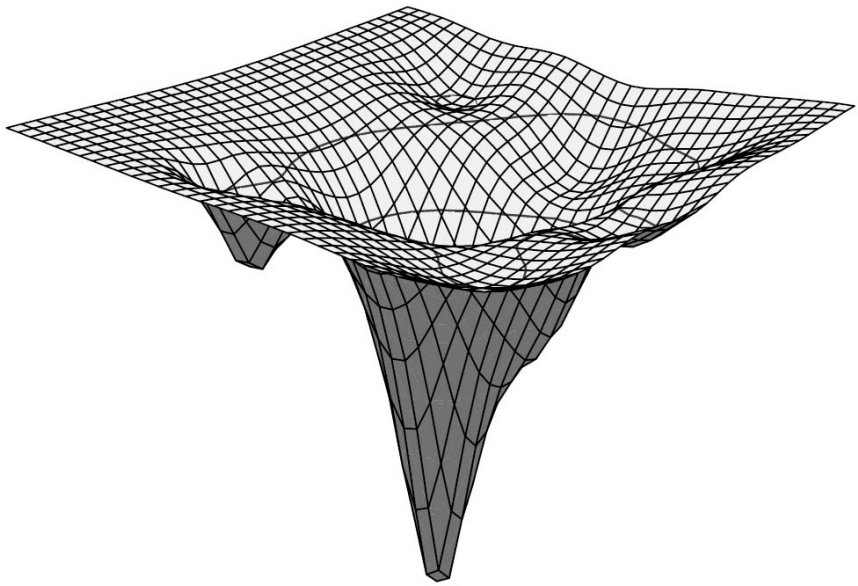


Figure 1. The folding funnel.

It has to be mentioned that the folding process *in vivo* often starts co-translationally. This means that fractions at the N-terminus of the protein start to fold before the completion of the synthesis of the C-terminus. Beside this, there are a number of helper proteins, called chaperones which facilitate the folding process. They also prevent aggregation of the freshly synthesized polypeptide in the crowded environment of the cell. A separate chapter of this book is dedicated to the effect of pressure on chaperones (Tölgyesi and Böde, 2009).

Misfolding and aggregation. Conformational diseases

Folding of the polypeptide chain into the native conformation is not always successful. There are intermediate conformations on the folding pathway where intermolecular interactions can become favorable, which leads to aggregation and misfolding of the protein. These aggregates can be deposited either in the cell or in the extracellular space. Such insoluble tissue deposits of water soluble proteins can be observed in several serious diseases (like Alzheimer, diabetes II, Creuzfeld-Jacob). In these diseases partially folded or misfolded proteins form amyloid fibers, which can lead to tissue death (Booth et al., 1997). The mechanism of the formation of the fibers is not yet fully understood. In mature fibers proteins are arranged in antiparallel □-sheet structure in such a way, that the polypeptide chains are perpendicular to the direction of the fiber (Fink, 1998; Dobson, 2003). The proteins attached to each other with hydrogen bonds typical in antiparallel □-sheets.

The 'conformational diseases' described above usually appear when the protein, which is aggregating, is present in a form with a reduced stability. The most probable reason for this is that the formation of the amyloid fibers is preceded by a partial unfolding of the protein. The destabilization of the native structure can be a result of a mutation (like in the case of lysozyme, two mutants of which known to cause familial amyloidosis), fragmentation or can be a consequence of interactions with other proteins (like in case of Creuzfeld-Jacob syndrome). Kendrick et al. (1998) showed that the aggregation of human interferon is preceded by the transient expansion of the molecule, which suggests that proteins should be partially unfolded or destabilized in order to be able to aggregate.

More than a dozen proteins and protein families are known today which cause conformational diseases due to aggregation of their metastable conformations (Carrell and Gooptu, 1998; Meersman and Dobson, 2006). These proteins already have (intramolecular) □-strands, which were thought of as precursors of the intermolecular structuring, but according to the current view all proteins can form fibers under appropriate conditions (Fändrich et al., 2001).

Formation of fibrous aggregatesis however the last step of a complex pathway. Aggregation prone protein chains usually form amorphous aggregatesfirst,followed by profilamentsand fibers.Theformation of fibrous aggregatesis usually very slow, it takes several days *in vitro*, but *in vivo* it can even take years. The stability of the aggregatesincreasesand the well packed fibers are then the most resistant onesboth against chemical and physical effects.

PRESSUREINDUCED UNFOLDING

Pressureis an intensive thermodynamic parameter, equally important to temperature. It is not surprising, that pressurehaspronounced effecton the folded–unfolded equilibrium of proteins(Heremansand Smeller,1998).The first observation about the unfolding effect of pressurewas published by Bridgman (1914).He pressurized the white of the eggand noticed that after the pressure cycle it had "an appearancemuch like that of a hard boiled egg". Of coursethis timeneither thestructurenor thefolding processof the protein wasknown, theobservation could not beinterpreted in theway we can seethe casenow. The next milestone was done by Brandts et al. (1970) and Hawley (1971),who performed a systematic investigation of the unfolding of ribonucleaseand chimotrypsinogen as function of pressure and temperature. Hawley also presented a short thermodynamic theory describing his observations. This is known today as the elliptic phase diagram of theproteins. Thetheory predictsan elliptic region on thepressure-temperature plane, inside which the native state is more stable than the unfolded, while outside of the ellipse the unfolded stateis more stable (Fig. 2).Thetheory wasbased on theequilibrium thermodynamic description of a system containing only two states,namely the folded and unfolded states.

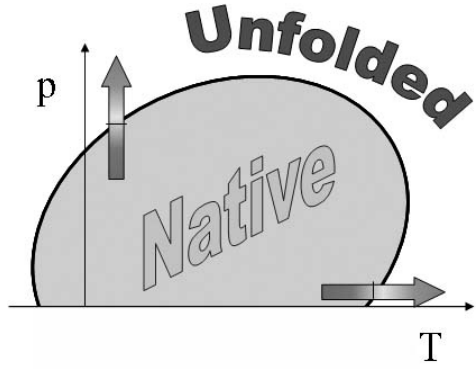


Figure 2. The elliptic phase diagram of the proteins (for further explanations, see text).

The Gibbs free energy difference

$$G=G_{\text{unfolded}}-G_{\text{folded}}$$

between the unfolded and folded state was written using the first and second order terms of p and T. The $G=0$ transition curve is mathematically a second order curve, because terms up to the second derivatives of G versus T and p were taken into account. This curve is an ellipse, in a certain range of the thermodynamic parameters, like the change in heat capacity (C_p), compressibility (κ) and thermal expansion (α) during the unfolding. However later it was pointed out that the Hawley theory has several serious limitations: 1) it assumes that C_p, κ, α are independent of the temperature and pressure, 2) the two-state system approach is too simple, there are intermediate and aggregated states, which have also to be taken into account. While the appearance of the third order terms due to the temperature and pressure dependence of C_p, κ, α does not change the elliptic shape drastically (Smeller and Heremans, 1997), the new states will change the phase diagram completely. The extension of the Hawley-diagram will be discussed later in this chapter.

There were several experimental investigations performed on a number of proteins to determine the actual shape of the elliptic curve (Zipp and Kauzmann, 1973; Heinisch et al., 1995; Zhang et al., 1995; Takeda et al., 1995; Panick et al., 1999; Torrent et al., 2001; Winter, 2002). The typical pressure for the protein unfolding is in the range of 5–10kbar (0.5–1GPa) at room temperature (Smeller, 2002). Some pressure sensitive proteins can denature at lower pressure values, even at 1–2kbar (Ruan et al, 2003). The pressure studies of aqueous protein solutions are limited by the freezing of water, which is slightly above 1GPa at room temperature. There are also extremely stable proteins, such as the heat shock protein MjHSP16.5 which was isolated from a thermophile (Tölgyesi et al., 2004).

The Hawley theory is a completely universal, phenomenological theory, without using any specific feature of proteins. It is not surprising therefore, that one can find similar elliptic phase diagrams for other systems, like liquid crystals (Cladis, 1988), polymers (Kunugi et al., 1997; Kato, 2005), and starch (Rubens and Heremans, 2000; Baks et al., 2008).

FOLDING EXPERIMENTS

The folding experiments all start with a sample, containing the aqueous solution of the folded protein. That is why the experiment must start with an unfolding step. The following (re)folding step is the actual subject of the investigation. Consequently there are two important technical parameters, the method of the unfolding (the nature of the unfolding 'agent') and the spectroscopic technique used in the observation of the folding process.

Themost widely used techniqueistheso called stopped flow technique (Table 1), where the protein is denatured by a chemical agent, usually urea or guanidine hydrochloride (GuHCl), and the solution is diluted by fast mixing of buffer and thedenatured protein containing solution (Volk, 2001). If theconcentration of thedenaturant decreasesbelowacritical limit, folding starts and can be observed by any appropriate spectroscopic methods. It hast to be mentioned, that stopped flow experiments are possible under pressure, (Heremans et al., 1980)but these equipments are used in study of enzyme kinetics rather than protein folding under pressure (Masson and Balny, 2005).

Alternatively onecan unfold theprotein by high pressureand thefolding can be observed after a fast decreaseof the pressure. This is known as the pressure-jump technique, which will be discussedlater in detail.

The fastest method to initiate protein folding or unfolding is the temperaturejump (Table1). In thiscasea fast heating of thesampleisinduced by a Nd-YAG laser pulse, which is Raman shifted in a hydrogen gascell, in order to obtain a pulse with the wavelength of 1.9 □m, which is absorbedby the water (falls in the range of the water vibration overtone). This way the sample is heated in its whole volume, avoiding delay and inhomogeneity coming from slow heat conduction. The typical temperature jump is 15°C which can be reachedin 10 ns, resulting in a heating rate over 10^9K/s. Since thisjump canonly beperformed in thedirection of theheating, thesubject of theexperimentsislimited to thoseproteinsthat can becold-unfolded above the freezing point of the solvent (Ballew et al., 1996aand b; Osvath et al., 2003).

Becauseof its high sensitivity fluorescence spectroscopy is used as a detection method in most cases.Detection of the fluorescence of intrinsic chromophores, mostly that of tryptophan residuesis widely used (Liu and Gruebele, 2007). Circular dicroism or infrared spectroscopy provide an alternative to fluorescence, but their time resolution is considerably lower. In Fourier transform infrared spectroscopy (FTIR) thereisa specialtechnique called 'step-scan', with a resolution in the range of ns, (Meilleur et al., 2004) but it can only be used with samples that can be unfolded-refolded in a reproducible way severalthousand times, which is not typical for proteins.

Thefolding timeof thevarioussecondary and tertiary structureelements is significantly different (Bieri and Kiefhaber, 1999). It is not surprising that the characteristicfolding time relatesstrongly to the size of the motif. The fastestfolding times are achieved by small loops. Alpha helicesformed by a nucleation process, require at least one turn formed at the beginning. Building up a beta sheet needs an order of magnitude longer time. The characteristicfolding timesof different polypeptidestructuresarepresented in the Fig. 3.

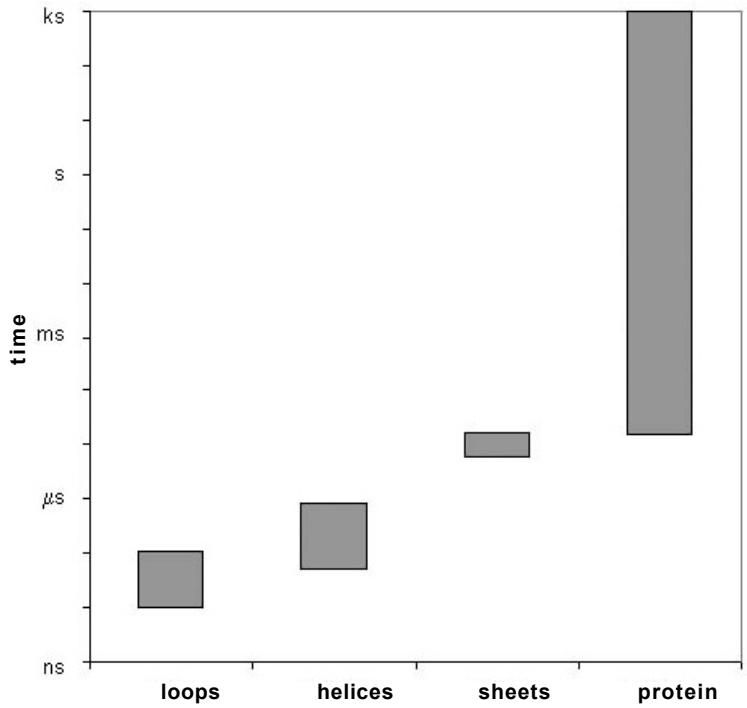


Figure 3. Characteristic folding times of different motifs.

PRESSUREJUMPTECHNIQUE

Pressure jump experiments have several advantages over the classical stopped flow technique. First of all, the pressure can be released completely, while the concentration of the chemical denaturant can never be diluted to zero. Unlike the temperature jump, pressure jump can be performed in both directions; one can observe both the unfolding and refolding kinetics (Ruan et al., 2003).

For applying pressure one can use two basically different methods. In the first approach one uses a strong pressure container, known as a thick wall cylinder, equipped with special windows usually made of sapphire for spectroscopic studies (Sherman and Stadmuller, 1987). The second approach is called diamond anvil cell (DAC), which compresses a very small sample between two diamond anvils (Jayaraman, 1986). There are several variants of these two main types with various types of pumps and windows for the cylindrical cell, or driving systems for the DAC.

The speed of the pressure jump is limited by the technique by which the pressure is created (Table 1). For pressure systems with the thick wall cylinder type pressure cell a pneumatic driven valve is used to change the pressure. In this way both positive and negative pressure-jumps can be achieved with the amplitude of 100 MPa in the pressure range from 0.1 to 600 MPa. The dead-time of the setup is about 5–10 ms (Woenckhaus, 2000; Winter, 2002; Ruan et al., 2003; Tan et al., 2006). Another group uses piezoelectric actuator for fast pressure jump (Jacob et al., 1999; Pearson et al., 2002). The cell used in this study is very simple; it contains a sapphire cylinder, with elastic sealing at the ends. The two ends of the cylinder are directly attached to a piezoelectric actuator and a pressure transducer, respectively. The first version of that cell could perform a pressure jump of a few bars (Clegg and Maxfield, 1976), but the design has been improved, the current limit is about hundred bars (few 10 MPa). This pressure step is performed within 50–100 □s (Jacob et al., 1999).

Table 1. Time ranges of the different techniques used in the folding studies.

Method	Time scale (sec)
Stopped flow	10^{-3}–10^{2}
Pressure jump	5 10^{-5}–10^{3}
Laser T-jump	10^{-8} –10^{-3}

A simple pressure jump system for FTIR spectroscopy has been recently constructed for pressures up to 10 MPa using the pumps of a high pressure liquid chromatograph (Schiewek et al., 2007) which resulted in a few ms decay time of the pressure.

For the study of the slow process of protein folding, or for the study of the aggregation, one can simply release the pressure by opening a valve by hand, or even turning the screw pump in a backward direction.

To my knowledge there are no fast pressure jump experiments published for the DAC technique. Several studies have been performed in DAC, however, on slow aggregation processes that followed the pressure release.

One needs to mention the adiabatic heating-cooling effect (depending on the direction of the jump) that accompanies pressure jumps. This has to be taken into account when the pressure jump is fast. The adiabatic heating-cooling causes a temperature change of less than 2°C/kbar (Winter, 2002). If the studied process is fast, one needs fast p-jump, and there is no time for temperature relaxation. The temperature, where the folding process occurs is different from the one before the p-jump, as a consequence of the adiabatic heating-cooling. Studying slow processes, it is possible to decrease the pressure slow enough to avoid significant temperature change due to the adiabatic effect. The first few seconds are in this case unuseable even if one uses fast p-jump, because the temperature is not constant in this range.

PRESURE-JUMPPROTEIN FOLDING EXPERIMENTS

A piezo-activated submicrosecond folding device was used by Jacobet al. (1999)to study the folding of the cold shockprotein of *Bacillussubtilis*.This is a small (66residue long) beta barrel protein, with no helices,showing a pure two-state folding behavior, which made it ideal for thesestudies.The limited amplitude of the pressurejump (up: 3□160 bar or down: 160□ 3 bar) made it necessaryto useadditional chemical denaturant. The pressure jump caused6% changein the fluorescencesignal if the jump was applied at the middle point of the unfolding curve, i.e.at 1.5M GuHCl. Comparing this change with the 50% fluorescence decreaseupon complete unfolding of the protein achieved by 3 M GuHCl, the pressureinduced equilibrium change was estimated as 78%–>54%. These values resulted in a rough estimate□V=60± 20cm^3/mol of theunfolding volumechange. In asystematic study of thewild typeand threemutant proteinsthey measuredthefolding and unfolding rate constants,repeating the pressure jump experiment at several temperatures. Theseexperiments allowed the determination of the thermodynamic parameters of the unfolding (□G, □H, □S, □C_p) and the corresponding activation parameters (□$G^{\#}$, □$H^{\#}$, □$S^{\#}$, □$C_p^{\#}$). In the three mutants one of the three phenylalanine residues of the central beta-sheet region was substituted by alanine. Thesesubstitutions did not changethe overall properties of the activated state relative to the unfolded state.The activated statewas found to be a native- type one.

Woenckhauset al. (2000)investigated thefolding and unfolding kinetics of a small single-domain protein called staphylococcal nuclease (SNase) using small-angleX-ray scattering.Thisprotein wasproven to fold reversibly, and served as a model protein in several previous stopped flow studies. In contrast to denaturant and pH-jump studies, pressure-jump folding was found to have a single exponential behavior (Vidugiris et al., 1995,Panicket al., 1998,1999;Woenckhaus et al., 2001).There was a big difference between the folding and unfolding ratesof SNase.While the folding after the jump from 400 MPa to 80 MPa proceeded with a short (4,5 s time constant, the unfolding at 300MPa had an almost 200 times longer time constant (14 min). The similarity of the folding activation volume (89cm^3/mol) and the equilibrium folding volume change (81 cm^3/mol) led the authors to the conclusion that the transition state is highly native-like.

Pressurejump experimentson the33 kDa protein of spinach photosystem (Ruan et al., 2001)showed folding and unfolding relaxation times in the same order of magnitude (100 and 125 s respectively). In a separate study thesamegroup investigated theeffect of sucroseand glycerol on thestability and kinetics of this protein (Ruan et al., 2003). All the rate constants decreased,but in both casesthe unfolding rate was influenced more than therefolding one.It wasproven earlier, that glycerol canlower theunfolding

volumechange(Oliviera et al., 1994)leading to thestabilization of thefolded state against pressure perturbation.

MISFOLDING, AGGREGATION AND PRESSURE

Pressuresensitivity of aggregatesand oligomers

Protein aggregates have a small surface compared to the same number of separate monomers, which means that the hydration shell is also smaller. This is the reason why these aggregates cannot form under pressure and tend to dissociate under pressure (Silva and Weber, 1988; Cheung et al., 1998; Gorovits and Horowitz, 1998; Smeller et al., 1999; Meersman et al., 2005).

In their seminal paper Silva and Weber (1988)reported the dissociation of thebromemosaic virusinto subunits. If thepressureduring thetreatment reached 140MPa the monomer units formed amorphous aggregatesinstead of re-associatinginto virus particles. This behavior was explained by the conformational drift theory, i.e. that thedissociated monomer unitsslightly changedtheir conformation, which preventedfrom forming a virusparticle again. Gaspar et al. (2001) compared the stability of enveloped and nucleocapsidparticlesof thealpha virusMayaro using hydrostatic pressure. Pressure dissociated the capsid particles but could not dissociate the ones with an envelope.

Pressure-dissociationof severalolygomer proteinswereobserved, among them hemoglobin (Schayet al., 2006)and the tetramer form of transthyretin (Ferraro -Gonzales, 2000). In caseof transthyretin, the pressure induced a conformation, in which the tryptophan residue become exposed to the solvent. Although only a small fraction of monomers was observed, the tetramer which appeared after thepressurecyclehad different conformation and increased aggregation capability (Ferraro-Gonzales, 2000). These aggregated conformations were characterized by Cordeiro et al. (2006)using FTIR spectroscopy.

An interesting application of the pressure sensitivity of aggregatesis the pressure assisted protein recovery (refolding) from inclusion bodies. (St John et al., 1999; Lee et al., 2006). Inclusion bodies are protein aggregates formed in thebacteria during biotechnological expressionof hardly soluble proteins. Pressure can dissociate these aggregates, and facilitate the correct refolding of theprotein under appropriateconditions(Randolph et al., 2002).

Infrared spectroscopy is a suitable tool for the simultaneous study of theconformation of theprotein and itsaggregation during and after pressure treatment. Myoglobin has been shown to have an increased aggregation tendency after a pressure unfolding-refolding cycle (Smeller et al., 1999). This was explained by a highly populated intermediate state, which was

folded or aggregatedcompetitively. Refolding of horseradish peroxidase,in contrary, did not populate aggregation prone intermediate states,unless,it was modified, in a way that decreased its stability (Smeller et al., 2003). Substitution of thehemegroup by a fluorescentporphyrine, or binding of a substrate did not lead to aggregation. Stronger destabilization, however, like the removal of the heme, cleavageof SS-bridgesor Ca^{2+}-removal, led to a metastableintermediate, and finally to aggregatedstates.

Similarly to myoglobin and destabilized horseradish peroxidases, lysozymewasalso found to form amorphousaggregatesafter an unfolding pressure cycle. Subdenaturing pressure treatment did not induce aggregation, which meansthat the intermediate state forms only after the unfolding of the protein. This conformation is metastableunder pressures lower than the denaturation limit (i.e. inside the elliptic shapeon Fig. 2).

Theseexperiments can besummarized by an energy landscape depicted in the Fig. 4.

At the end of this section it should be noted, that the term 'pressure induced aggregation' can cause confusion. It could give the impression that pressure induces aggregation, as it is inducing unfolding, i.e. the proteins aggregate *under* pressure. This is however not the case,becauseif one reads these publications carefully, the aggregation is detected after

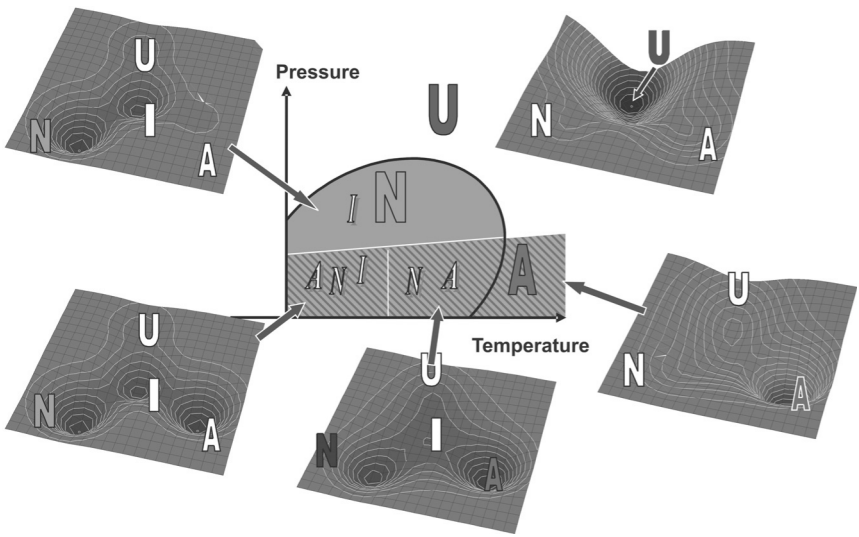


Figure 4. Pressure–temperature phase diagram of lysozyme and the corresponding Gibbs-free energy landscapes. Open letters show metastable states, corresponding to local minima on the landscape, while closed letters indicate stable thermodynamic states, which belong to global minima.
A: Aggregated; I: Intermediate; N: Native; U: unfolded.
(Reprinted from Smeller et al. (2006) with permission from Elsevier)

pressure release. The pressure treatment converts the protein into an aggregation pronestate, which isaggregating only *after*thepressuretreatment. Thename 'pressuretreatment induced aggregation' would bea moreprecise name for this phenomenon.

Pressuresensitivity of fibrous protein aggregates

Contrary to amorphous aggregatesand olygomers, mature fibers are quite pressure resistant. Dirix et al. (2005) studied the 105–115fragment of transthyretin using FTIRspectroscopyand AFM (atomic forcemicroscopy). While the early (amorphous) aggregatescould be dissociated by pressures ashigh as220MPa, the mature fibrils were resistant to 1.3GPa.This supports the idea that fiber formation needsmore flexible structure, but when the fibers are formed, their close packed nature makes them resistive against most physical and chemical intervention (Meersman and Dobson, 2006).

The high pressure tolerance of the matured fibrils observed in conformational diseases,limits the possible use of pressure as preventive treatment for foodstuff against the proliferation of the conformational diseases(Dubois et al., 1999;Heindl et al., 2006).Inactivation of the pressure resistant subpopulation of highly infectious prion subpopulations needed three cycles of pressurization at 800MPa and at 60 C, (Heindl et al., 2008) conditions which would denature almost all proteins.

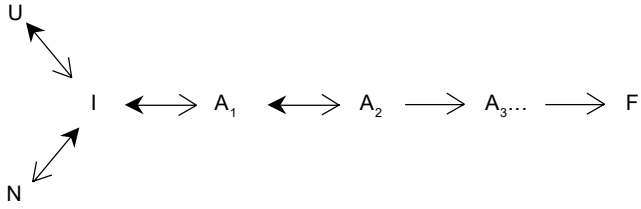
Radovan et al. (2008)studied a 37-amino acid peptide hormone, called islet amyloid polypeptide (IAPP), which is the principal component of the islet amyloid in type II diabetesmellitus. The deposition of theseamyloids in theextracellular matrix of beta-cells,leadsto islet cell death. Thefragments 1–19and 1–29were resistant to pressure treatment, which was explained by a more densely packed aggregatestructure with lessvoid volume and strong cooperative hydrogen bonding. They also observed that the 30–37 amino acidsregion of the peptide is lessdensely packed, leading to pressure sensitivity of thefull-length IAPP.

Sawayaet al. (2007)observeda 'steric zipper' like structure formed by segments of various amiloid proteins. This very efficiently packed, low volume structure can also be a key to the understanding of high pressure resistivity.

Protofibrilsof intrinsically denatured disulfide-deficient variant of hen egg white lysozyme have been found to have increased volume and compressibility (Akasaka et al., 2007). The volume increase during protofilament formation was found to be570ml/mol, which is a few times larger than the typical volume changeduring pressureunfolding. This can explain the increased pressure sensitivity of the nonmature aggregatesin accordancewith the FTIR studies on TTR peptide (Dirix et al., 2005).

CONCLUSION

High pressure can unfold proteins which consequently can be used for (re)folding studies. During therefolding, metastablestatescanbepopulated, which have increased aggregation propensity. The aggregation of proteins has a multistep nature, ending in very stable fibrous aggregates, which play a role in many serious conformational diseases, like BSE, CJD, Alzheimer's and Parkinson diseases. Mild pressure can reverse the amorphous aggregation, but the mature fibers are even more stable against pressurethan thenativefolded structure. Thefollowing schemesummarizes the possibleconformations of the proteins including folding / unfolding and aggregation pathways and the pressure effect on the conformational changes.



U: unfolded, I: intermediate, A_1A_2, A_3: aggregated to different extent, F> fibrous aggregate, N: Native pressure promotes the transitions in the direction of the full arrow.

As it can be seenfrom the scheme, pressure can be used to guide the protein on the folding-unfolding-misfolding pathway. Also somethermodynamic parameters, that can only be determined from pressure studies also have relevance in the chemical-biochemical processes occurring at ambient pressure.

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