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Pressure-induced amorphization in biopolymers

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Abstract

Pressure-induced unfolding of proteins in solution shows analogies to the pressure-induced amorphization observed in some inorganic and polymer systems. More specifically, pressure gives rise to conformations that show a strong tendency to form supramolecular aggregates that have some relevance to molecular diseases. Hydrostatic pressure can tune the conformation of the intermediates along the unfolding/aggregation pathway. Pressure can also be used to probe the stability of the aggregate, and thus the interactions that are responsible for it. In particular, we demonstrate that pressure might be an interesting tool to study the fibril formation. Fourier transform infrared spectroscopy reveals the presence of fibril secondary structures other than random coil and intermolecular β-sheet.

1. Introduction

Proteins can be denatured and/or unfolded by high hydrostatic pressure and this gives rise to an elliptic phase diagram in the temperature–pressure plane [1, 2]. The diagram gives a description of the conditions under which the protein is in the native or the denatured state at a given temperature and pressure with a specified solution composition.

The elliptic phase diagram does not give any information about the mechanism of the unfolding and the aggregation of the unfolded state. Nor does it give information on the possible role of intermediates in the unfolding process. Our previous studies, as well as those of other groups, indicate that the differences between the structural changes induced by temperature and pressure can be studied with FTIR spectroscopy [1–4]. The infrared spectra of most heat-unfolded proteins show two specific bands for the aggregated protein in the amide I′ band range at 1615 and 1685 cm^{-1}. These bands have been assigned to an intermolecular antiparallel β-sheet structure stabilized by hydrogen bonding. These aggregation-specific bands are not observed in the case of the pressure-unfolded protein.

In many *in vitro* studies of protein folding the formation of aggregates is usually considered as an undesirable side effect which obscures the folding process as such. However, because of its important role in a number of diseases, the mechanism of the formation of aggregates, and the possible role of folding intermediates, deserves closer attention. *In vivo* protein aggregates in the form of fibrils or amyloid plaques are the pathological hallmark of molecular diseases such as Alzheimer's disease and the transmissive spongiform encephalopathies (scrapie, mad cow disease and Creutzfeldt–Jakob) [5]. Their human and economic importance is immense. Recent studies on muscle myoglobin indicate that studies on model systems may help in obtaining a better insight into the molecular mechanisms underlying the formation of aggregates [6]. The general picture that is emerging from a number of studies is that under certain conditions proteins may acquire intermediate conformational states that have a strong tendency to aggregate.

Pressure- as well as temperature-induced effects are closely connected with the presence of water. However, pressure-induced amorphization has been observed in inorganic substances, liquid crystals and synthetic polymers [7] and starch [8] and this gives some new directions for the interpretation of the observed effects in water-soluble biopolymers. The relation to the water activity has important consequences for the mechanism of pressure-induced protein unfolding. The elliptical shape of the pressure temperature stability phase diagram connects the pressure-, cold- and heat-denaturation of proteins with the difference in compressibility, thermal expansion and heat capacity between the folded and the unfolded state. But whether the shape of the diagram can be entirely attributed to the properties of water is questionable. Although this has been suggested for the temperature effects on proteins, it has long been known that the physical properties of water become less anomalous under pressure.

In particular, the use of pressure to investigate the behaviour of biopolymers has received considerable attention. Several reasons can be found for this interest. Since high pressure affects different interactions than heat it represents an interesting way to tune the interactions that (de)stabilize a protein or an aggregate. On the one hand the formation of inclusion bodies in biotechnology is often undesired, whereas aggregation as a step towards gelation of food materials is needed in a controlled way. These are some of the processes that require a full understanding of aggregation and fibrillogenesis.

2. Experimental methods

Pressure- and temperature-induced changes in the proteins are studied with infrared spectroscopy (Bruker IFS 66). High-pressure studies are performed in the diamond anvil cell (Diacell products, UK).

Atomic force images were taken using a dimension 3000 microscope (Digital Instruments) equipped with a J-scanner. This microscope is provided with a built-in optical microscope which makes imaging possible after inspection of the sample. The scans were carried out in the tapping mode [9].

The ultrasonic measurements were performed using an acoustic interferometer [10] which was provided as a prototype by Resonic Instruments GmbH, Ditzingen, Germany. It is constructed as a twin interferometer consisting of two identical resonator tubes. The length of a tube is 7 mm and it contains a sample volume of about 250 μl. The wavelength of the ground wave is $\lambda_0 = 14$ mm and this corresponds to a ground frequency of about $f_0 = 107$ kHz. For the determination of the sound velocity we used overtones of the 65th up to the 70th order. The resonance frequencies are between $f = 7.0$ and 7.5 MHz and the averaged wavelength is $\lambda \approx 0.2$ mm. The readability of the measurements is $\Delta U = 0.001$ m s^{-1} and the reproducibility is $\Delta U = 0.020$ m s^{-1}. The temperature of the cell was adjusted at

25 ± 0.01 °C using a home-made thermostat chamber which enabled a temperature stability of $\Delta T \approx 10^{-3}$ K.

3. Results and discussion

Previous studies in our laboratory have shown that the pressure-induced unfolding of proteins in solution shows analogies to the pressure-induced amorphization observed in some inorganic and polymer systems. More specifically, pressure gives rise to conformations that show a strong tendency to form supramolecular aggregates that have some relevance to molecular diseases. Hydrostatic pressure can tune the conformation of the intermediates along the unfolding/aggregation pathway. Pressure can also be used to probe the stability of the aggregate, and thus the interactions that are responsible for it. In particular, we demonstrate that pressure might be an interesting tool to study the fibril formation. FTIR spectroscopy reveals the presence of fibril secondary structures other than random coil and intermolecular β-sheet.

Our previous work on the effect of temperature on the pressure-induced denaturation of myoglobin suggested that pressure-induced partially unfolded states may play a very important role in the aggregation of proteins. The high-pressure unfolding of horse heart metmyoglobin results in an intermediate form that shows a strong tendency to aggregate after pressure release. These aggregates are similar to those that are usually observed upon temperature denaturation [3].

3.1. The pressure–temperature stability diagram

The first phase diagram was established by Suzuki [11] who studied the kinetics of denaturation of ovalbumin and carbonylhemoglobin. Connecting the different experimental points with the same rate constant he found an elliptically shaped p, T, k-diagram. Since then an immense number of phase diagrams have been determined for many proteins, using different unfolding methods such as urea, pH and pressure. For an overview of elliptical phase diagrams determined so far we refer to reviews [1, 12]. Such diagrams are not only found for proteins, but also for starch [8], micro-organisms [13] and water-soluble synthetic polymers [14]. In contrast, they are not found for lipids, which show a linear behaviour [15].

The elliptical shape of the pressure–temperature phase diagram not only indicates the existence of the pressure and heat unfolding. It also shows an intersection with the temperature axis at low temperature and 0.1 MPa. This is the so-called cold-induced unfolding. A thermodynamic description is derived by integration of the change in the Gibbs free energy as a function of pressure and temperature, assuming that ΔV and ΔS are both pressure and temperature dependent. The equation contains the change in compressibility, thermal expansion and heat capacity between the native and the unfolded state. These are correlated with the volume, entropy and the coupling between entropy and volume fluctuations, respectively [1]. One of the limitations of this equation is the fact that only differences in the heat capacity, compressibility and thermal expansion between the native and the denatured state can be determined. In order to obtain absolute quantities, calorimetry, ultrasound velocimetry and densitometry experiments are required.

It should also be mentioned that, in regard to the following paragraphs, the phase diagram gives no information on possible folding/unfolding intermediates nor on the aggregation. It is a purely thermodynamic description, but can nevertheless be extended to kinetic data.

The shape of the phase diagram shows a likeness with the dome-shaped curves one obtains when plotting the Gibbs free energy change for proteins versus temperature. Based on this

agreement Klotz [16] argued that changes in protein structure are due to the temperature-dependent changes in water structure. However, such dome-shaped curves can also be found for pressure unfolding. Moreover, the behaviour of water becomes less anomalous at higher pressures. Thus, the suggestion that protein unfolding is due to changes in water structure is an insufficient explanation. Nevertheless, water is an important factor in the unfolding process. Dry proteins require very high pressures to induce conformational changes [17], whereas in solution pressures of 400–800 MPa are more than sufficient to cause protein unfolding [1, 18]. Several authors have emphasized the need of pressure-induced water penetration into the protein in order to achieve its unfolding [11]. This model is supported by high-pressure molecular dynamics computer simulations [19].

3.2. Pressure-induced amorphization

Pressure-induced disordering is contrary to the behaviour that one expects for most materials. However, in recent years it has been observed in a number of one-component systems such as H_2O, SiO_2, some other inorganic systems and synthetic polymers [7]. For water this can be interpreted as a struggle between the desire for compactness and the inclination for optimal hydrogen bonding between the water molecules. Similar mechanisms may play a role in other systems. In general they may be the crucial factor in systems where directional interactions play a dominant role.

For two-component systems it has been observed in water-soluble synthetic polymers [14], proteins [1] and starch [8]. The stability phase diagram for starch is similar to that of proteins. Infrared spectroscopy is particularly suited for *in situ* studies of these systems. If one considers the temperature and pressure effects on water and the relation to the stability diagram of biopolymers together with recently reported results on synthetic polymers in the absence of any solvent [7] then one may conclude that pressure, as well as temperature, causes changes in the interaction of the biopolymer with the solvent (hydration) but also that the imperfect packing effects in the interior (cavities) contribute to the re-entrant behaviour of aqueous solutions of proteins and starch.

Thus the pressure-induced amorphization in inorganic substances, liquid crystals and synthetic polymers gives new directions for the interpretation of the observed effects in water-soluble biopolymers.

3.3. Unfolding and aggregation

In what follows we shall discuss some factors that can influence the aggregation behaviour. In the next paragraph the emphasis will be on the role of pressure in understanding aggregation. We shall demonstrate that FTIR spectroscopy is particularly well suited for the study of aggregation processes. Firstly, because of the relatively long wavelength of the infrared light there will be no scattering when the particle size increases. Secondly, some aggregates are characterized by specific infrared bands.

3.4. Effect of pressure/cold pre-treatment

It is now generally accepted that the aggregating species is only a partially unfolded protein. Smeller *et al* [3] showed that the pressure unfolding of myoglobin results in a state that still contains secondary structure. After return to 0.1 MPa and 25 °C this state aggregates at a lower temperature than the native protein. This suggests that the pressure-unfolded state might represent an intermediate on the pathway to unfolding/aggregation. Likewise, Ferrão-Gonzales *et al* [20] found that pressure can turn transthyretin into its amyloidogenic state.

They referred to the pressure-unfolded protein as the pre-aggregated state. In this respect pressure can be a way to study aggregation intermediates that are not populated under other experimental conditions.

In a recent study we compared the pressure and the cold unfolding of myoglobin. We found that although not identical, both processes proceed in a similar manner and result in a partially unfolded state. In the case of lysozyme the cold-unfolded state was suggested to resemble an early folding intermediate. Thus, given the fact that the cold-and pressure-unfolded states are very alike, the pressure-unfolded state might represent some folding intermediate as well. This is what we found in the case of myoglobin. Also, when heated after return to ambient conditions the cold-unfolded state also showed an increased aggregation tendency, providing further evidence for the existence of partially unfolded states on the aggregation pathway.

3.5. Chemically reduced proteins

Chemical modifications of proteins (e.g. the cleavage of the disulfide bridges) or changes in the solvent composition (e.g. the pH) have a strong effect on the stability of a protein. This in turn affects the population of the intermediate conformation that shows a stronger tendency to aggregate.

We have investigated the effect of reduction of disulfide bridges in lysozyme on its pressure stability. We found that after pressure release the pressure-unfolded state readily aggregated. The spectrum of the amide I band (1600–1700 cm^{-1}) of the aggregated lysozyme shows the presence of the two bands at 1616 and 1683 cm^{-1} typical for intermolecular anti-parallel β-sheet aggregation. It may be assumed that the reduction of the disulfide bonds does not significantly change the conformation of the denatured state, but that it rather increases its flexibility. Thus these experiments indicate the role of flexibility in the aggregation process. Information on the flexibility of the reduced protein may be obtained from the compressibility.

3.6. Ultrasound as a tool for the characterization of intermediates

The impact of pressure on geometric properties is described by a number of elastic quantities. In the case of fluids a frequently used elastic property is the isotropic compressibility β which can be expressed as relative, specific or molar compressibility. To extract the contribution of the hydrated solute one can consider its partial molar adiabatic or specific adiabatic compressibility. The compressibility obtained from a solution is a quantity composed of contributions arising from the solutes as well as from free and bound water. One can divide the compressibility of a solution into several contributions [21, 22]. The intrinsic compressibility gives a positive contribution and arises from cavities created by the imperfect packing of the peptide chains. Hydration gives a negative contribution that arises from the change of the hydration shell during compression as a result of e.g. the pressure dependence of electrostriction. Finally, the relaxational contribution exceeds mostly not more than 10% of the intrinsic compressibility. Because it is assumed that hydration is correlated with the accessible molecular surface its contribution depends on the relation between molar volume and molar surface. Therefore, for small molecules the hydration part is the dominating contribution and for very large molecules its influence gets negligible and the measured compressibility equals the intrinsic compressibility.

If the compressibility is difficult to measure by direct methods one has to determine physical quantities, which have a straightforward relationship to compressibility. One of these quantities is the sound velocity U, which is related to the relative adiabatic compressibility β_S and the density ρ by the well known Laplace equation.

The determination of the sound velocity for liquid samples is usually done by ultrasound measurements because of the appropriate range of the wavelengths with respect to the size of the sample on the one hand and the size of the solute molecules on the other hand. Ultrasound velocimetry is thus a tool to access elastic properties of matter. Usually one applies acoustic interferometry [10] or pulse methods. One obtains thus an adiabatic quantity and if it is required to have the isothermal value β_T this can easily be calculated with the thermal expansion, the isopiestic heat capacity and the density. For proteins with a molar mass of $M_r < 100\,000$, an ultrasound frequency $f < 10$ MHz and a weight concentration $<2\%$ the difference between adiabatic and isothermal compressibility is negligible.

A further interesting relationship from statistical mechanics is the relation between structural flexibility (volume fluctuations) and the isothermal compressibility. In preliminary investigations we have indeed observed a change in compressibility between the native structure and the reduced conformation that shows a strong tendency to aggregate after pressure treatment.

3.7. Protein aggregation and molecular diseases

Molecular diseases are characterized by the presence an aggregated species under the form of fibrils. X-ray diffraction studies on these fibrils are found to have the same cross-β structure, whatever the native structure of the amyloidogenic protein. Furthermore, it has been suggested that under the right destabilizing conditions every protein is capable of forming such fibrils.

3.8. FTIR spectroscopy of fibrils

The diffraction pattern mainly shows the intermolecular β-sheets that constitute the fibril, as well as the spaces between the protofilaments that twist around each other to form the fibril. However, it is unlikely that the aggregated molecules consist only of sheet structure that is entirely involved in the intermolecular connections. Here FTIR spectroscopy might yield more information regarding the structure of that part of the molecule that is not involved in the intermolecular aggregation. Figure 1 shows the infrared spectrum of insulin fibrils between 1600 and 1700 cm^{-1}. The large band at 1627 cm^{-1} is normally assigned to β-sheet structure, but FTIR investigations of fibrils indicate that this intense band can be correlated with the presence of fibrils. Further evidence for this assignment comes from atomic force microscopy. This technique reveals other fibril details such as length, width, length of the twist, and whether the fibrils are branched or not. Absorption bands other than the 1627 cm^{-1} band can be seen, indicating the presence of other secondary structures. Structural analysis of these bands can reveal the presence of other structures other than random coil and intermolecular β-sheet.

3.9. Pressure effect on insulin fibrils

We have investigated the pressure stability of these fibrils. Figure 1 shows that the fibril band at 1627 cm^{-1} decreases already at pressures below 300 MPa. Pressure is known to cause dissociation of protein oligomers and protein aggregates [3] in this pressure region, but what is happening here still needs further investigation. Since increasing urea concentrations can cause the dissociation of the fibrils into their protofilaments it is often assumed that the interactions between the protofilaments are hydrophobic. Pressure affects these interactions as well, causing the reversible dissociation of the fibrils. This would make pressure an ideal tool for probing the protofilament interactions as well as for studying the formation of fibrils.

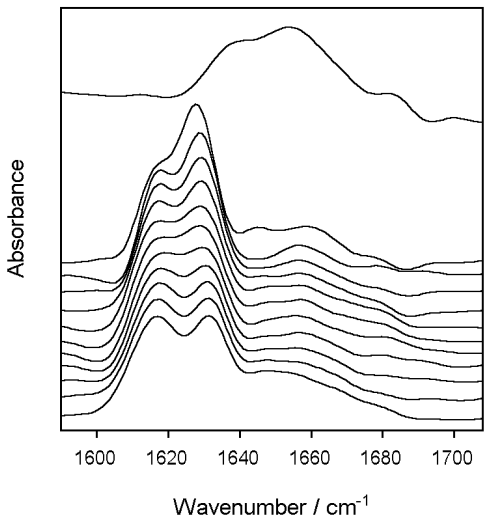

Figure 1. Amide I′ band of insulin. The top spectrum is the native state, characterized by the α-helix absorption at 1650 cm^{-1}. The stacked plot shows the effect of pressure on the insulin fibrils. Pressure increases approxmately 100 MPa/step from top to bottom.

4. Conclusions

The pressure unfolding results in the formation of a partially unfolded state. Its conformation resembles an early folding intermediate. This seems to be more aggregation prone than the native state. Thus pressure studies on proteins allow the population of certain intermediates on the pathway to folding/aggregation. Furthermore, the pressure effect on fibrils enables the study of fibril formation, as well as the interactions that are responsible for maintaining the fibril structure. Compressibility experiments would be of interest to further characterize the intermediate states. FTIR spectroscopy in combination with the diamond anvil cell is found to be particularly useful for these *in situ* high pressure studies.

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