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High pressure induces the formation of aggregation-prone states of proteins under reducing conditions

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Abstract

The pressure stability of ribonuclease A and bovine pancreatic trypsin inhibitor has been investigated with Fourier transform infrared spectroscopy in the presence of the disulfide bond reducing agent 2-mercaptoethanol. The secondary structure of the reduced proteins at high pressure (1 GPa) is not significantly different from the pressure-induced conformation of the native form. Upon decompression under reducing conditions, amorphous aggregates are formed. Such aggregates are not formed upon decompression of the native proteins. Our data demonstrate that high pressure populates, and thus allows the potential characterization of highly aggregation-prone conformations. The relevance of these findings with regard to fibril formation is discussed and the possible role of conformational fluctuations of intermediates on the aggregation pathway is emphasized.

Keywords: High pressure; Aggregation; BPTI; RNase A; Conformational fluctuations; Disulfide bond reduction

1. Introduction

Considerable effort has been put in gaining a better insight into the mechanism of protein aggregation, and in particular the formation of amyloid fibrils. Because of its human and economic consequences, of which Alzheimer's disease and the transmissible spongiform encephalopathies such as BSE are the best known, protein aggregation has attracted the attention of biotechnologists, pharmacologists and biochemists [1,2]. The general picture that has emerged so far is that a partially unfolded conformation is the aggregation-prone species [1,3–6]. One approach to characterize this species is to create solvent conditions in which the protein is destabilized. The right conditions can be achieved by reducing the pH, varying the ionic strength or by addition of, for instance, trifluoroethanol [7,8].

Recently, it was demonstrated that high pressure can be a valuable tool in the formation of a partially unfolded conformation. Smeller et al. [4] showed that pressure-treated myoglobin, when heated, aggregated at significantly lower temperatures than the untreated protein. Ferrão-Gonzales et al. [9] were able to convert native transthyretin after a compression–decompression cycle into a

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; RNase A, ribonuclease A; FTIR, Fourier transform infrared spectroscopy; H/D-exchange, hydrogen–deuterium exchange.

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species that forms amyloid fibrils under conditions where the untreated protein is stable. They also indicated that this species might well be an intermediate that is actually on the aggregation pathway. Both investigations suggest that in order to achieve the aggregating species, pressure treatment of proteins is a useful tool.

Why would an intermediate conformation have a greater tendency to aggregate than the native fold? Chalikian and Breslauer [10] investigated the change in partial specific adiabatic compressibility β_S^o accompanying conformational transitions. They found that the transition between the native and partially denatured state is characterized by a small decrease in β_S^o, whereas the transition from the native to the fully denatured state results in a large decrease of the compressibility. This suggests that the partially denatured state has a higher β_S^o than the fully denatured state. In other words, partially denatured states seem to be more dynamic than fully denatured ones.

In this paper we describe the effect of pressure on bovine pancreatic trypsin inhibitor (BPTI) and ribonuclease A (RNase A) under reducing conditions in the presence of 2-mercaptoethanol. Both proteins are paradigms in folding studies investigating the role of disulfide bonds [11]. Their pressure stability in the absence of any reducing agent is well documented [12–14]. Fourier transform infrared (FTIR) spectroscopy is our method of choice because it is highly sensitive to β-sheet [15], and because the most common form of aggregation displays a well-defined infrared pattern. The emphasis of this work is on the aggregation behavior and the role of the flexibility of the aggregating species.

2. Materials and methods

2.1. Sample preparation

RNase A and BPTI were purchased from Sigma (Bornem, Belgium) and used without further purification. All proteins were dissolved at 50 mg ml^{-1} in 10 mM deuterated Tris–HCl buffer (pD 7.6). Reducing conditions refer to the same buffer but in the presence of 300 mM 2-mercaptoethanol (Merck, Darmstadt, Germany). The samples were stored overnight to ensure complete H/D-exchange of all solvent accessible protons. Mercaptoethanol was present during overnight storage. All samples were centrifuged for 10 min at 12 100×g before use.

2.2. Pressure unfolding

High hydrostatic pressure is achieved using a diamond anvil cell (DAC) in combination with the MINICELL (Diacell Products, UK), which is the drive mechanism. The pressure is generated by means of a hand-driven knob and screw, acting on spring washers, moving a lever and presser plate mechanism. The lower presser plate drives a piston, on which a diamond anvil is mounted. The opposing anvil is held fixed on a plate, adjustable for tilt (www.diacell.co.uk). The choice of diamond anvils is based on the fact that diamonds are the hardest substance known, and because they are transparent to photons over a wide energy range [16]. Type IIa diamonds, especially, are used in infrared studies because they have a low absorption outside the relevant protein absorption bands. A stainless steel gasket, with a hole in it, is squeezed between the two diamonds, and has two functions: (i) it provides support to the anvils, (ii) it creates a chamber in which the sample can be contained [17]. The usual gasket thickness before compression is 50 μm, and the diameter of the hole is 0.5 mm. So the final volume of protein solution in the DAC is in the order of several nanolitres. The use of such a small volume has the advantage that there is no adiabatic heating during compression. Moreover, the MINICELL is connected to a thermostat, which allows adjustment of the temperature. In the present case all experiments were performed at 25 °C. After pressure increase/decrease the protein solution was allowed to equilibrate for 10 min before taking the infrared spectrum. Barium sulfate was used as an internal pressure standard [18].

Note that the short path length (50 μm) explains why we use a high protein concentration. The use of lower protein concentrations would require subtraction of the solvent contribution (even in a deuterated buffer). This is not a straightforward procedure and may result in artefacts. In addition,

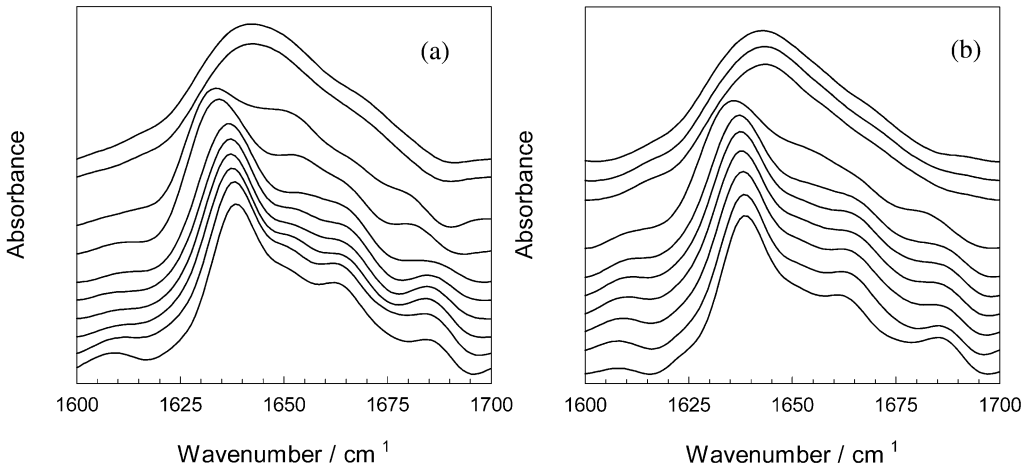

Fig. 1. The effect of increasing pressure on the deconvoluted amide I′ band of RNase A at 25 °C: (a) native protein, (b) protein in the presence of 300 mM mercaptoethanol. Pressure increases from bottom to top between 0.1 MPa and 1 GPa in steps of approximately 1 MPa.

this procedure would require solvent spectra at exactly the same pressure as the corresponding protein solution which is impossible to achieve with a DAC. Remark that under the given solvent conditions both RNase A and BPTI readily dissolve.

2.3. FTIR spectroscopy

The infrared spectra were obtained with a Bruker IFS66 FTIR spectrometer equipped with a liquid nitrogen cooled broad band mercury–cadmium–telluride solid state detector. Two hundred and fifty interferograms were co-added after registration at a resolution of 2 cm^{-1}.

Resolution enhancement was achieved by Fourier self-deconvolution, a mathematical technique of band narrowing, and was performed using the BRUKER software. The assumed line shape was Lorentzian. A half bandwidth of 21 cm^{-1} and an enhancement factor of 1.7 were used [19].

3. Results

3.1. Ribonuclease A

We investigated the pressure stability of RNase A in the absence and in the presence of the reductant 2-mercaptoethanol. Fig. 1 compares the deconvoluted amide I′ band of RNase A as a function of pressure under native and reducing conditions. The amide I′ band (1600–1700 cm^{-1}) is mainly due to the C=O stretching vibration of the protein backbone, and therefore sensitive to the secondary structure [15]. It can be seen that the spectra at 0.1 MPa and 25 °C are very similar. This suggests that not all disulfide bonds are reduced at ambient conditions.

In both cases the component bands at 1638, 1652, 1662, 1675 and 1685 cm^{-1} can clearly be seen at ambient pressure. These have been assigned to β-sheet, α-helix, turns, turns and β-sheet, respectively [13,14,20]. At 1 GPa, a broad band with a maximum at approximately 1644 cm^{-1}, typical for the disordered structure [13,14,20], can be found in both cases. This indicates that the overall structure of the conformational ensemble that makes up the unfolded protein is essentially the same for the reduced and the native RNase A. However, upon decompression the behavior of the reduced RNase A differs from the native protein. Whereas the native protein refolds to its native form, the reduced protein remains as its disordered structure down to 200 MPa. Below this pressure two distinct bands at 1612 and 1683 cm^{-1} start developing. These bands are characteristic of inter-

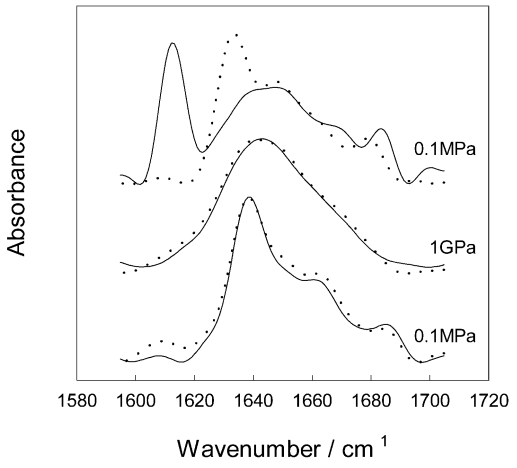

Fig. 2. The deconvoluted amide I' band of non-reducing (dotted line) and RNase A under reducing conditions (full line) at 25 °C and 0.1 MPa, 1 GPa and 0.1 MPa after decompression (from bottom to top). The spectra were normalized for the same overall intensity.

molecular anti-parallel β-sheet aggregation [21,22]. It should be emphasized that this is an amorphous, non-fibrillar form of aggregation as observed by electron microscopy [7,23].

For better comparison Fig. 2 shows the superposition of the spectra at 0.1 MPa, 1 GPa and 0.1 MPa after decompression. These correspond to the native and the denatured state and to the conformation obtained after pressure treatment, respectively.

Fig. 3a shows the position of the amide I′ band maximum of native RNase A as a function of pressure. The maximum is situated at approximately 1640 cm^{-1}, indicative of the high degree of β-sheet present [13,14,20]. Two transitions, with midpoints ($p_{1/2}$) at 600 ± 11 and 750 ± 2 MPa, can be observed. The first transition coincides with the H/D-exchange transition (Fig. 3a; $p_{1/2} = 605 \pm 4$ MPa), which causes a shift of the composing amide I′ peaks to lower wavenumbers [15]. The H/D-exchange can be followed by the disappearance of the amide II band at 1550 cm^{-1} and the increase of the amide II″ band at 1450 cm^{-1} [24]. The former band is mainly (60%) caused by the N–H bending vibration, while the latter is due to the N–D bending vibration [15]. The second transition is the actual unfolding transition, shifting the band maximum to approximately 1644 cm^{-1}, characteristic for disordered structure [13,14,20]. The completion of the H/D-exchange before the main unfolding can be explained by a transient global unfolding mechanism [25]. Under reducing conditions we can observe only one transition in Fig. 3b ($p_{1/2} = 640 \pm 0.8$ MPa), which roughly coincides with the H/D-exchange transition

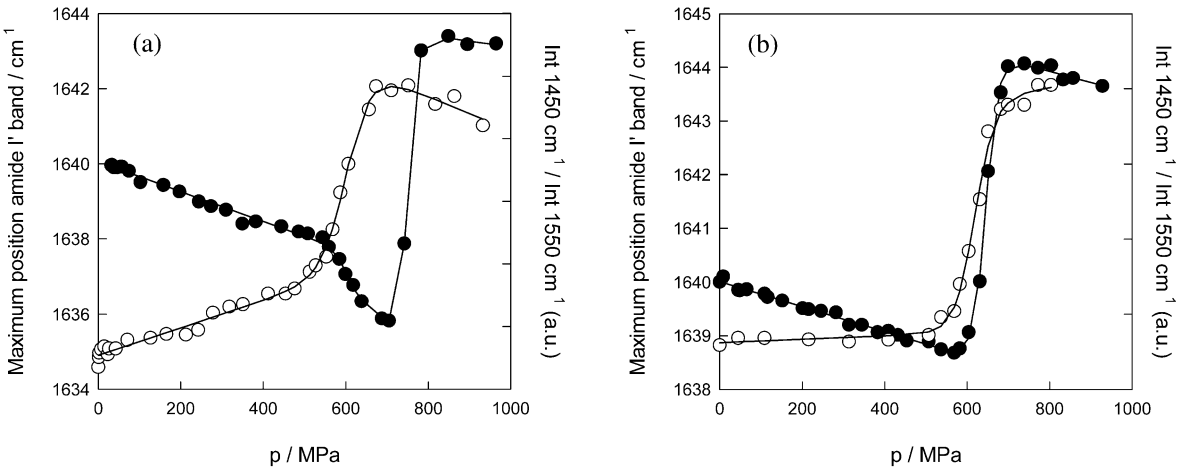

Fig. 3. The pressure dependence of the RNase A amide I′ band maximum (●), and of the ratio of the intensities at 1450 and 1550 cm^{-1} (○) (at 25 °C): (a) native protein, (b) protein in the presence of 300 mM mercaptoethanol.

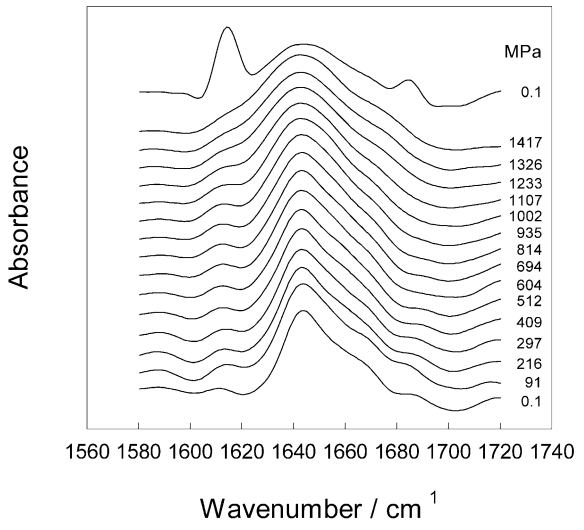

Fig. 4. The pressure-induced changes in the amide I′ band of BPTI in the presence of 300 mM mercaptoethanol at 25 °C (deconvoluted spectra). The top spectrum was taken at 0.1 MPa after decompression. Note the presence of the two bands at 1612 and 1683 cm^{-1}, indicating aggregation after decompression.

($p_{1/2} = 618 \pm 0.1$ MPa). This suggests that in the presence of 2-mercaptoethanol, RNase A unfolds as soon as the disulfide bonds become accessible to the solvent due to a transient global unfolding and are subsequently reduced.

At this point we would like to emphasize that under the experimental conditions used in this work only the solvent accessible protons will be exchanged for deuterons after overnight storage. The buried protons do not become exchanged until the protein unfolds, either transiently or non-transiently. Our approach, compared to fully exchanging the protein as suggested by Haris et al. [26], has the advantage that one can monitor other processes preceding or accompanying the unfolding by means of the H/D-exchange. However, note that the completion of the exchange will cause a shift of the amide I′ bands to lower wavenumber [26], but this does not affect our analysis.

3.2. Bovine pancreatic trypsin inhibitor

Fig. 4 shows the pressure dependence of the amide I′ band of BPTI in the presence of 2-mercaptoethanol. Two pressure regions can be distinguished. Up to approximately 400 MPa the width of the amide I′ band decreases, while all spectral features remain clearly distinct. The intense band at approximately 1643 cm^{-1} has been assigned to disordered structure which is also present in the native protein [12]. Above 400 MPa a gradual broadening of the amide I′ band takes place and the intensity of the 1643 cm^{-1} band decreases. The broadening is mainly due to the increase of the intensity of the bands at approximately 1624 and 1636 cm^{-1}, characteristic of β-sheet structure [13,14,20]. Above 1.0 GPa the unfolding of the reduced BPTI is complete. Pressure release results in the formation of two bands at 1612 and 1683 cm^{-1}, respectively, indicating aggregation of the protein (Fig. 4, top spectrum). These were absent in the pressure-treated native protein [12].

4. Discussion

RNase A contains four disulfide bonds, two of which are buried in the hydrophobic core of the protein, and two that are only partially buried [27]. Whereas the latter two may be exposed to the reducing solvent upon local unfolding, the former two require a global unfolding in order to become reduced. Thus, we expect that under the present experimental conditions at least two disulfide bonds are still present at 0.1 MPa. However, a correlation between the H/D-exchange in the absence of reductant (Fig. 3a) and the unfolding in the presence of mercaptoethanol (Fig. 3b) provides evidence that at approximately 600 MPa a transient global unfolding takes place. At that point the disulfide bonds are reduced and RNase A concomitantly unfolds. Our data also indicate that the secondary structure of the pressure-unfolded protein under reducing conditions does not significantly differ from the pressure-unfolded state of the native protein. This is also observed in studies of isothermally prepared nonnative protein states, using $H_2^{17}O$ magnetic relaxation dispersion [28], small angle X-ray scattering [29] and fluorescence resonant energy transfer [30], that have shown that even the unfolded states of reduced proteins are not random coils and that they bear significant

resemblance to the unfolded state of native proteins.

The pressure stability of BPTI in the absence of a reducing agent using FTIR spectroscopy was investigated by Goossens et al. [12]. They found that up to 500 MPa all observed changes in the FTIR spectrum are related to changes in bond length, hydration and cavities, rather than to a loss of structure [31]. This was confirmed by high pressure computer simulations [32]. Above 500 MPa a gradual transition from α-helix and disordered structure to β-sheet takes place. However, a cooperative unfolding transition could not be observed and all changes were reversible. In agreement with these observations we find that the pressure dependence of the amide I′ band of the reduced BPTI is also characterized by two phases below and above 500 MPa. But in contrast to the native BPTI we observe a complete unfolding rather than a structural re-arrangement. The assumption of a complete unfolding is based on the fact that at 1.4 GPa the amide I′ band is more or less symmetrical at approximately 1644 cm^{-1}, lacking a distinct shoulder at lower wavenumbers (1624–1636 cm^{-1}). Also, a plot of the pressure-induced structural changes indicates completion of the transition (not shown).

Goossens et al. [12] concluded that the native BPTI was not unfolded at 1.5 GPa based on the fact that secondary structural features, in particular the newly formed β-sheet, still existed at this high pressure. However, there is now sufficient evidence that for many proteins a persistent amount of secondary structure remains present in the unfolded state [33–36]. Thus, we suggest that even the native BPTI is unfolded at 1.5 GPa, albeit that the presence of the disulfide bonds poses some constraints on the remaining structure. This might explain the minor spectral differences between the native and the reduced protein at high pressure. Finally, an amorphous aggregate is formed upon decompression of the reduced protein.

Denisov et al. [28] argued that although the average structure of the denatured state of reduced proteins is not very different from the denatured state of the native proteins, their flexibility is likely to be affected to a larger extent. In addition, H/D-exchange studies have indicated that reduced proteins have a higher exchange rate [37]. This is also suggestive of an increased flexibility, in the sense of conformational fluctuations. Thus, we assume that, in the absence of any structural difference between the pressure denatured protein under native and reducing conditions, the latter undergoes larger conformational fluctuations, because it lacks the constraints imposed by the disulfide bonds. As a result the hydrophobic core of the reduced protein becomes likely more exposed, and therefore the reduced protein tends to aggregate more easily. It is generally assumed that exposure of hydrophobic patches that otherwise remain buried within the domains of persistent secondary structure allows the formation of intermolecular associations [1,38]. Evidence for this exposure comes from 8-anilinonaphthalene-1-sulphonate binding studies [37,39]. Moreover, the present type of aggregation is normally not observed after pressure treatment, unless at higher temperatures (>40 °C). Higher temperatures are also known to increase the amplitude of conformational fluctuations [40].

The fact that the aggregation does not take place above 200–300 MPa is due to the dissociating effect of pressure at higher pressures [4,41]. In contrast, temperature would cause such flexible structures to aggregate immediately. This shows that pressure is a useful way of conformational perturbation that allows the population and, potentially, the characterization of flexible, aggregation-prone species. This adds further support to the earlier high pressure work on myoglobin [4] and transthyretin [9].

It is interesting to consider the effect of disulfide bond reduction from the viewpoint of fibril formation. Recently, it was shown that a reducing environment is not a prerequisite for the conversion of the cellular form of the prion protein PrP^{C} into its pathological isoform PrP^{Sc}, that can associate into fibrillar structures [42]. Another study concluded that the reduction of the disulfide bridge in recombinant human prion protein at neutral pH results in the formation of amorphous aggregates [39]. Similarly this was also shown to be the case for the amyloid protein β_2-microglobulin [37]. However, at more acidic pH and in the presence of salts amyloid fibrils of β_2-microglob-

ulin did form. These results suggest that disulfide bond reduction is indeed no prerequisite for fibril formation. They also seem to indicate that the β-sheet-rich intermediate on the fibrillation pathway is not very flexible, since this gives rise to amorphous aggregates. On the other hand, a certain degree of conformational fluctuation may allow processes such as domain swapping to take place. It has been suggested that this phenomenon might be a first step in fibril formation [43,44]. Additionally, the amyloid-associated light chain of human immunoglobulin was shown to have a faster rate of H/D-exchange than the homologous non-pathological light chain, indicative of stronger conformational fluctuations in the former [38]. Thus, the flexibility of intermediate states may be an additional factor determining the outcome between refolding/unfolding, amorphous aggregation and fibril formation. The conditions (pH, salt, temperature and pressure) for the formation of these flexible intermediates and their experimental characterization requires further attention.

5. Conclusion

We have shown that complete reduction of disulfide bonds requires an unfolding that can be achieved by pressure. Moreover, the secondary structure of the reduced pressure-unfolded state does not significantly differ from that obtained under non-reducing conditions. However, under reducing conditions, decompression results in the formation of an amorphous aggregate. This shows the potential of high pressure to populate and characterize aggregation-prone species. It also highlights the possible importance of flexible intermediates in the aggregation mechanism of proteins

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