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High pressure as a tool for investigating protein–ligand interactions

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Abstract

In recent years, the application of pressure on biological systems has gained increasing interest. Pressure-induced destabilization of electrostatic and hydrophobic interactions is currently exploited to study conformational protein stability and macromolecular assemblies of proteins. Due to links between severe human pathologies and ordered protein oligomerization into aggregates, which have become apparent, a better knowledge of the molecular and structural determinants that ensure the packing efficiency and stability of such complexes has taken on special importance.

Here, we report the effect of pressure on the property of human ataxin-3 of aggregation. The results indicate the importance of its polyglutamine chain length in the stability and the tendency of the protein to form spheroids. Partial unfolding of the protein leading to solvent exposure of hydrophobic domains appears to be a prerequisite in the aggregation process of ataxin-3.

1. Introduction

Protein–ligand interactions are crucial for numerous biological processes—for example, cell signalling, transcription, translation, inter-membranous exchanges and enzymatic reactions. Protein self-assemblies can also be considered as protein–ligand interactions. Since the discovery of links between neurodegenerative diseases and features such as amyloidosis, Alzheimer's dementia, prions, ataxia and highly structured complexes of specific proteins (see for review [1–4]), the molecular basis of these reactions has also taken on special importance, in recent years. In most cases, non-covalent bonds such as electrostatic and hydrophobic interactions ensure the formation of the protein–ligand complexes. From a chemical point

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of view, this process can be represented as a reversible reaction, where the equilibrium state between reactants (A), (B) and the complex A:B is related to the thermodynamics of the system. However, in fact, protein–ligand interactions do not strictly follow this simple scheme. Non-exhaustive examples in literature show that molecular events such as local and long-range conformational changes of the protein (and potentially the ligand, depending on its nature (small molecules, nucleic acids or protein)) are often a prerequisite to the interaction of the two partners. From this observation, it is easy to realize that protein–ligand interactions are not restricted to a basic biological process but rather consist of a multistep reaction.

Within several methods used to study such interactions, hydrostatic pressure can be considered as the most appropriate perturbation for obtaining a detailed thermodynamic and kinetic description of biological phenomena. Indeed, pressure affects chemical equilibrium by favouring the dissociation of electrostatic bonds and the solvation of hydrophobic contacts. Thus, the fact that pressure-induced conformational changes are usually reversible offers the opportunity to trap putative intermediates and to determine the kinetic parameters [5–9].

To illustrate our proposal, we used the property of hydrostatic pressure to study the mechanism of the aggregation process of human ataxin-3, a protein involved in Machado–Joseph/SCA 3 disease [10].

2. Materials and methods

2.1. UV/visible spectroscopy

For all experiments, we used a thermostated high-pressure cell, equipped with sapphire windows, allowing experiments from atmospheric pressure (0.1 MPa) to 650 MPa [11]. For absorbance, the cell was connected at 180° through focused optical quartz fibres (200 μm diameter) to a Jobin-Yvon-Spex 30 W deuterium lamp and to a Jobin-Yvon-Spex 270 M spectrograph coupled to a UV-coated back-thinned Spectrum ONE^{TM} 512/512 array detection CCD 3500 camera (Jobin-Yvon-Spex Inc.). Another fibre served as a reference beam. A grating of 1200 grooves mm^{-1} was used, producing dispersion from 266 to 303 nm. The pixels were fully binned vertically (for intensity) and as 2 by 2 horizontally (for dispersion). The set-up produced data points separated by 0.15 nm with a spectral bandwidth of 0.6 nm. For each measurement, fifty transmission spectra (1.5 s integration time each) were accumulated. The absorbance was computed with the reference beam and corrected for the baseline (buffer). High-pressure experiments were performed in 25 mM Tris-HCl pH 7.5. The concentration of proteins was 1.5 mg ml^{-1}. Fourth-derivative spectra were computed as described in [11]. The fluorescence experiments were carried out at 25 °C using an SLM Series 2 luminescence spectrometer (Aminco Bowman) modified to accommodate a high-pressure cell. 8-anilinonaphthalene-1-sulfonate (ANS) and thioflavin T (ThT) binding were studied by exciting them at 350 and 450 nm (4 nm slit), respectively, and emission spectra (8 nm slit) were recorded from 400 to 600 nm and from 460 to 600 nm, respectively. The concentrations were 0.35 mg protein ml^{-1}, 700 μM ANS and 80 μM ThT.

2.2. Electronic microscopy

Aggregates were obtained by pressurization (650 MPa) or heating (90 °C) of human Q26 ataxin-3 protein solution at 2 mg ml^{-1}. The aggregates were diluted to a protein concentration of 0.2 mg ml^{-1}, deposited onto Formwar-carbon-coated grids and negatively stained with 2% aqueous uranyl acetate. The grids were examined using a JEOL 1200EX^2 electron microscope at an accelerating voltage of 80 kV.

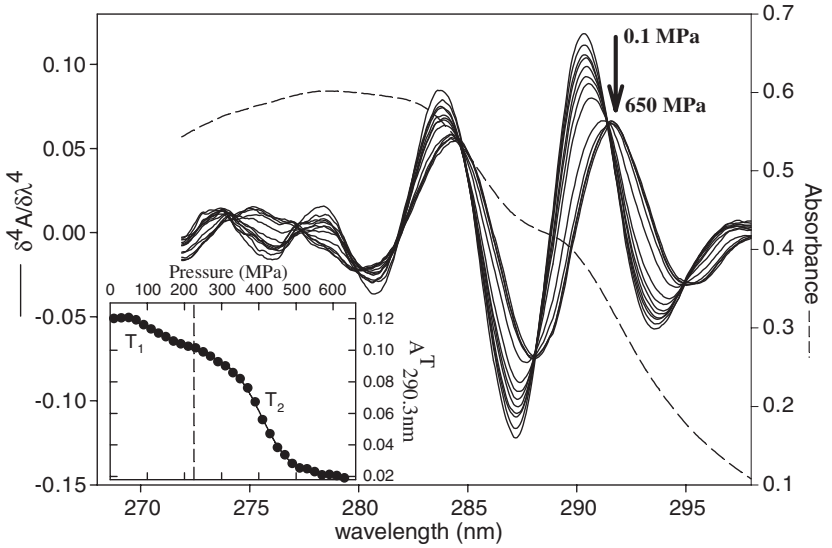

Figure 1. The effect of hydrostatic pressure on the conformational stability of ataxin-3 shown by UV spectroscopy at 25 °C. A representative view of fourth-derivative UV spectra of human Q26 ataxin-3 as a function of hydrostatic pressure between 0.1 and 650 MPa. Protein concentration: 1.5 mg ml^{-1} in 25 mM Tris-HCl buffer, pH 7.0. The pressure was raised in steps of 20 MPa from 0.1 to 650 MPa. Only intervals of 60 MPa are shown. For a comparison, the original zero-order spectrum at 0.1 MPa of the human protein is shown (dashed line, right-hand scale). Inset: the effects of pressure on the fourth-derivative amplitude in the tryptophan region. T1 and T2 refer to the first and the second transitions, respectively. The solid curve is the nonlinear least-squares fit of the experimental results against a multistate model.

3. Results and discussion

Ataxin-3 is the product of the gene responsible for Machado–Joseph disease (MJD)/spinocerebellar ataxia (SCA) type 3, one of the dominantly inherited cerebellar ataxias [12]. An abnormally expanded polyglutamine (polyGln) tract within the C-terminal part of the protein destabilizes the native α-helix-rich protein, which aggregates into amyloid-like β-fibrils [13] and forms neuronal intranuclear inclusions in the affected brains [14, 15]. This event is considered to play a crucial role in early pathological steps leading to the disease [4, 16, 17]. However, recent studies showed that the normal, non-expanded form of ataxin-3 also appears to be involved in aggregation processes. These observations prompted us to investigate the role of the polyGln chain length and its protein environment in the structural stability and aggregate formation of the normal, non-pathological ataxin-3. In the present study, we compared the conformational stability of a non-pathological variant of human ataxin-3, which includes a tract of 26 consecutive glutamines (Q26), with its murine counterpart, which has only six residues (Q6). These proteins display high sequence homology with a conserved N-terminal part, called the Josephin domain, and a C-terminal region where the polyGln sequence is located.

First, we used UV absorbance spectroscopy in the fourth-derivative mode to evaluate the pressure-induced conformational changes in the vicinity of tryptophan residues. As shown in figure 1, increasing pressure up to 650 MPa induced a significant red-shift and a decrease of the fourth-derivative maximum in the area (290–295 nm) corresponding to tryptophan residues for human Q26 (figure 1). Plots of the spectral amplitude at 290.3 nm as the signal of the transition against pressure exhibited a bi-sigmoidal unfolding profile, suggesting a

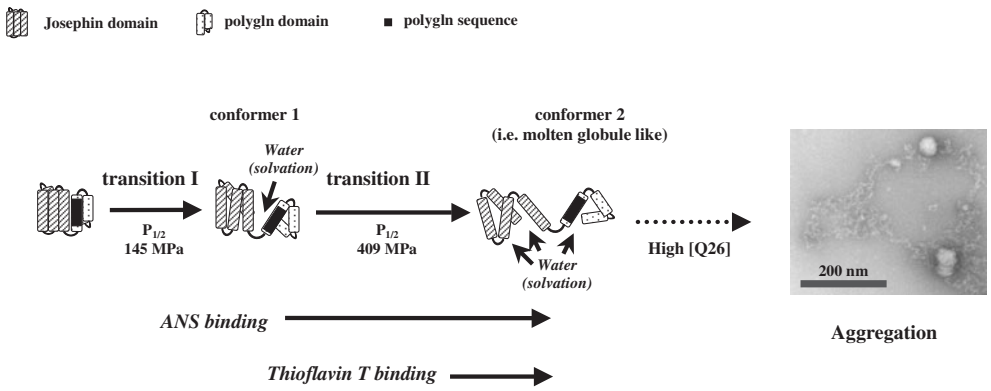

Figure 2. A mechanistic model of ataxin-3 unfolding. Pressure-induced unfolding of ataxin-3 is characterized by a complex mechanism, implying at least two steps and distinct unfolding intermediates within the experimental pressure range.

complex mechanism of unfolding for human protein (figure 1, inset). Indeed, using the environment-sensitive fluorescent dye 8-anilinonaphthalene-1-sulfonate (ANS) as a probe to reveal the solvent exposure of hydrophobic regions of ataxin-3, we observed that the pressure-induced structural changes affected the protein conformation at low pressure but remained incomplete even at 650 MPa (data not shown). In addition, ataxin-3 bound thioflavin T at high pressure, suggesting that the partly unfolded state of human protein may form soluble metastable structures. This observation was confirmed by the pressure-induced aggregation of highly concentrated Q26 ataxin-3 samples. In the case of murine Q6, the UV absorbance profile is consistent with a two-state transition and matched the transition T2 observed for the human counterpart (see figure 1, inset). Thus ANS bound to the protein at higher pressure than to human Q26, indicating that the murine protein was more resistant to pressure.

From the results described above, the pressure-induced unfolding of ataxin-3 can be schematized as illustrated in figure 2. Using fourth-derivative UV spectroscopy [11, 18], fluorescence and electron microscopy, we showed that two factors appear to govern ataxin-3 unfolding and aggregation: the length of the polyglutamine tract and its protein context. First, applying pressure up to 200 MPa induces a partial unfolding of the Q26 ataxin-3 (transition 1) in which tryptophan residues become more exposed to solvent. ANS binds to human Q26 from 200 MPa, indicative of solvent exposed hydrophobic domains of the protein. This suggests that the conformational stability of ataxin-3 in the low-pressure range depends mainly on the length of the glutamine chain. Pressure-induced weakening of the interactions at the interface between the Josephin and polyGln domains promotes the entry of water molecules. The local destabilizing effect of pressure is strengthened by the fact that the H-bonding potential of polar side-chain amide groups of glutamine residues favours interactions with water that reduce protein stabilization through H-bonded self-interactions [19]. Increasing pressure up to 650 MPa resulted in the detection of a new pressure-induced conformational state (conformer 2). An increased solvent exposure of tryptophan residues and the hydrophobic core of ataxin-3 mainly characterize this conformer. Thus, ANS binding experiments suggest that the high-pressure unfolded state resembles a molten globule-like structure, as already observed for several proteins at high pressure [20, 21]. In addition, this conformer binds thioflavin T at high pressure, suggesting that the partly unfolded state of ataxin-3 may form soluble metastable structures and aggregate at high concentration of human protein. Electron micrographs of negatively stained protein samples showed the simultaneous presence of

unstructured aggregates and of spheroid structures (100 nm in diameter). In some cases, these spheroids formed protofibrils of various lengths and orders.

Altogether, our results show that partial unfolding of the protein environment of the polyGln domain is a prerequisite in the aggregation process of ataxin-3. They are also in good agreement with the hypothesis of Perutz and Windle [4] who pointed out that the length of the polyglutamine sequence determines the tendency of ataxin-3 to aggregate. Thus, they suggest that the protein has a rigid and virtually undenaturable scaffold consisting of secondary structure elements, along with a flexible, overall tertiary structure. Therefore, these properties might represent the structural prerequisites that explain the capability of human ataxin-3 to modify its conformation without undergoing denaturation as a result of interactions with well-defined molecular partners found in the nucleus [22]. Placing our short report in the context of protein–protein interactions, the self-assembly of macromolecules into large complexes (fibrils, amyloids) is one important step, but this process is by no means a simple aggregation of macromolecules with predefined, rigid complementary structures [23]. In almost all cases the component molecules undergo either domain rearrangements or foldings of disordered portions (polar zippers, 3D domain swapping), which occur only following binding to the correct partners [24, 25]. As a consequence, the area in which the pressure technique may prove particularly fruitful in the future is in the study of the molecular basis of diseases of protein misfolding (see for a recent review [26]).

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