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High-pressure NMR techniques for the study of protein dynamics, folding and aggregation

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ABSTRACT

High-pressure is a well-known perturbation method used to destabilize globular proteins and dissociate protein complexes or aggregates. The heterogeneity of the response to pressure offers a unique opportunity to dissect the thermodynamic contributions to protein stability. In addition, pressure perturbation is generally reversible, which is essential for a proper thermodynamic characterization of a protein equilibrium. When combined with NMR spectroscopy, hydrostatic pressure offers the possibility of monitoring at an atomic resolution the structural transitions occurring upon unfolding and determining the kinetic properties of the process. The recent development of commercially available high-pressure sample cells greatly increased the potential applications for high-pressure NMR experiments that can now be routinely performed. This review summarizes the recent applications and future directions of high-pressure NMR techniques for the characterization of protein conformational fluctuations, protein folding and the stability of protein complexes and aggregates.

1. Introduction

Hydrostatic pressure has been widely used over the past decades to characterize the stability of globular proteins and protein complexes, mostly by fluorescence spectroscopy but also small angle X-ray scattering and infrared spectroscopy. The combination of high-pressure perturbation with NMR spectroscopy emerged in the mid-50s with the development of "autoclave" high pressure probes for which the entire radiofrequency transmitters and detection coils are placed in a high-pressure vessel [1]. This method was later developed by Jonas and coworkers for the study of biomolecules under pressure [2] but the incompatibility of this probe design with modern NMR probe electronics and thermal shielding has limited the further application of this approach. These limitations were largely circumvented by the development of pressure-resistant capillary cells that could be used with standard NMR probes and allowed the measurement of any multidimensional experiments [3], an approach that was then popularized by Akasaka and coworkers who used high-pressure perturbation to characterize the folding mechanism of numerous globular proteins [4]. Nevertheless, the capillary cell method also suffers from certain limitations, including the hand-made manufacturing of the cells and the small sample volume (about 40 μL). A large volume NMR tube capable of kilobars of pressure was first introduced by Wand and coworkers in 1996 using a novel method for joining a sapphire tube to a pressure manifold [5,6]. A subsequent shift in materials (to aluminum-toughened zirconia) and manufacturing process led to the development of the high-pressure NMR tubes currently commercially available (Daedalus Innovation™), rated to pressures up to 3 kbar [7]. These ceramic tubes with an inner diameter of 2.75 mm (3.0 mm for the tubes rated at 2.5 kbar) can be used with any commercial NMR probe and maintain a sensitivity of ~50% of a standard Shigemi™ tube with a similar sample volume [7].

When combined with NMR spectroscopy, high-pressure has been shown to be a very sensitive and perfectly reversible method of perturbation, allowing a detailed characterization of the factors governing the stability of globular proteins and protein complexes. Pressure can also be combined with other perturbation methods such as pH, temperature, or chemical denaturants to provide an in-depth description of protein free-energy landscapes. An overview of the high-pressure NMR techniques will be presented here, from the thermodynamic aspects of pressure perturbation to the detection and structural characterization of high-pressure conformers and the effects of pressure on protein folding equilibrium and kinetics. We will finally present a brief overview of the possible applications of pressure to study the stability of protein complexes and aggregates.

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2. Thermodynamic aspects

An increase in pressure shifts the thermodynamic equilibrium toward the states with the lower molar volume. When applied to proteins, pressure in a range of a few thousand bar tends to destabilize or completely unfold proteins by increasing the relative population of the lower volume unfolded states compared to the higher volume folded state [8]. Assuming a two-state folding reaction, the difference in free-energy can be expressed through a 2nd order Taylor series expansion around the reference pressure p_0:

$$\Delta G_u(p) = \Delta G_u^0 + \Delta V_u(p - p_0) - \frac{\Delta\beta_u}{2}(p - p_0)^2$$

where ΔG_u^0 stands for the free energy difference between the unfolded and folded states at atmospheric pressure, ΔV_u the volume change upon unfolding and $\Delta\beta_u$, the difference in compressibility between the unfolded and folded states.

Because the difference in compressibility upon unfolding is small for globular proteins [9], it is often assumed that the relative stability of the folded state with respect to the unfolded states changes linearly with pressure in the typical pressure range used in NMR experiments (1–3 kbar) (Fig. 1). Nevertheless, it has been observed in several cases that a non-null difference in compressibility is necessary to fully explain the experimental data [10,11].

The magnitude of ΔV_u values measured for globular proteins typically lies around 50–100 ml/mol, which represent only 0.5–2% of the protein's molar volume [8]. The intriguingly small magnitude of ΔV_u has generated a large number of different interpretations over the last 40 years. Brandts et al. pointed out that the small ΔV_u values measured for proteins likely originate from an almost perfect compensation of large magnitude negative and positive contributions [12]. They also noticed that due to the numerous negative contributions, as (i) the transfer of apolar groups (−23 ml/mol from methane model), (ii) the transfer of polar groups (−4.5 ml/mol from propanol), (iii) the exchange of a intrapeptide to a peptide-water hydrogen bond (−2 ml/mol), (iv) the ionization of amino and carboxyl groups (∼−10 ml/mol) and (v) the elimination of cavities and void volume in the folded states, the resulting ΔV_u values should be at least one order of magnitude larger than the actual values. The large difference between the expected ΔV_u and the experimental values, suggesting the existence of a missing positive contribution, has been termed the "protein volume paradox" by Chalikian and Breslauer [13].

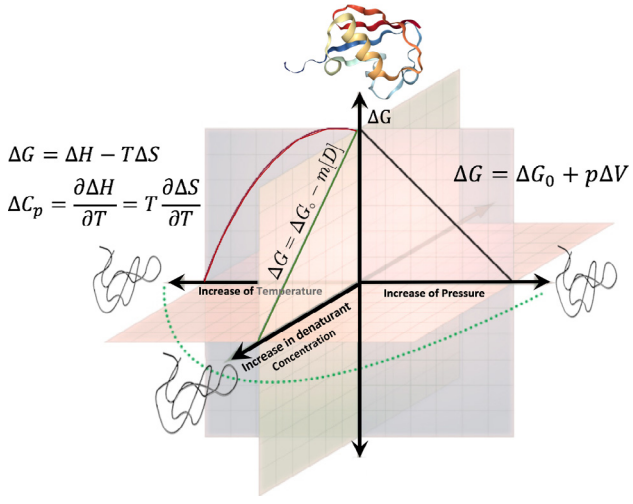

Fig. 1. Effects of pressure, temperature, and chemical denaturant on the unfolding free-energy of a globular protein. Because the difference in compressibility between the folded and unfolded states is very small, the unfolding free-energy, ΔG, decreases quasi linearly with pressure. The volume change upon unfolding, ΔV, corresponds to the steepness of the decrease of ΔG with pressure. The degree of pressure sensitivity of a globular protein (i.e. the magnitude of the ΔV value) is largely dependent on the amount of packing defects and internal cavities present in the core of the structure. Similarly, the unfolding free-energy decreases also linearly with an increase of chemical denaturant concentration. The so-called *m*-value is correlated with the difference in solvent-accessible surface area exposed upon unfolding. On the other hand, the temperature dependence of ΔG is more complex due to the fact the second-order parameter ΔC_p, the difference in heat capacity between the unfolded and folded states, is rather large for globular proteins.

Recent high-pressure NMR and computational studies have provided evidence that the elimination of the solvent-excluded internal voids due to imperfect protein packing, rather than the differential hydration of individual atoms, likely represents the largest contribution to the magnitude of ΔV_u [8,14,15]. Under the influence of high pressure, water molecules are believed to penetrate into internal cavities of the protein core and to induce the destabilization of hydrophobic interactions [14–16]. De Oliveira and Silva recently proposed a push-and-pull hypothesis to describe the opposite mechanisms of chemical and pressure denaturation of proteins [17]. In this model, urea molecules preferentially bind to the protein backbone creating a pulling effect, whereas pressure favored the hydration of the solvent-excluded cavities creating a pushing effect [17].

3. Pressure-induced chemical shift perturbation for the detection of low lying conformational states

The pressure dependence of chemical shifts has been recognized since the late 80's as exquisitely sensitive parameters to monitor subtle structural changes occurring within the folded state ensemble [18]. Akasaka and Li compiled the pressure dependence of ^{1}H chemical shifts for a set of 8 globular proteins and observed that the mean value of the chemical shift linear pressure dependence was similar for all the proteins under study [19]. This observation suggests a general, non-specific, downfield shift of the ^{1}H chemical shifts resulting from the compression of the hydrogen bonds at high-pressure. On the other hand, the non-linear pressure response of the ^{1}H chemical shifts showed much more variations among the 8 proteins and was attributed to the presence of low lying conformational substates within the folded states basin. Interestingly, the authors found a slight correlation between the mean value of the non-linear pressure response and the cavity density calculated from the protein structures [19].

Many efforts have since been directed at confirming the presence of these high-energy conformational substates which are commonly referred to as "low-lying" excited states because they lie within about 10 kJ/mol just above the lowest energy conformation at the bottom of the folding funnel [19–24]. As mentioned above, high-energy conformers are usually detected indirectly, by observing non-linear changes in the ^{1}H chemical shifts as a function of pressure. A general framework for the interpretation of these experimental data was proposed by Akasaka and coworkers through the "protein volume theorem" stipulating that *the partial molar volume of a protein decreases in parallel with the decrease of the conformational order* [25,26] (Fig. 2).

Further analysis on the effect of pressure on ^{15}N chemical shifts of different proteins by Kitahara et al. revealed that residues around water-excluded cavities exhibit large deviations from the average values, indicating again that cavities can be an important source of conformational fluctuation in globular proteins [27]. An example of such structural fluctuations around internal cavities has been recently reported in a study of the human prion protein, showing a correlation between the xenon binding sites and the regions exhibiting non-linear chemical shift perturbation as a function of pressure [28]. It has also been demonstrated for several globular proteins that high-pressure was able to stabilize partially

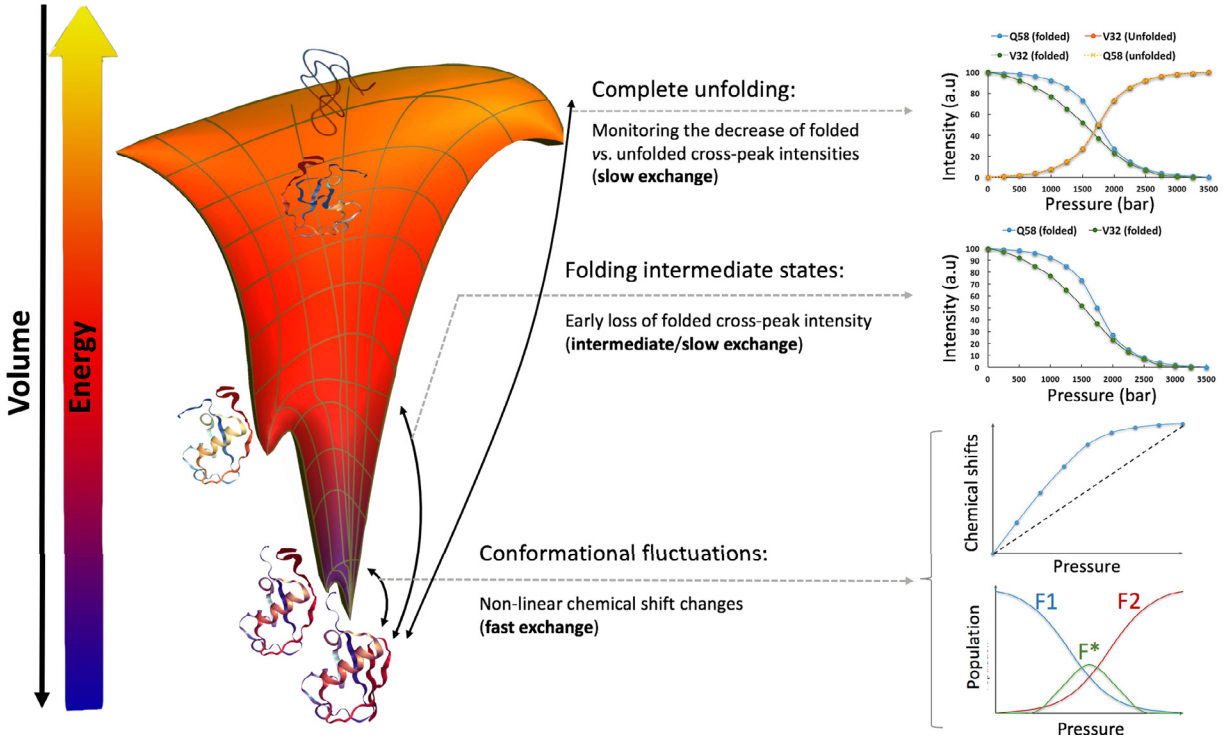

Fig. 2. Representation of the "protein volume theorem" described by Akasaka [25,26]: the higher energy conformational substates occupy a smaller molar volume than the lower energy conformers. Therefore, an increase in pressure tends to destabilize the native states and shifts the equilibrium toward the partially or completely unfolded states. Small pressure-induced conformational fluctuations around the folded state occur on a fast NMR time scale and are usually detected by monitoring non-linear chemical shift changes (usually ^{1}H or ^{15}N) as a function of pressure. This is illustrated here by the low-lying conformer F* populated at intermediate pressure. The presence of a distinct folding intermediate on an intermediate/slow exchange time scale can be detected by monitoring the decrease in the folded cross peaks intensity or volume as a function of pressure. Residues located in the regions that partially unfold in the intermediate states (V32 here) will show a greater pressure sensitivity than the residues located in the non-affected regions (Q58 the present example). Large variations in the residue-specific ΔV values are a strong indication of a non-cooperative unfolding process, underlying the presence of intermediate states. Ultimately, the complete characterization of the pressure-induced unfolding requires the assignment of the unfolded chemical shifts and the comparison for each residue of the intensity profiles measured for the folded cross peak with that of the unfolded cross peak (illustrated here in the top panel).

folded conformers prior to complete unfolding. The thermodynamic properties of such on-pathway molten globules or intermediate states have been characterized for the Ras binding domain of RalGDS [16], rPrP prion protein [29], ubiquitin [25], sperm whale apomyoglobin [30] and outer surface protein OspA [31]. In the case of β-lactoglobulin [32] the free-energy difference between the native state and an excited conformer was found to be in good agreement with ΔG_{HX} values measured from H/D exchange experiments [33], suggesting that the high-pressure stabilized conformers were similar to the "open" conformations sampled at atmospheric pressure.

Analysis of the effect of pressure was later extended to the ^{1}Hα and ^{13}C chemical shifts by Wilton et al. on protein G and barnase revealing that pressure induces a general upfield shift for these two nuclei [34], a tendency that was also observed in the case of the intrinsically disordered protein α-synuclein [35]. More recently, Kalbitzer and coworkers reported a series of careful parametrizations of the linear and non-linear factors (B_1 and B_2 respectively) for the pressure dependence of the ^{1}H, ^{13}C, and ^{15}N chemical shifts using simple model peptides [36–38]. These first order and second order parameters measured on model peptides are particularly useful in order to distinguish real pressure-induced conformational changes on a globular protein from the non-specific effects of pressure on chemical shifts. In addition, Kalbitzer and coworkers also recently published a more in-depth interpretation of non-linear chemical shifts changes based on protein structural fluctuations, showing that the ratio of the first- and second-order pressure coefficient B_1 and B_2 was related to the ratio of the compressibility $\Delta\beta'$ and partial molar volumes ΔV [39].

The origin of the ^{1}H chemical shift changes induced by pressure have been studied extensively and shown to be strongly correlated with the change of the hydrogen-bond lengths [19,40]. For an amide proton hydrogen bonded to an amide carbonyl, both the electric field effect and the bond magnetic anisotropy of the carbonyl double bond contribute to the chemical shift [27,41]. Thus, the downfield shifts of amide ^{1}H are thought to originate from the compression of hydrogen bonds induced by pressure. This effect has been directly measured by Grzesiek and coworkers with through-hydrogen bond $^{3}J_{NC'}$ couplings, describing the pressure and temperature stability of the hydrogen bond network of ubiquitin [42]. The opposite effect of pressure (downfield shift) and high temperature (upfield shift) on the ^{1}H chemical shifts, associated with a compression and elongation of the hydrogen bonds respectively, has also been recently highlighted in the case of α-synuclein [35]. For the ^{15}N and ^{13}C chemical shifts, the interpretation is more complex. ^{15}N chemical shifts, in theory, depend on hydrogen bonding to both amide nitrogen and amide carbonyl, backbone dihedral angles, and side chain orientations. La Penna et al. have recently published an interpretation of pressure effects on ^{15}N chemical shifts based on molecular modeling, correlating the chemical shift changes with the change in the population of hydrogen bonds involving the backbone amides [43]. On the other hand, pressure-induced changes of the ^{13}Cα shifts are thought to originate from slight compressions of covalent bonds [27,34].

In contrast to chemical shifts, much less is known about the effect of pressure on *J*-couplings. The effect of pressure on $^{3}J_{HN\text{-}H\alpha}$ couplings, which are very sensitive reporters of the backbone torsion angle ϕ, was first reported for α-synuclein, showing a general decrease of the $^{3}J_{HN\text{-}H\alpha}$ couplings by about 0.2 Hz at 2.5 kbar compared to atmospheric pressure [35]. These results suggest that as for chemical shifts, an increase in pressure might have an opposite effect to an increase in temperature on $^{3}J_{HN\text{-}H\alpha}$ couplings [35]. In contrast to $^{3}J_{HN\text{-}H\alpha}$ couplings, Kalbitzer et al. observed a general increase of $^{1}J_{N\text{-}H}$ couplings at higher pressure for the protein HPr from *Staphylococcus carnosus* [44]. A more recent study by Koehler et al. on $^{1}J_{N\text{-}H}$ response to pressure in model peptides and proteins revealed significant variations among different types of amino acids, with a larger effect of pressure measured for residues with a higher β-propensity [45].

4. Structural characterization of high-pressure intermediate states

Even if pressure is able to significantly stabilize conformational substates with a lower molar volume, a precise structural characterization of these non-native states represents a considerable challenge. Williamson and Akasaka first proposed a method to describe the structural changes occurring at high-pressure from the pressure dependence of the chemical shifts. This method was successfully applied to BPTI [46] and hen lysozyme [47], revealing a global shortening of H-bonds and large structural changes for residues close to buried water molecules. In 2005, Kitahara et al. reported the first de novo structure determination of a high-pressure stabilized conformer for ubiquitin [22]. Increasing pressure from 1 bar to 3 kbar was known to shift the equilibrium between the native states (N) and a low lying excited states (N2), as a consequence of the lower molar volume of N2 (i.e. $\Delta V_{N2\text{-}N} = -24$ ml/mol) [20]. By collecting more than a thousand distance constraints and 80 torsion angle constraints at 3 kbar, the authors were able to determine an atomic structure of the low lying excited states N2, which they later found to be close to the structure of the mutant Q41N [48]. The structure of the high-pressure conformer N2 is characterized by a partial opening of the protein core at the C-terminal side and a 5% increase of the surface area with respect to the native structure. Additionally, spin relaxation analysis revealed that the conformational exchange between N and N2 populations occurs on a time scale of about ten microseconds, with an activation volume of +18.5 ml/mol for the N2 to N reaction and −4 ml/mol for the N to N2 reaction [22]. Several MD simulation studies have later shown that this conformational change promotes the penetration of water molecules in the protein core [49–51].

Residual dipolar couplings (RDC), which are very sensitive reporters of the time-averaged orientation of inter-nuclear vectors, represent also a promising strategy for the characterization of high-pressure conformational substates. Several groups have shown that various alignment media, including Pf1 filamentous phage, C12E5/n-hexanol mixture and dinucleotide d(GpG), can be used to measure RDC in high-pressure conditions [52–54]. RDCs measured at 600 bar in a weakly oriented solution obtained by the addition of squalamine were recently used as restraints in all-atom molecular dynamics simulations in order to characterize the pressure-induced structural changes in the mature HIV-1 Protease [55]. The results showed that the glycine-rich flaps, known to be critical to the catalytic activity of the protease, tend to adopt a wide-open conformation at high-pressure, which promotes the penetration of water molecules into the subunit hydrophobic cores [55].

5. Use of high-pressure NMR to characterize protein folding cooperativity

An important application of high pressure NMR spectroscopy is the investigation of protein folding cooperativity and the detection of folding intermediates. The atomic resolution offered by NMR experiments provides an intrinsic multi-probe approach to assess the degree of protein folding cooperativity, which is otherwise difficult to characterize using techniques such as circular dichroism or fluorescence. In addition, the high reversibility of pressure unfolding/refolding experiments ensures a proper thermodynamic characterization of the process, which is often problematic to asses by heat denaturation because of excessive aggregation. The pressure-induced unfolding reaction, which generally occurs on a slow NMR time-scale, is usually analyzed by monitoring the decrease in cross-peak volume or intensity in a series of $^{1}H\text{-}^{15}N$ experiments recorded at increasing pressure. This type of experiments can be analyzed by individually fitting the intensity profile of each residue as function of pressure, yielding residue-specific apparent ΔV_u values [15]. Large variations in the ΔV_u values measured for different residues of a given protein typically reflect departure from an ideal cooperative unfolding transition and inform on the potential presence of intermediate states [15,56].

A problem often faced when monitoring such intensity profiles is that the observation is typically limited to the cross-peaks of the folded state. Whether the loss of intensity is due to local unfolding or to the presence of a high-energy substrate with a distinct structure remains in most cases undetermined. This has led to a recent controversy on the nature of the pressure-induced unfolding pathway of T4 lysozyme [57,58]. The first study by Wand and coworkers reported that the cavity-containing C-terminal domain of the L99A mutant of T4 lysozyme was completely unfolded at moderate pressures while the N-terminal domain still remained largely folded even at a pressure as high as 2.5 kbar [57]. Shortly after, Kitahara and coworkers published a study of the same mutant L99A of the T4 lysozyme but reached a slightly different conclusion [58]. The authors proposed that the loss of signal intensity with increasing pressure observed for the methyl groups of the C-terminal domain was due the presence of a low-lying excited state rather than caused by a partial or complete unfolding of the domain [58]. In favorable cases, a way to address such ambiguities in interpretation is to compare for each residue the intensity profiles of the folded and unfolded cross peak, as recently demonstrated for the mature HIV-1 Protease [56]. However, the complete assignment of the pressure denaturated state is often very challenging.

Residues-specific unfolding data can also be analyzed in terms of fractional contact map, defined as the product of the normalized intensities measured at a given pressure for the amide resonances of the two residues forming a native contact [15,17,59]. This approach was inspired by the analysis method used by Munoz and coworkers to characterize the thermal unfolding of the downhill protein BBL [60,61]. Fractional contact maps determined from pressure unfolding experiments can also be combined with Go-model simulations to obtain a structural representation of the conformal ensemble populated at high pressure [15].

6. Effect of pressure on protein dynamics and kinetics

Under pressure, the rate of any conformational exchange is exponentially dependent on the magnitude of the volume of activation, $\Delta V^{\ddagger}$ (i.e. the difference in volume between the ground states and the height of the free-energy barrier). It has been consistently observed that exchanges occurring on very fast time scale, such as picosecond or nanoseconds backbone fluctuations, are

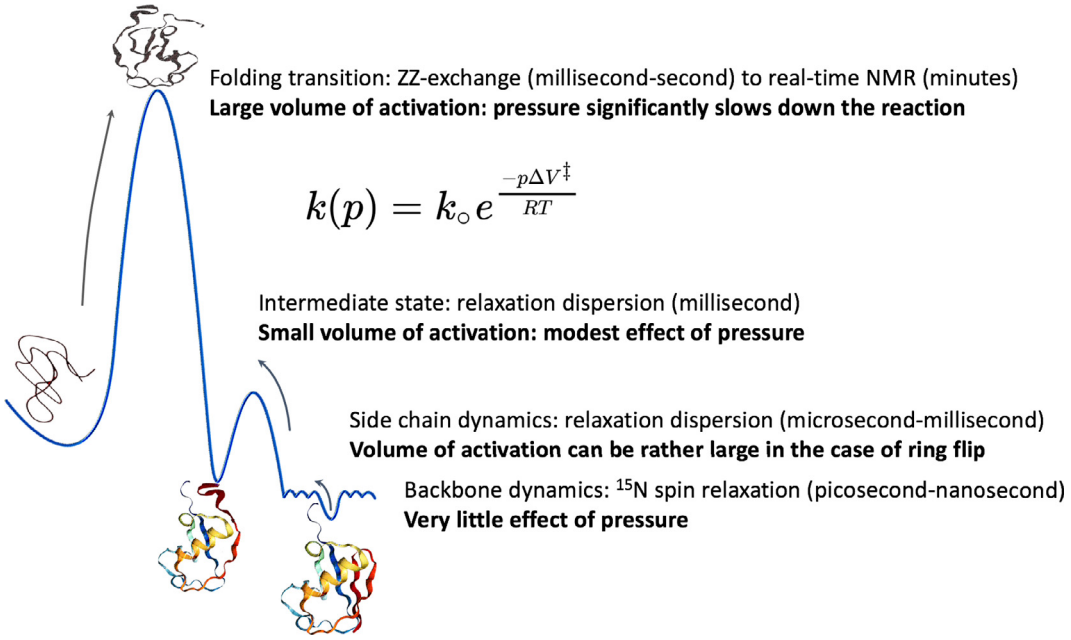

Fig. 3. Illustration of the effects of pressure on the rate of conformational exchanges. Backbone fluctuations taking place on a picosecond to nanosecond time scale are usually not sensitive to pressure, while conformational exchanges occurring on a millisecond time scale can potentially be slowed down significantly by pressure, depending on the magnitude of the volume of activation involved in the reaction. The volume of activation for folding is usually large for most globular protein, which can result into a slowdown of the folding reaction by several orders of magnitude under high-pressure conditions [70]. Therefore, folding transitions that were too fast for a direct detection by standard NMR techniques at atmospheric pressure can become accessible to ZZ-exchange or real time NMR experiments at a 1–2 kbar pressure range.

not very sensitive to pressure while the rate of conformational exchanges occurring on a much longer time scale, (i.e. folding or unfolding reaction) can be extremely sensitive to pressure (Fig. 3). Indeed, ^{15}N spin relaxation measurements have shown no effect of pressure on the ps-ns backbone dynamics of an isolated alpha helix [62], a globular protein such as ubiquitin [22] or an intrinsically disordered protein as α-synuclein [35]. Fu et al. reported the first study of sub-nanosecond motion of side chains in ubiquitin at 1 kbar using a combination of ^{2}H methyl side chain relaxation and ^{15}N relaxation and interestingly observed a non-homogeneous response to the application of pressure, reflecting the differences in compressibility within the protein structure [11]. In a subsequent study, Wand and coworkers combined ^{13}C with ^{15}N relaxations to characterize the sub-nanosecond internal motion of aromatic-ring systems in ubiquitin as a function of pressure and temperature [63]. A sharp thermal dynamical transition was observed at 312 K for the aromatic side chains and the amplitude of motion was shown to decrease at higher pressure [63].

The pressure dependence of the aromatic ring flip rate, occurring on the microsecond-millisecond time scale, was first determined for BPTI through line shape analysis by Wagner [64]. Later on, Li et al. reexamined in more details the effect of pressure on these motions and reported a 3–7 times slowdown of the flip rates of aromatic ring of Y35 and F45 in BPTI at 1 kbar, revealing large positive volumes of activation for the rotational motion of these side chains [65]. Pressure has also been used to probe the volumetric parameters accompanying the two-state folding of the F61A/A90G mutant of apocytochrome b_{562} using relaxation dispersion experiments [66]. More recently, high-pressure relaxation dispersion NMR was also used to uncover the energetic and volumetric properties of a three-state folding process of a metastable variant of the Fyn SH3 domain by introducing a slight perturbation of the equilibrium between the folded, the unfolded and the intermediate states [67]. On a much slower time scale, Fuentes and Wand examined the local stability and dynamics of apocytochrome b_{562} by recording H/D exchange experiments under high pressure conditions and found three regions of local cooperative stability similar to those detected by perturbation with chemical denaturant [68]. These observations contrast with a more recent study of the effect cavity-creating mutations on the folding free-energy landscape of Staphylococcus nuclease (SNase), which shows that chemical denaturation and pressure perturbation affect differently the folding cooperativity of the protein and the relative population of intermediate states [59].

When applied to the complete unfolding/folding transition, high-pressure can slow down the rate of folding, and also possibly unfolding, by several orders of magnitude due to the very large volumes of activation involved in these reactions. The activation volume for folding, $\Delta V_f^\ddagger$ is usually large and positive (i.e. the molar volume of the transition state ensemble is larger than that of the unfolded states) [69], while the activation volume for unfolding, $\Delta V_u^\ddagger$, can be either negative or positive. A remarkable example of such a dramatic effect of pressure was reported for a series of variants of SNase, with a decrease of the folding rate by more than 5 orders of magnitude at 1000–2000 bar compared to atmospheric conditions [70]. In this study, relaxation after pressure-jumps was monitored at a residue-specific resolution by real-time NMR using the fast 2D experiments introduced by Schanda and Brutscher [71]. The data revealed that SNase variants with cavities in the central hydrophobic core showed highly heterogeneous transition-state ensembles, while those containing a cavity in the peripheral region of the protein only showed slight differences in comparison to the wild type SNase. Additionally, these results further confirmed the more local effect of pressure perturbation as opposed to the global effect of temperature and chemical denaturants [70].

Very recently, Zhang et al. introduced a high-pressure ZZ-exchange NMR method to investigate the transition states of protein folding [72]. By combining the effect of high pressure with a ZZ-exchange NMR experiment, the authors were able to obtain residue-specific folding rates for the two autonomous N-terminal and C-terminal domains of the ribosomal protein L_9, indicating that N-terminal (NTL_9) folding is a two-state process. Deviations from this two-state process, however, were observed for the C-terminal

region (CTL_9). More importantly, large positive activation volumes for folding were reported for both NTL_9 and CTL_9, indicating that their transition states still contain the majority of the solvent-excluded voids found in the cores of the native ensembles [72]. In addition, high-pressure ZZ-exchange experiments provide a relatively easy method to assign the $^{1}H/^{15}N$ chemicals shifts of the pressure-induced denatured states.

7. Pressure-induced dissociation of protein complexes and aggregates

Because high-pressure shifts the thermodynamic equilibrium of a system toward its lower volume states, pressure tends to dissociate protein complexes and aggregates in favor of the monomeric species [73,74]. High-pressure NMR was, for example, recently used to characterize the monomer-dimer equilibrium of the mature HIV-1 protease and estimate the change of volume associated with the dissociation of dimer [56]. In the case of amyloid aggregates, the susceptibility to pressure is largely governed by their degree of compaction; mature fibrils being less sensitive to pressure because of their tight packing, while early aggregates are often more rich in internal cavities and therefore more sensitive to pressure-induced dissociation [75]. A nice example of the application of high pressure to probe the stability of a protein aggregate was recently demonstrated by Zweckstetter and coworkers [76], who used a combination of high-pressure NMR and molecular dynamics simulations to study the effect of Ser 8 phosphorylation on the stability of Aβ aggregates. They observed that aggregates formed by phosphorylated peptides were more compressible than the non-phosphorylated species. The conformational fluctuations of amyloid peptides, Aβ and hIAPP, have also been analyzed by high-pressure NMR using non-linear chemical shift changes to detect the presence of partially ordered substates [77,78].

8. Conclusion

Pressure perturbation offers unique opportunities to finely tune the stability of a globular protein or to modulate the rate of a conformational exchange in a completely reversible manner. The brief overview presented here shows that high pressure NMR techniques can be successfully applied to gain a better understanding of a large variety of fundamental processes, ranging from protein dynamics to protein folding and protein-protein interactions. Future research directions will likely lead to a broader application of high-pressure RDC measurements, which offer very exciting prospects for the detailed characterization of intermediate states, but also of kinetic methods such as ZZ-exchange or relaxation dispersion for a precise determination of the rates of conformational exchange and folding under high-pressure conditions.

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