Perspectives in Magnetic Resonance

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.

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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 $p_0$: 

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

where $\Delta G_u^0$ stands for the free energy difference between the unfolded and folded states at atmospheric pressure, $\Delta V_u$ the volume change upon unfolding and $\Delta b_u$, 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 $\Delta V_u$ 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 $\Delta V_u$ has generated a large number of different interpretations over the last 40 years. Brandts et al. pointed out that the small $\Delta V_u$ 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 tripeptide 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 $\Delta V_u$ values should be at least one order of magnitude larger than the actual values. The large difference between the expected $\Delta V_u$ and the experimental values, suggesting the existence of a missing positive contribution, has been termed the “protein volume paradox” by Chalikian and Breslauer [13].

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 $\Delta V_u$ [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
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 DHX 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 1H and 13C 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 (B₁ and B₂ respectively) for the pressure dependence of the 1H, 13C, and 15N 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 shift changes based on protein structural fluctuations, showing that the ratio of the first- and second-order pressure coefficient B₁ and B₂ was related to the ratio of the compressibility ΔP and partial molar volumes ΔV [39].

The origin of the 1H 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 1H 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 3JHN 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 1H 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 15N and 13C chemical shifts, the interpretation is more complex. 15N 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 15N 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 13Cα shifts are thought to originate from slight compressions of covalent bonds [27,34].
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 assay 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-$^{15}$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 Δνu values [15]. Large variations in the Δνu 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 peaks, 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 u values [15]. Large variations in the 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].

### 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, ΔV^c (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
in intrinsically disordered protein as a α-helix [62], a globular protein such as ubiquitin [22] or an no effect of pressure on the ps-ns backbone dynamics of an isolated in ubiquitin at 1 kbar using a combination of 2H methyl side chain reported the first study of sub-nanosecond motion of side chains motion of aromatic-ring systems in ubiquitin as a function of pres- was observed at 312 K for the aromatic side chains and the ampli- 

dynamics of α-synuclein [35]. Fu et al. examined the local stability and dynamics of apocytochrome b562 experiments [66]. More recently, high-pressure relaxation disper- of the Fyn SH3 domain by introducing a slight perturbation of and chemical denaturants affect differently the folding cooperativity of the protein and the relative population of intermediate states [59].

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 b562 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 interme- 

dia of the protein only showed slight differences in comparison to the hydrophobic core showed highly heterogeneous transition-state ensembles, while those containing a cavity in the peripheral region of the protein showed 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 L9, indicating that N-terminal (NTL9) folding is a two-state process. Deviations from this two-state process, however, were observed for the C-terminal
region (CTls). More importantly, large positive activation volumes for folding were reported for both NTLs and CTls, 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 $^1H^15N$ chemical 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 AjI aggregates. They observed that aggregates formed by phosphorylated peptides were more compressible than the non-phosphorylated species. The conformational fluctuations of amyloid peptides, AjI and hAPP, have also been analyzed by high-pressure NMR using non-linear chemical shift changes to detect the presence of partially ordered structures [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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References
