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Revealing protein dynamics with high-pressure NMR

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Review

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Abstract

While the structure of proteins can now be predicted from sequence with high certainty, the prediction of protein functional dynamics remains to be achieved. Progress towards this goal will require a much larger experimental database of the relationships among sequence, dynamics, and function than currently available. Dynamic transitions that are key to protein function and turnover remain difficult to access and characterize because they have significantly higher free energy than the folded states of proteins and hence are not populated. To access these higher free energy states, proteins must be perturbed. High temperatures often lead to aggregation, while chemical denaturants, because they interact with the entire protein backbone, tend to smooth protein conformational landscapes. In contrast, high hydrostatic pressure represents a continuous and reversible variable that can perturb protein structure locally around internal cavities, leading to partial structural disruption, populating these higher energy states sufficiently for their characterization.

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Introduction

Protein dynamics across multiple timescales are essential for biological function. And while the prediction of protein structure from sequence has made stunning progress in recent years (Evans *et al.*, 2021; Jumper *et al.*, 2021; Abramson *et al.*, 2024), prediction of protein functional dynamics based on sequence, as recently noted (Rennella *et al.*, 2023), remains a major challenge. Progress towards this goal will require a much larger experimental database of the relationships among sequence, dynamics, and function than currently available. Unfortunately, the characterization of all but the fastest protein dynamics under native or non-denaturing conditions is generally quite difficult, as these dynamics implicate states of significantly higher free energy than the folded states of proteins, which are not highly populated. NMR relaxation methods, as described below, can be used to characterize such states provided they are populated at levels of at least 1–2%. This detection threshold limits the accessible range of dynamics that can be characterized. To access a broader range of motion, linked to higher free energy states, including functional dynamics or even complete unfolding, the systems must be perturbed, either by temperature, chemical composition, or, as we discuss below, pressure. Because of the changes in heat capacity associated with protein conformational order/disorder transitions, the interpretation of temperature perturbation can be complex. In addition, proteins tend to aggregate at high temperatures. Chemical denaturants interact favorably with exposed protein surfaces, which are similar regardless of which region of a protein becomes exposed. Thus, denaturants act equally on all regions of

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proteins, leading to a smoothing of conformational landscapes. Moreover, the addition of denaturants is not a continuous or easily reversible perturbation. In contrast, pressure is a continuous and reversible variable that, for reasons explained below, perturbs protein structure locally, allowing for structural characterization of energetically excited states and for their simultaneous thermodynamic and kinetic characterization.

Physical basis for pressure effects on protein conformational dynamics

Equilibria shift toward lower volume states under pressure

The first result of pressurization on any system is compression; all atoms in the system move closer together, decreasing the system's volume. Locally, however, some interatomic distances can increase, for example, when water molecules penetrate internal cavities and rearrange hydrogen-bond networks, so that the detailed structural response can be highly heterogeneous (Williamson *et al.*, 2003). For systems in equilibrium between multiple states, pressurization will result as well in a shift in these equilibria towards the lower partial molar volume states, which for proteins are states that are less structured than the native state. The lower system volume of the less structured states of proteins stems from the fact that folded proteins are not perfectly packed (Frye and Royer, 1998; Eriksen *et al.*, 2001; Williamson *et al.*, 2003; Ando *et al.*, 2008; Roche *et al.*, 2012; Nucci *et al.*, 2014; Maeno *et al.*, 2015; Chen and Makhatadze, 2017). There exist within protein structures void spaces that are not occupied to any statistically significant degree by solvent (water) molecules. When these cavity-containing regions are disrupted, either the void volumes are eliminated and the volume taken up by solvent, or in some cases, the protein opens up and water fills the cavities. In both cases, void volume in the native state is occupied by water, lowering the partial molar volume of the system.

Although changes in internal cavities and packing defects appear to be the dominant contributors to the volume changes observed for protein unfolding and local structural transitions, other effects can also contribute. In particular, electrostriction of water around newly exposed charged or polar groups provides an additional negative contribution to the partial molar volume upon unfolding or local rearrangements (Murphy *et al.*, 1998; Caro *et al.*, 2023). In most of the systems considered here, the magnitude and sequence-dependence of ΔV are best explained by the creation or elimination of internal voids; nonetheless, electrostrictive effects should be kept in mind, especially for conformational transitions that substantially alter solvent-exposed charge.

The differences in volume between native and fully unfolded states of proteins are relatively small, 1–2% of the total molecular volume of the protein (Royer, 2002), necessitating significant pressures (1 bar-4 kbar is the typically accessible range) to elicit an effect. The pressure range over which protein unfolding occurs also depends upon the global stability, which can eventually be tuned by adjusting pH or temperature, adding denaturant, or even mutation in order to observe unfolding over the accessible pressure range. Volume changes for partial structural disruption or local conformational changes are even smaller than those for global unfolding, although the free energy differences are generally much smaller as well, such that intermediates are often detected. Moreover, locally less stable regions often are those that contain cavities and hence are more susceptible to pressure than better-packed regions. Note that, as discussed below, the volume change for protein unfolding (partial or global) is a strong function of temperature.

Activation volume ($\Delta V^{\#}$) as an analytic probe of protein conformational transitions

Analysis of the kinetics of conformational transitions provides information about the transition state of the reaction, and thus the mechanism for the transition. Pressure-jump relaxation experiments generally result in complete or partial disruption of protein structure. For simplicity, we will refer to these transitions as folding and unfolding. The pressure dependence of the observed rate constants can be analyzed to obtain the rate constants for folding and unfolding at atmospheric pressure, k°_{F} and k°_{U}, and the activation volumes for folding and unfolding, $\Delta V^{\#}_{F}$ and $\Delta V^{\#}_{U}$. This allows one to place the partial molar volume of the transition state ensemble (TSE) relative to the folded and unfolded forms. We have referred to this as the *Vi*-value (Mitra *et al.*, 2007), in analogy with the Φ-value for the activation m-values of denaturant-jump relaxation experiments (Fersht and Sato, 2004). Although Φ-values have provided many important insights into folding transition states, this approach also has limitations, as underscored by the Ψ-value analysis introduced by Sosnick and co-workers, which highlights cases where Φ-values can be misleading or ambiguous (Krantz and Sosnick, 2001). Moreover, the interpretation of Φ-values is predicated on the notion that the structure of the TSE remains the same, with only the stability changing upon mutation. *Vi*-value analysis offers a complementary volumetric perspective that can be particularly sensitive to cavity formation and hydration in the TSE. Indeed, *Vi*-value is a more structural as opposed to energetic parameter. We have found in several instances that the *Vi*-value and hence the volumetric (structural) properties of the TSE can be strongly modulated by mutation (Roche *et al.*, 2013).

In most cases studied to date (i.e., Vidugiris *et al.*, 1995; Mohana-Borges *et al.*, 1999; Pappenberger *et al.*, 2000; Roumestand *et al.*, 2001; Seemann *et al.*, 2001; Rouget *et al.*, 2011), the partial molar volume of the TSE is situated between that of the folded and unfolded states, and generally closer to that of the folded state, as in the schematic derived from fluorescence-detected p-jump relaxation experiments on staphylococcal nuclease (Figure 1*a*). A relatively large positive activation volume for folding leads to significant slowing of folding under pressure, facilitating detection of the transition. A large, positive activation volume for folding also indicates that most of the cavities in the native state have formed at the barrier, corresponding to a relatively 'late' TSE. However, as noted for equilibrium volume changes associated with protein structural transitions (Seemann *et al.*, 2001), the value of the activation volumes is strongly temperature dependent (Figure 1*b*) (Rouget *et al.*, 2010). Thus, the placement of the volume of the TSE can change relative to the folded and unfolded states due to differential thermal expansion of the different states. In the case of the ankyrin repeat domain of the Notch protein, at higher temperature, the partial molar volume of the TSE was found to be higher than that of the folded state (Figure 1*b*), corresponding to a positive activation volume for unfolding as well. Likewise, small changes in sequence can modulate the absolute and relative volumetric properties of protein states.

Backbone fluctuations and side-chain dynamics

On the picosecond to nanosecond timescale, protein dynamics are generally not expected to be significantly perturbed by hydrostatic pressure, as the associated activation volumes are typically close to zero for rapid vibrational motions (Figure 2). This has been consistently demonstrated by ^{15}N spin relaxation measurements,

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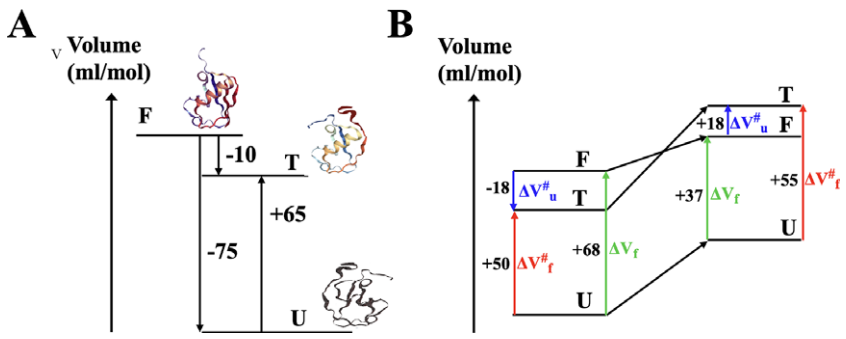


Figure 1. Volumetric diagram of protein folding reactions. (a) The volume diagram for the unfolding/folding reaction of staphylococcal nuclease (redrawn from Mitra *et al.*, 2007) indicating the overall volume change between the folded and unfolded states ΔV_F (−75 ml/mol) and its decomposition into the activation volume for folding, $\Delta V^{\#}_F$, (+65 ml/mol) and the activation volume for unfolding, $\Delta V^{\#}_U$, (−10 ml/mol). (b) Temperature dependence of the volume diagram for the unfolding/folding reaction of the ankyrin repeat domain of the Notch receptor protein (redrawn from Rouget *et al.*, 2011). Equilibrium volume changes, ΔV_F, are green, activation volumes for folding, $\Delta V^{\#}_F$, are red, and activation volumes for unfolding, $\Delta V^{\#}_U$, are blue. Note that the $\Delta V^{\#}_U$ is negative at low temperature and positive at high temperature due to the differences in thermal expansion of the folded state, α_F, with respect to the transition, α_T, and unfolded, α_U, states.

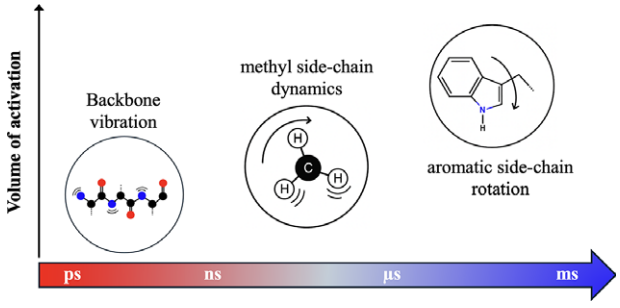


Figure 2. Volume of activation and protein dynamics. Schematic representation of the relative volume of activation associated with different ranges of protein motion. On the left, backbone N–H bond vibrations occur on the picosecond to nanosecond timescale and exhibit near-zero activation volumes, reflecting their insensitivity to hydrostatic pressure. Methyl side-chain dynamics typically occur on nanosecond to microsecond timescales and display modestly larger activation volumes, indicative of localized compressibility within the protein core. In contrast, aromatic side-chain rotations, which take place on microsecond to millisecond timescales, can involve substantially larger activation volumes (on the order of 10–15 mL mol^{-1}), resulting in pronounced pressure effects.

which report negligible pressure effects on the backbone dynamics for systems ranging from an isolated α-helix (Orekhov *et al.*, 2000), to a globular protein such as BPTI (Sareth *et al.*, 2000), and to intrinsically disordered proteins (Roche *et al.*, 2013). For instance, R1ρ relaxation rates measured for α-synuclein at 1 bar and 2.5 kbar show no substantial changes beyond a uniform 5% increase in the relaxation rates attributed to the small increase in solvent viscosity at higher pressure.

Although pressure has little effect on fast internal motions, it can influence amide chemical shifts through compression-induced modulation of the local electronic environment. A general downfield shift of H^N amide protons is generally observed as a function of pressure for both folded and disordered protein systems. This downfield shift is commonly attributed to compression of the N–H···O=C hydrogen bond, with bond length reductions of up to ~1% at 2 kbar in globular proteins (Li *et al.*, 1998). The linear component of HN chemical shift changes can be compared to reference values obtained from model tetrapeptides (Koehler *et al.*, 2012). This enables separation of purely local compression effects from larger-scale conformational changes. Beyond the linear response, the non-linear pressure dependence of chemical shifts provides additional insight into protein conformational heterogeneity. Akasaka and Li have notably correlated the magnitude of the non-linear pressure response in globular proteins to the volume of internal cavities, suggesting that proteins with large internal void volumes are more likely to populate low-lying excited states under high-pressure (HP) (Akasaka and Li, 2001).

In contrast to backbone vibrational motions, side-chain dynamics, particularly those of methyl-bearing and aromatic residues, can exhibit pressure sensitivity that varies across the protein structure (Figure 2). Early high-pressure NMR work by Wagner on BPTI demonstrated that aromatic ring flips can be significantly slowed down by elevated pressure, highlighting the large activation volumes associated with side-chain dynamics and their sensitivity to local packing and hydration (Wagner, 1980). In a landmark study, Fu *et al.* (2012) used a combination of ^2H relaxation of methyl groups and ^{15}N relaxation to probe sub-nanosecond side-chain dynamics in ubiquitin at 1 kbar. While backbone dynamics were unaffected, they reported heterogeneous changes in methyl group mobility, with more pronounced attenuation in the protein core.

This non-uniform response was interpreted as a manifestation of differential local compressibility within the folded structure. In a subsequent study, Kasinath et al. (Kasinath *et al.*, 2015) extended these observations to aromatic side chains by analyzing ^{13}C relaxation of ring carbons under varying pressure and temperature conditions. They identified a sharp thermal transition near 312 K, beyond which the amplitude of ring rotational motions decreased markedly with pressure, again highlighting localized dynamic sensitivity to volume perturbation. Most recently, Caro *et al.* (2023) investigated the Barnase–Barstar interaction and showed that pressure-induced changes in methyl side-chain dynamics were spatially correlated with functionally relevant regions, underscoring the role of dynamic adaptability in molecular recognition. Altogether, these studies suggest that while backbone fluctuations are largely pressure insensitive, side-chain dynamics can provide a sensitive readout of localized compressibility and structural adaptability (Wilton *et al.*, 2009).

Local substates and large-scale protein dynamics

Intermediate timescale conformational exchange

While fast internal motions are for the most part largely pressure-insensitive, slower conformational transitions, on the microsecond to millisecond timescale, are exquisitely sensitive to pressure

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modulation, providing a window into protein substates and folding intermediates. Indeed, while the energetic barriers to these transitions are larger than those associated with ps-ns motions, the more extensive disruption involves larger differences in partial molar volume. Pressure-dependent ^{15}N CPMG relaxation dispersion experiments have proven to be exceptionally powerful in dissecting the thermodynamics and kinetics of complex folding pathways. Bezsonova *et al.* (2006) first demonstrated this approach using a millisecond-folding G48M mutant of the Fyn SH3 domain. By analyzing how relaxation dispersion profiles vary between 1 and 120 bar, they quantified the volumetric properties of both the unfolded and transition-state ensembles, revealing a substantial positive activation volume for folding ($\Delta V^{\#}_{f}$) that reflects significantly decreased hydration in the transition state relative to the unfolded state. This pioneering work established the principle that pressure modulation of exchange rates in relaxation dispersion experiments affords precise insight into packing and solvation changes during folding transitions and underscores the utility of pressure perturbation to populate such excited states such that they can be characterized (Figure 3).

Building on this foundation, Tugarinov *et al.* (2015) investigated a metastable mutant of Fyn SH3 with a three-state folding mechanism using HP relaxation dispersion. By globally fitting dispersion data across pressures, they extracted pressure-dependent rate constants, activation volumes for folding and unfolding, and changes in isothermal compressibility, enabling a fine reconstruction of the protein's volumetric energy landscape. The results revealed that the on-pathway folding intermediate state, though energetically similar to the unfolded ensemble, has a partial volume close to the volume of the folded protein. Interestingly, the compressibility of the intermediate state was found to be larger than that of the folded state, indicative of an intermediate ensemble that is more structured than the unfolded ensemble but less rigid than the folded state.

More recently, Zhang *et al.* (2022) applied pressure-dependent CPMG and CEST experiments to investigate conformational dynamics in the leucine-rich repeat protein pp32 (Figure 3). Mutational insertion of cavities at distinct locations revealed how pressure modulates exchange timescales and structural features of excited states. Specifically, a cavity introduced in the N-terminal capping motif (I7A) induced pressure-dependent exchange on the 500 μs-2 ms timescale detectable by CPMG experiments, while a cavity in the core region (L60A) led to slower exchange events (>2 ms) detectable by CEST. ^{15}N chemical shift values obtained from the CEST experiments for the pressure-populated excited states revealed that the N-terminal region was unfolded, while the central core of the protein retained native-like structure. Very slow exchange for residues in the C-terminus of this variant of pp32 allowed mapping of the pressure-induced population of an intermediate with native-like structure in the C-terminal region by quantifying the corresponding peak intensities (Jenkins *et al.*, 2018). CEST was also useful in revealing the folded-like nature of the pressure-induced functional molten globule state of a small GTPase, Arf1 (Koduru *et al.*, 2024). A note of caution: in the cases of the pp32 variants and the Arf proteins, the pressure-induced excited ensemble was very heterogeneous, with multiple transitions overlapping across the pressure range. This complexity precludes a full analysis of either the CPMG or CEST experiments to obtain exchange rates or from CPMG, excited state frequencies. In this case, it was useful to compare the T_2 relaxation time obtained from Hahn Echo experiments to that obtained via CPMG at a single, relatively high frequency, with differences being indicative of intermediate exchange.

As in the case of the Arf proteins, the enzymatic function of adenylate kinase involves a large conformational change. In the case of Adk this change corresponds to the opening of a lid obstructing the active site in the rate-limiting step. Comparison of the pressure effects on lid opening dynamics of Adk enzymes from mesophilic and piezophilic showed agreement between volumetric terms obtained from HP enzyme turnover and HP relaxation dispersion NMR (Stiller *et al.*, 2019). Mutation of the mesophilic enzyme at three positions converted its pressure-independence to an equivalent negative activation volume for lid opening found for the piezophile, establishing the sequence determinants for the differential effects of pressure on dynamics. Generally, the studies discussed

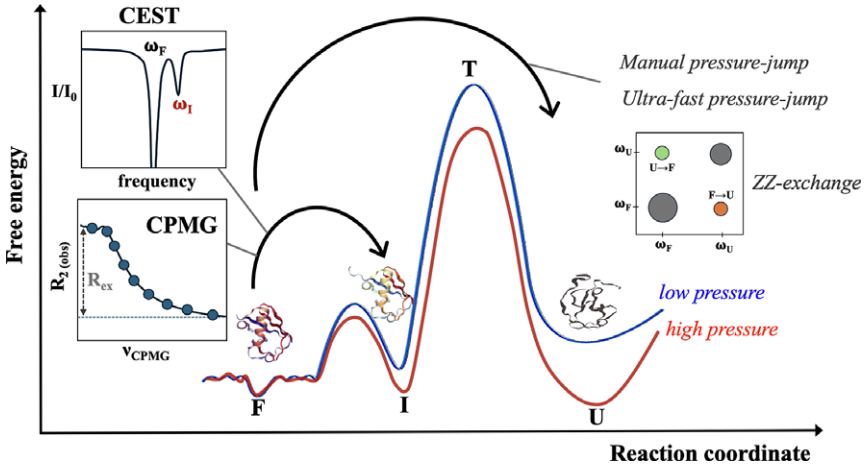


Figure 3. High-pressure NMR methods for probing protein conformational exchange and folding kinetics. Schematic representation of how different high-pressure NMR approaches access distinct regions of the protein energy landscape. Local conformational exchange within the folded ensemble (F ↔ I) can be characterized using ^{15}N CPMG relaxation dispersion and CEST experiments, which provide rate constants and populations of excited states. In contrast, real-time pressure-jump experiments – performed manually for slow transitions or using ultra-fast instrumentation for rapid events – along with ZZ-exchange spectroscopy, enable direct measurement of folding and unfolding kinetics (F ↔ U). Pressure shifts the free energy surface, stabilizing lower-volume states and altering the relative barriers between folded (F), intermediate (I), transition (T), and unfolded (U) states.

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here illustrate how internal packing defects, when combined with pressure modulation, can unmask functionally relevant conformational exchange processes with distinct kinetics and spatial localization, reinforcing the sensitivity of relaxation dispersion to local energetic frustration and cooperative dynamics in repeat protein architectures. Although these excited states are preferentially populated under pressure, comparisons with temperature and denaturant perturbations, as well as with functional readouts, indicate that they correspond to low population substates that are already sampled at 1 bar and that modulate folding, allostery, and catalysis. Work from Akasaka, Kitahara and co-workers provides particularly clear examples of how pressure-stabilized states map onto the conformational landscape at 1 bar. In their PNAS study (Kitahara and Akasaka, 2003), a pressure-stabilized intermediate was shown by HP NMR and thermodynamic analysis to correspond to the on-pathway folding intermediate inferred from kinetic experiments at ambient pressure, and subsequent work on lysozyme and ubiquitin generalized this picture to additional systems (Akasaka et al., 2013). A series of HP NMR studies on ubiquitin further quantified the pressure-dependence of exchange rates and activation volumes between native-like substates, linking the amplitudes and timescales of backbone and side-chain fluctuations directly to functional motions (Kitahara et al., 2005). Finally, in dihydrofolate reductase, pressure-dependent changes in NMR observables related low-population conformational excursions to specific steps in the catalytic cycle, demonstrating that the same fluctuations amplified by pressure underpin enzyme function at 1 bar (Kitahara et al., 2000). Together, these studies make a strong case that HP NMR detects genuine folding intermediates and functional substates on the ambient-pressure landscape and provides quantitative access to their thermodynamic and kinetic parameters.

Pressure-dependent unfolding on a slow timescale monitored by NMR

When the pressure-induced unfolding of globular proteins occurs on a slow NMR timescale, the folding/unfolding equilibrium can be monitored by recording a series of ^{1}H-^{15}N HSQC spectra at increasing pressure. Under such conditions, unfolding typically manifests as a progressive decrease in the intensity (or volume) of folded-state cross-peaks, and an increase in intensity for peaks of the unfolded state. Since locally, each residue backbone atom (NH or Cα) or side chain methyl group is either in its native environment or it is not, the residue-specific pressure dependence of these decreases in native state peak intensities can be fitted to a two-state transition to extract apparent residue-specific values of the folding free energy (ΔG_f) and volume change (ΔV_f). For an ideally cooperative two-state transition, cross-peak intensity curves for all residues should overlap and converge on a narrow range of ΔG_F and ΔV_F values within experimental uncertainty. Conversely, large variations in ΔG_F and/or ΔV_F across residues indicate deviations from cooperative behavior and suggest the presence of partially folded intermediates. Such analyses have revealed pressure-stabilized substates with reduced partial molar volume in multiple globular proteins, providing a sensitive probe of local folding cooperativity and packing defects (Roche and Royer, 2018).

However, interpretation of intensity loss can be complicated when the unfolded state remains unassigned or invisible. In such cases, it is difficult to distinguish global unfolding from local unfolding events or from the transient population of structurally distinct high-energy substates that are broadened beyond detection. This challenge was exemplified in studies of the L99A mutant of T4 lysozyme. Wand and colleagues (Nucci *et al.*, 2014) observed pressure-dependent disappearance of cross-peaks from the C-terminal domain, which they attributed to complete domain-scale unfolding. In contrast, Kitahara and coworkers (Maeno *et al.*, 2015) proposed that these residues instead populated sparsely populated, pressure-stabilized high-energy states driven by the flipping-in of a surface aromatic side chain, reducing local cavity volume. By tracking both amide and methyl cross-peaks across pressure, they argued for the presence of a locally unfolded substate rather than a globally denatured form (Kitahara and Mulder, 2015). This interpretation was rebutted by Wand and Nucci (2015) who cited SAXS and reverse micelle experiments to support the global unfolding hypothesis. Subsequent work by Xue *et al.* (2019) employed pressure-dependent CPMG relaxation dispersion and hydrogen exchange NMR to uncover multiple partly disordered excited states whose populations were tunable through internal cavity engineering.

An example of how HP NMR can be integrated with complementary structural techniques to resolve hidden folding intermediates is provided by Koduru *et al.* (2024), who investigated the nucleotide-switch mechanism of the small GTPase Arf1. Using a combination of HP NMR, SAXS, FTIR, computation and site-directed mutagenesis, they identified a molten globule-like intermediate that primes Arf1 for GDP release and GTP binding. Pressure-induced changes in chemical shifts and peak intensities revealed a loosely packed, partially structured, and functionally relevant ensemble that remained undetectable at ambient pressure. Integration of the HP NMR data with SAXS, computation, and FTIR enabled a detailed view of the conformational landscape underlying functional switching. This study highlights the power of pressure perturbation, especially when combined with orthogonal techniques, to characterize low-lying intermediates involved in complex allosteric transitions and nucleotide-driven signaling cycles.

Folding/unfolding kinetics with HP NMR

Real-time pressure-jump NMR

Beyond equilibrium measurements of folding intermediates and their interconversion dynamics, pressure perturbation can also be used to initiate and monitor time-resolved global un/folding reactions, offering a direct probe of folding kinetics and pathway selection. The large and positive activation volumes for folding ($\Delta V^{\#}_F$) observed in many globular proteins can slow refolding by several orders of magnitude under elevated pressure. This kinetic deceleration offers a unique advantage for HP NMR spectroscopy: when the refolding timescale extends into the seconds-to-minutes range, real-time NMR measurements can be performed using conventional 2D experiments such as ^{1}H-^{15}N HSQC or SOFAST-HMQC. Under such conditions, moderate pressure-jumps (typically 100–300 bar) enable direct, residue-specific monitoring of folding kinetics across the pressure-induced unfolding transition, permitting the dissection of folding pathways with near-atomic resolution (Figure 3).

Roche *et al.* (2013) applied this strategy to cavity-containing variants of Staphylococcal nuclease (SNase). Folding kinetics were tracked following pressure-jumps, using time-resolved series of 2D ^{1}H-^{15}N spectra. Under optimized folding conditions, relaxation under pressure was slow enough (minutes to hours) that detailed residue-specific refolding profiles could be extracted from HP

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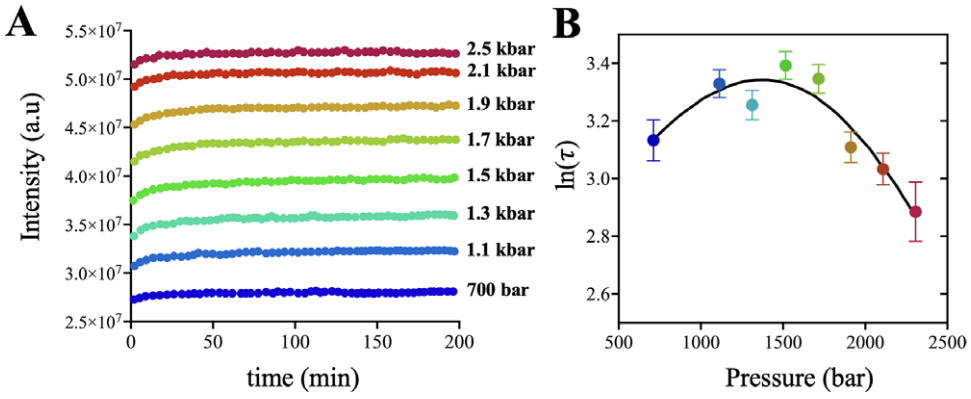


Figure 4. Real-time 1H pressure-jump unfolding kinetics of the DEN4-ED3 domain from the Dengue virus protein envelope. (a) Time evolution of the unfolded-state methyl resonance intensity following a series of positive 200-bar pressure-jumps conducted between 700 and 2500 bar. The pressures indicated on the right correspond to the final pressures reached after each jump (e.g., "700 bar" represents a jump from 500 to 700 bar). (b) Chevron plot of the logarithm of the observed exchange times [ln(τ)] as a function of pressure, used to extract the global folding and unfolding rate constants (k_F and k_U), as well as the apparent activation volume of folding ($\Delta V^{\#}_F$). Data adapted from Herrada *et al.* (2018).

^{1}H-^{15}N HSQC or SOFAST HMQC spectra over time across multiple SNase variants and denaturant concentrations. This comprehensive dataset (>18,000 p-jump relaxation profiles) enabled precise mapping of the transition state ensemble (TSE) for the different variants, revealing how individual structural elements refold at distinct rates and how specific core mutations alter folding rates and routes. For instance, the Δ + PHS background variant, like WT SNase (Figure 1*a*) (Roche *et al.*, 2013), folded through a TSE featuring an ordered hydrophobic core and an unstructured C-terminal helix, while the Δ + PHS I92A mutant exhibited multiple parallel refolding pathways. Interestingly, the TSE for the folding of the I92A variant was very early, with only a small positive activation volume for folding compared to the WT (see Figure 1*a*). These results provided direct evidence that even subtle perturbations to packing can reshape the energy landscape and diversify folding mechanisms.

Analyzing the folding pathway of the immunoglobulin-like titin I27 domain, both as a single module and in tandem, Herrada *et al.* (2018) took advantage of the time dependent increase of the methyl proton resonances corresponding to the unfolded state of the protein in a series of 1D spectra recorded after P-jumps, and found a very late, dehydrated, native like transition state. A subsequent study on another IgG domain, DEN4-ED3 from the Dengue virus, exploiting residue-specific unfolding rate constants determined using a similar approach (Figure 4), revealed that despite the structural homology to the titin I27 domain and the presence of a similar folding intermediate in the folding pathway, folding proceeded via a very early, wet TSE close in volume to the unfolded state (Saotome *et al.*, 2019).

Together, these studies illustrate how real-time pressure-jump NMR leverages the naturally slow folding kinetics under high pressure that arise from positive activation volumes to provide mechanistic and structural insights into protein folding pathways. Crucially, this approach does not require specialized rapid-mixing equipment, making it broadly accessible for high-resolution dissection of folding landscapes in both simple and multidomain proteins.

Ultra-fast pressure-jump NMR

While conventional real-time pressure-jump NMR has proven effective for capturing slow folding kinetics, the structural interrogation of faster events, particularly those occurring on the millisecond to second timescale, requires much faster and precisely synchronized pressure changes. This technical challenge was first addressed by Kremer *et al.* (2011), who developed a pioneering HP NMR system capable of delivering precisely controlled pulsed pressure perturbations within the NMR pulse sequence dead time. Their system, based on a microprocessor-controlled hydraulic pump, was integrated directly into the NMR pulse sequence and achieved jumps of ~80 MPa (800 bar) in under 30 milliseconds. Importantly, these pressure cycles could be applied repetitively and synchronized with spectroscopic acquisition, enabling kinetic studies and line shape analysis of pressure-perturbed protein transitions.

Bax and colleagues later introduced an improved system optimized for ultra-fast pressure drop experiments and applied it to monitor the folding of a pressure-sensitive ubiquitin variant (V17A/V26A) via hydrogen/deuterium exchange. In a landmark study, Alderson *et al.* (2017) combined a rapid pressure release (from 2.5 kbar to 1 bar) with pulse-programmable acquisition to monitor hydrogen exchange protection within ~150 ms. By recording a series of 2D ^{1}H-^{15}N spectra at defined delay times following the pressure drop, they tracked for individual backbone amides the switch of the exchange rates from unfolded to folded values, thereby mapping the sequence at which structural elements are formed during folding (Figure 3).

Charlier *et al.* (2018) further refined the approach, adapting the pressure-jump protocol to monitor ^{15}N chemical shift changes with high temporal resolution. This allowed them to follow the evolution of individual amide chemical environments immediately after pressure release via a pseudo-3D NMR experiment, enabling the identification of intermediate states transiently populated during the folding process. An interrupted folding protocol was subsequently introduced in a complementary study (Charlier *et al.*, 2018) in which the protein was cycled between high- and low-pressure states within the duration of a single experiment. This approach successfully trapped a transient folding intermediate of ubiquitin that forms within the pressure cycle. The recovered ^{1}H, ^{15}N, and ^{13}C' backbone chemical shifts revealed partial structuring of β-sheet elements and incomplete hydrogen-bond protection in terminal regions, consistent with the presence of a partially ordered folding intermediate that precedes full native-state acquisition. Importantly, this partially folded intermediate, only readily detectable under high pressure, is proposed to be an on-pathway species at

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atmospheric pressure, providing structural and mechanistic insight into the overall folding process. Furthermore, analysis of the kinetics suggested that the protein makes approximately 100 transitions to this intermediate state for every productive fold to the native state, highlighting the role of this high-energy state in kinetic partitioning.

In one recent study, Masoumzadeh *et al.* (2024) applied the pressure-jump NMR approach to investigate proline isomerization as a rate-limiting step in folding of the L50A mutant of ubiquitin. By cycling between high- and low-pressure conditions, the authors could monitor proline isomerization kinetics in both model heptapeptides and the pressure-denatured protein. The data revealed site-specific differences in activation energies and pointed to metastable, partially folded states, notably a cis-E18-P19 isomer with unfolded C-terminal residues and exchange-broadened N-terminal signals, consistent with a transient, partially folded state.

Building on this body of work, Bax and colleagues solved the atomic-resolution structure of the transient, on-pathway folding intermediate of the pressure-sensitized ubiquitin mutant (Masoumzadeh *et al.*, 2025). Using an extensive set of structural restraints, including chemical shifts, NOEs, and RDCs measured during synchronized pressure-jump cycles, the authors determined that the intermediate adopted a non-native β-sheet registry involving strands β1 and β5. This metastable conformation, which acts as a kinetic trap during folding, was previously implicated in the PINK1 mitophagy pathway, which underscores its functional relevance under ambient conditions. Analysis of long-timescale MD trajectories further revealed that such register-shifted β-sheet intermediates are visited repeatedly along the folding pathway, reinforcing the view that the pressure-populated intermediate corresponds to a genuine on-pathway state that is also sampled, at very low population, under native conditions.

Together, these studies established ultra-fast pressure-jump NMR as a cutting-edge method for accessing folding kinetics on the millisecond timescale, bridging the gap between traditional stopped-flow techniques and slower NMR-based pressure protocols. By combining pressure perturbation with advanced pulse programming and dedicated hardware, this approach offers real-time, residue-level snapshots of transient conformational states.

ZZ-Exchange NMR under pressure

When protein folding and unfolding transitions occur on the millisecond to second timescale and remain in the slow exchange regime on the NMR chemical shift timescale, two-dimensional ZZ-exchange spectroscopy (also called EXSY for exchange spectroscopy) offers a powerful route to directly measure forward and reverse folding rate constants. In an example of this approach (Zhang *et al.*, 2016), Zhang et al. applied HP ZZ-exchange spectroscopy to the N-terminal domain of the ribosomal protein L9 (NTL9) that folds within 1 ms at atmospheric pressure and a destabilized variant of L9 C-terminal domain (CTL9 I98A) that folds within 70 ms. By tracking exchange between assigned folded and unfolded state resonances, residue-specific folding and unfolding rate constants were extracted as a function of pressure, allowing for the calculation of apparent residue-specific activation volumes for folding $\Delta V^{\#}_{F}$ and unfolding $\Delta V^{\#}_{U}$. The results revealed that NTL9 undergoes folding through a near-ideal two-state mechanism with uniformly large $\Delta V^{\#}_{F}$ and relatively small $\Delta V^{\#}_{U}$ values. These findings are indicative of a compact, dehydrated transition state, with volumetric properties close to those of the folded state. While a similar pattern was observed for CTL9 I98A, several residues near the C-terminal β-strands exhibited faster-than-average folding rates, which points to transient intermediate states that may play a role in initiating folding. These findings underscore the utility of ZZ-exchange under pressure as a structurally sensitive probe of folding cooperativity and kinetic heterogeneity through direct observation of state-to-state transitions at equilibrium.

Concluding remarks

In the ongoing quest to understand how sequence determines protein dynamics and ultimately function, pressure perturbation coupled with NMR spectroscopy has provided deep insight into protein energy landscapes. High pressure selectively increases the population of excited protein states, while NMR provides exquisitely detailed, residue-specific information on their conformational dynamics and stability. By comparing natural or engineered variants of proteins of interest, high-pressure NMR has revealed detailed sequence determinants of transitions to and among protein excited states, ranging from low-lying, locally disrupted structures to completely unfolded states. The amplitudes and rates of these dynamic transitions depend on the local energetics of the conformational landscape, which in turn are determined by the specific sequence. Across the examples discussed here, the excited conformers populated under pressure often correspond to folding intermediates, functional molten globules, or allosteric substates that are also sampled, albeit at low population, under native conditions. In the case of titin I27, for example, the intermediate detected by high pressure, involving detachment of the first strand of the parallel β-sheet from the remainder of the β-sandwich, is very similar to that predicted by computational modeling and characterized by single-molecule force spectroscopy (AFM) (Herrada *et al.*, 2018). High-pressure NMR, therefore, offers a powerful way to magnify and quantify the same dynamic processes that govern protein stability, catalysis, and regulation at atmospheric pressure.

Interestingly, the titin I27 intermediate state detected under high-pressure and atmospheric conditions has never been observed by chemical denaturation. Similar behavior is seen for cavity-containing variants of staphylococcal nuclease, where high-pressure NMR revealed multiple intermediates and locally disrupted conformers that remain largely hidden in chemical denaturation experiments; in that system, guanidinium chloride reshuffles the folding landscape by suppressing some pressure-populated intermediates while stabilizing alternative partially unfolded states (Roche *et al.*, 2012). These observations underscore that pressure and chemical denaturants perturb distinct physical quantities (*i.e.*, internal void volume versus solvent-exposed surface area) and therefore favor the population of overlapping but non-identical ensembles of excited states (all of which are inherent to the conformational landscape), making high-pressure NMR uniquely complementary to traditional denaturation-based approaches.

When combined with other biophysical methods, pressure-perturbation further enriches our understanding of protein energy landscapes. High-pressure SAXS, FTIR, and fluorescence have been used alongside HP NMR to cross-validate global and local structural changes, while HP NMR hydrogen-exchange measurements have provided residue- or segment-level maps of protection factors as a function of pressure (Fuentes and Wand, 1998; Roche *et al.*,

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2012). These studies show that regions that become labile under pressure are often the same elements that control folding nucleation, allosteric switching, or catalysis at ambient pressure. Going forward, integration of HP NMR with HP H/D-exchange mass spectrometry (HP H/D XMS), while offering lower resolution information than HP NMR (small peptides, rather than individual residue amides or sidechains), will extend these insights to larger systems that exceed current NMR size limits.

One interesting area of future research for such approaches would be to decipher how evolution has allowed for protein function in the diverse, and often extreme environments on Earth, including combinations of low or high temperatures, with high pressure, low and high pH, high salt or other extreme chemical conditions. Another possible direction could be found in drug design programs aimed at stabilizing or destabilizing the protein excited states characterized by HP NMR. Depending on their role in protein function or dysfunction in specific disease states, computational docking of the excited state structures derived from HP NMR could yield possible compounds which could be tested experimentally. Finally, understanding which residues control function and which control stability will allow for more targeted protein design for a variety of applications in human health and green chemistry.

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