This is a repository copy of *Characterization of low-lying excited states of proteins by high-pressure NMR*.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/140628/

Version: Accepted Version

Article:
Williamson, M.P. and Kitahara, R. (2019) Characterization of low-lying excited states of proteins by high-pressure NMR. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1867 (3). pp. 350-358. ISSN 1570-9639

https://doi.org/10.1016/j.bbapap.2018.10.014

Article available under the terms of the CC-BY-NC-ND licence (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Reuse
This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

Characterisation of low-lying excited states of proteins by high-pressure NMR

Mike P Williamson[a,*] and Ryo Kitahara[b]

[a]Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

[b]College of Pharmaceutical Sciences, Ritsumeikan University, Nojihigashi 1-1-1, Kusatsu 525-8577, Japan

*Corresponding author. *E-mail address*: m.williamson@sheffield.ac.uk

Abstract

Hydrostatic pressure alters the free energy of proteins by a few kJ mol^{-1}, with the amount depending on their partial molar volumes. Because the folded ground state of a protein contains cavities, it is always a state of large partial molar volume. Therefore pressure always destabilises the ground state and increases the population of partially and completely unfolded states. This is a mild and reversible conformational change, which allows the study of excited states under thermodynamic equilibrium conditions. Many of the excited states studied in this way are functionally relevant; they also seem to be very similar to kinetic folding intermediates, thus suggesting that evolution has made use of the 'natural' dynamic energy landscape of the protein fold and sculpted it to optimise function. This includes features such as ligand binding, structural change during the catalytic cycle, and dynamic allostery.

Key words: pressure, NMR, volume, allostery, energy landscape, cavity

1. The effect of pressure on proteins

Since the first demonstration over 100 years ago that high hydrostatic pressure causes egg white albumen to coagulate in a similar way to boiling [1], it has been clear that pressure does interesting things to proteins. At constant temperature, the effect of pressure p on protein stability (ie the free energy difference between ground and excited states) can be approximated as:

$$\Delta G = \Delta G^0 + \Delta V^0 (p - p^0) - 0.5\Delta\beta V^0 (p - p^0)^2 \quad (1)$$

Here, ΔG^0 is the free energy difference between ground state and excited state at a pressure p^0 of 0.1 MPa [1 bar = 0.9869 atm = 10^5 Pa, so standard atmospheric pressure is 0.1 MPa], ΔV^0 is the change in partial molar volume between the conformational states at p^0, and $\Delta\beta$ is the change in compressibility of the protein between ground state and excited state at p^0. For small globular proteins, ΔV^0 between ground state and unfolded is variable, but is around -100 ml/mol (0.1 kJ mol^{-1} MPa^{-1}) [2]. In other words, at 100 MPa, the ground state is destabilised by about 10 kJ mol^{-1} compared to the unfolded state. Given that many proteins have an overall unfolding free energy in the range 20 – 60 kJ mol^{-1} [3], many proteins can be unfolded by relatively mild pressures of a few hundred MPa. For comparison, hydrostatic pressure in water increases by 10 kPa m^{-1} depth, meaning that at the bottom of ocean trenches (10 km), the pressure is around 100 MPa or 1 kbar. The standard current commercially available high-pressure NMR system [4] goes up to 250 MPa, implying that global unfolding of some proteins and partial unfolding of many proteins can be observed straightforwardly by NMR. There are cells for crystallography or spectrophotometry that go up close to 1 GPa, meaning that a wide range

of investigations of protein structural change up to unfolding can be carried out on commercially available systems.

At lower pressures, proteins do not unfold completely, but often display a range of partial unfolding behaviour and excited states. The stability of these states depends on the partial molar volume of the state compared to the denatured state. Partial molar volume is the volume of a protein solution compared to the volume of the same solvent but without the protein. There has been much debate over the factors controlling partial molar volume, but the emerging consensus is that compression of the van der Waals volume is negligible, implying that the total volume change compared to the denatured protein can be described simply as [5]

$$\Delta V_{tot} = \Delta V_{cavity} + \Delta V_{hyd} \quad (2)$$

where ΔV_{cavity} is the change in the volume of cavities, and ΔV_{hyd} is the change in volume of hydration. Folded and unfolded proteins both contain cavities, but there are more and larger cavities in folded proteins so that ΔV_{cavity} is always negative: a folded protein always has a larger partial molar volume than an unfolded protein. ΔV_{hyd} is positive and is more or less proportional to the exposed surface area of the protein [5]. It is not entirely clear why this should be so. However, solvation of charges generally leads to a reduction in overall volume, a phenomenon often called electrostriction, whereas solvation of hydrophobic surfaces generally leads to an increase in volume because of the ice-like directional hydrogen bonding properties of water, which are strengthened adjacent to hydrophobic surfaces. ΔV_{cavity} and ΔV_{hyd} are of similar magnitude, meaning that the change in partial molar volume of a protein on unfolding is small. In almost all cases it is negative [2, 5]: a decrease in partial molar volume with pressure, implying that the protein tends to unfold with pressure.

All proteins contain cavities. Many of these are large enough to contain a water molecule: there is roughly one buried water molecule per 40 amino acid residues [6]. When observed by X-ray crystallography, some of the cavities are hydrated and some not. The apparent lack of water could be because the water in the cavity is disordered, but a range of studies suggest that many cavities are genuinely empty. Buried waters are often well conserved between orthologues [7], typically occurring as single water molecules forming around 4 hydrogen bonds [6]. Buried waters typically stabilise proteins, by a small amount [8]. They are often well ordered, with crystallographic *B* factors similar to those of surrounding protein atoms. However, an interesting study [9] demonstrates that the most stably buried water in bovine pancreatic trypsin inhibitor (BPTI) produces an overall increase in conformational flexibility of the protein. It is suggested that this may be because librational motion of the water softens delocalised (large-scale) motional modes of the protein [10]: in other words that rotation of the buried water 'drags' local parts of the protein with it, and therefore facilitates conformational change in the protein. This concept will become important below, when we consider conformational exchange in proteins at high pressure.

When proteins are subjected to high pressure, two kinds of conformational change happen simultaneously. First, there is a *compression* of the protein. This has been studied using crystallography, NMR, ultrasound and many other methods. The compression is relatively small: a roughly 1% decrease in protein volume at 200 MPa [11]. Although the pressure is isotropic, the compression is not [12]. The most compressible parts are the cavities, followed by hydrogen bonds. Van der Waals distances are almost incompressible [12]. Helical domains compress fairly uniformly, but β-sheet regions are relatively incompressible, and tend to twist. Interestingly, in lysozyme, this twisting seems to rotate around two cavities containing buried water molecules [13], again highlighting the importance of hydrated cavities in protein fluctuation under pressure. Some parts of proteins even expand locally [13-15], and BPTI undergoes a small overall volume expansion [14],

although a reduction in partial molar volume due to changes in hydration. Even more interestingly, the regions of greatest volume change tend to cluster around the hydrated cavities [11], suggesting that the cavities are important for volume change and therefore ultimately for function. This connection arises because of the thermodynamic relationship [16]

$$\beta V k_B T = <(\delta V)^2> \quad (3)$$

where β is the compressibility (ie the amount of structural change with applied pressure), and k_B is Boltzmann's constant. In other words, the mean square volume fluctuation is proportional to compressibility. We shall return to this later, because of the connection between volume fluctuation and allostery.

Second, high pressure *stabilises alternative states*, ultimately resulting in unfolding (Figure 1). This review focuses on the nature of these alternative states, which lie typically within about 10 kJ mol^{-1} of the ground state, making them populated to a few percent at atmospheric pressure. High-pressure NMR is a good technique to characterise such states. Whereas increase in temperature adds a large amount of kinetic energy (and simultaneously alters both thermal energy and volume), and addition of chemicals such as urea or guanidine introduces complications because of binding of the denaturants to proteins, high pressure reversibly alters the energy by only a few kJ mol^{-1}, selectively destabilising the native state and increasing the population of alternative states. It therefore offers the possibility of characterising alternative states at thermodynamic equilibrium. Akasaka has provided a framework for understanding this effect by postulating a 'volume rule' [17, 18], which states that the partial molar volume decreases in parallel with the loss of conformational order. This has not been rigorously proven, but seems reasonable. It suggests that pressure may stabilise a range of different states, stabilising states of increasing disorder as the pressure increases. The evidence discussed here is that such states are functionally relevant.

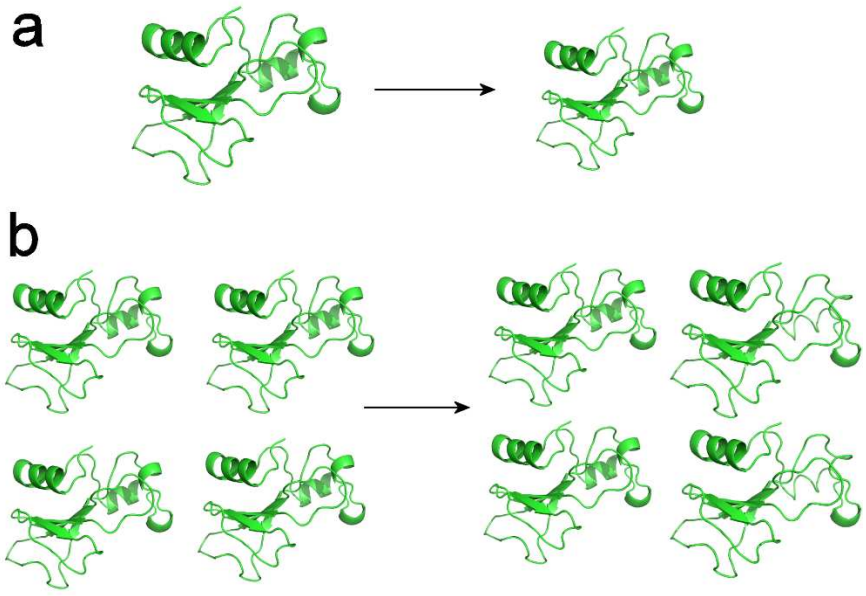


Figure 1. Pressure has two effects on proteins. (a) Compression. Not necessarily isotropic, and only about 1% reduction in volume at 200 MPa. (b) An increase in population of alternative states with smaller partial molar volume, often partly unfolded (the helix at centre right is unfolded).

2. The nature of pressure-stabilised alternative states

2.1 NMR methodology

The first attempt to study NMR at high pressure was by Jonas. In about 1971, he constructed a high-pressure cell, which contained sample and detector coil inside a pressure-resistant vessel [19]. It was at first used to study organic solvents, though he later went on to study proteins, and in particular pressure denaturation of proteins, partly in collaboration with Markley and Royer [20, 21]. This was an impressive achievement, but essentially means building your own NMR probe. It is therefore difficult to achieve good linewidth and water suppression, and this method is no longer used for biological applications. The record for a custom-built low-resolution pressure cell for NMR is 90 GPa [22], so there is a potential for obtaining useful data on proteins at very high pressure.

At around the same time, Yamada produced a high-pressure cell on a quite different design [23]. This was a thick-walled quartz tube, pulled out to a capillary. The capillary can then be glued into a holder, which can be attached on-line to a pressure pump and lowered into a standard high-resolution spectrometer. Spectral resolution is excellent, although the active volume is only about 30 μl, meaning that sensitivity is low. It is also difficult to fill, empty and clean. It was first used for investigating solvent structure [24], and later used by Akasaka for measurement of proteins [25]. A very similar device was constructed and used by Wagner to study the pressure dependence of ring flips in BPTI [26], and by Morishima to study heme [27]. Because it uses a standard NMR probe, this is a much more versatile system than that of Jonas.

However, the technology that has had the greatest impact is the recent introduction of a commercially available high pressure NMR system. This was developed by Wand for the formation of reverse micelles in liquid ethane [4], and uses a ceramic zirconia tube that can be connected up to a standard high-pressure pump, and goes up to 250 MPa. The inner diameter is 3 mm, providing an active sample volume of about 300 μl and thus good signal intensity. It is a safe and reliable system, easy to set up, and suitable for programmable pressure changes including pressure-jump experiments [28]. Technical aspects have recently been reviewed [29]. A similar system was developed by Kalbitzer using a sapphire cell [30].

As these NMR techniques developed, they were adopted for studying proteins under pressure, and a wide range of techniques have been applied under pressure, including standard NOE-based structure determination [31, 32], amide exchange [33-35], ^{15}N relaxation [36, 37], relaxation dispersion [38], measurement of *J* coupling through amide hydrogen bonds [39, 40], residual dipolar couplings [41], zz exchange [42] and ^{31}P NMR [43, 44].

2.2 Pressure denaturation and unfolding intermediates

Most of the early protein studies at high pressure had limited resolution and sensitivity, and studied protein unfolding, because it was about the only biologically interesting observation possible. Folding at high pressure is often at least ten times slower than it is at atmospheric pressure [42, 45, 46] as a consequence of the large positive activation volume [47], meaning that the folded and unfolded forms are often in slow exchange on the chemical shift timescale. It is also frequently observed that signals become exchange broadened due to unfolding [48, 49]. Because the freezing point of water at 200 MPa is -22 °C, one can also use pressure to follow cold unfolding, which is also typically in slow exchange [50, 51]. The spectra of the pressure and cold-denatured forms are not random coil, unlike those of the heat-denatured form, providing another example of how pressure is a more 'gentle' method than heat [20]. Interestingly, H/D exchange protection factors are similar for the cold and pressure denatured states, implying that they have similar residual structure [52]. Pressure also seems to be more 'gentle' than chemical denaturation: it is usually reversible, and for

example when applied to the protein pp32 one can see intermediates in pressure unfolding whereas urea unfolding is apparently two-state [53].

There has been much discussion over the origins of pressure denaturation, but it now seems clear that because pressure favours states with a lower partial molar volume, one effect of pressure is to squeeze water molecules into cavities [41, 54], which takes a water out of the bulk phase and inserts it into what previously was effectively vacuum [7, 11]. Molecular dynamics and magnetic relaxation dispersion [55] have shown that this generally occurs by the opening of transient channels, typically open for no more than 5 ps. This has been shown occasionally to increase the volume locally (because the original cavity was not quite large enough for a water molecule) but overall lowers the partial molar volume [56]. This hydrates the interior of the protein [57], and leads eventually to unfolding [58]. Crystallography has shown the same result [17, 56, 59]. The same effect occurs with protein oligomers and amyloid: pressure inserts waters into cavities and leads to their dissociation [36, 45, 51, 60].

An interesting observation made very early on [45, 48] was that pressure unfolding is in most cases not a two-state process: there are intermediates visible, as seen both from the appearance and disappearance of signals at different chemical shifts, and from the patterns of loss of signal intensity with pressure, which are different in different regions [21, 45, 61-64] (Figure 2). One can map out where for example the loss in signal intensity with pressure is larger, and thus form ideas as to what is happening. An interesting recent example involved rapid depressurization from 2.5 kbar to 1 bar, permitting characterization by ^{15}N relaxation of partially structured states shown to be 'failed folding events' [65], which provides a direct experimental demonstration of parallel folding pathways. One can also calculate free energies and volumes for the intermediate states [48, 49, 66, 67], as well as (in favourable cases) rates and transition state energies [38, 68, 69] and even chemical shifts [70]. It is possible to calculate the apparent volume of activation for unfolding, with different residues showing different values [53, 69, 71]. High pressure NMR therefore provides a large number of measures of the structure of the intermediate.

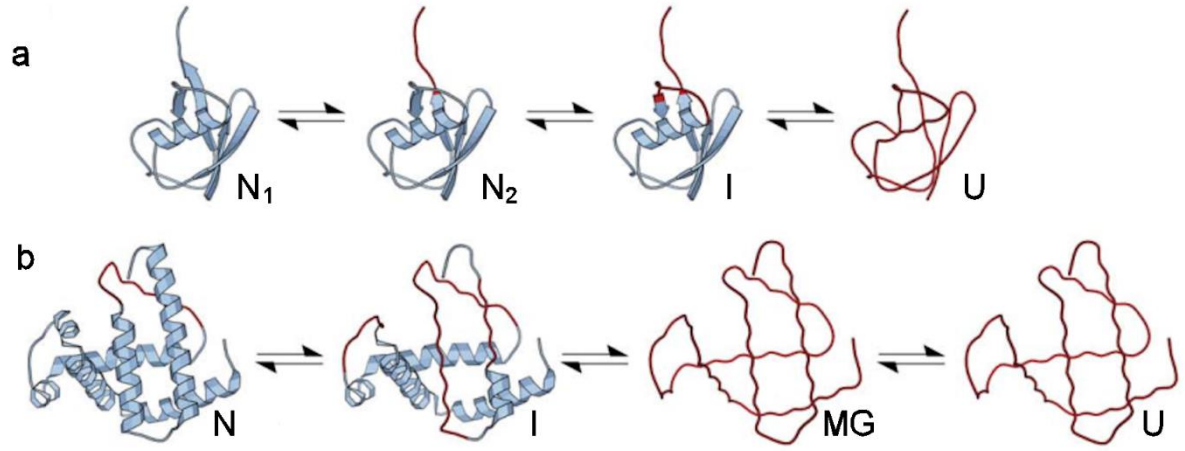


Figure 2. Examples of local pressure-induced unfolding. (a) Ubiquitin. (b) Sperm whale apomyoglobin. Native structure is in blue and unfolded structure in crimson. Adapted from Y. O. Kamatari *et al.*, *Methods* (2004), *34*:133-143 with permission. N: native; I: intermediate; MG: molten globule; U: unfolded.

Protein folding can also be followed kinetically by measuring spectroscopic changes on changing conditions, such as rapid dilution of denaturant. Intermediates have often been seen during kinetic refolding, and there has been a wide variety of ingenious experiments to work out the structure

of these kinetic intermediates. All the data published so far are consistent with the idea that these kinetic intermediates are the same as those partially unfolded forms stabilised by high pressure. This has been suggested for lysozyme [45, 72], ribonuclease A [73], ubiquitin [66], $P13^{MCTP1}$ [74] and a large number of other proteins [18, 50, 75]. A particularly convincing case was made for ubiquitin [66], where the structural changes seen in the pressure-stabilised conformer exactly matched those found in a proline-trapped kinetic intermediate found by pulse labelling.

This suggestion is fully consistent with the prevailing energy landscape view of protein folding. Proteins are envisaged as falling down a folding funnel, at the bottom of which they are folded to the native state, with a small number of thermally accessible alternative states close to the bottom. There is general agreement that evolution has smoothed out the folding funnel and removed many potential local minima, to ensure that proteins do not get trapped ('frustrated') on the way down, which would create a risk of aggregation or proteolytic destruction. Nevertheless, there must be some local minima, because most proteins need to adopt alternative states as part of their function, for example in catalysis or ligand recognition. All the states on the folding funnel must be attainable under suitable conditions, from the bottom of the funnel as well as the top, and it must therefore be the case that the minimum number of folding intermediates (the local minima on the way down) must be at least as large as the number of alternative states required for function (the local minima on the way up) [76]. It could of course be that there are more folding intermediates than merely those needed for function, but this would seem to be risky and unnecessary – one might reasonably expect that evolution should have been able to smooth out all local minima other than those that are in some way important. (However, Kalbitzer points out [76] that evolution has to simultaneously optimise function and folding, with function being more important. There are indeed examples where folding appears to result in nonfunctional trapped states, presumably because function dominates [77].) A similar argument was made by Akasaka, who concluded that the alternative conformations seen for a wide range of proteins at high pressure 'are designed for function and are closely similar to kinetic intermediates' [75]. Thus *a priori* we should expect any alternative conformations or identifiable metastable states to have functional significance.

An important consequence of this argument is that the states identified in one set of experiments as folding intermediates could be essentially identical to those identified in other experiments as functionally important alternative states, or in other words 'the energetic distinction between a low-lying excited state and a locally unfolded state is often obscure' [78]. A similar observation was made for reduced horse cytochrome *c*, where an alternative state significantly populated at normal conditions was identified as closely similar both to the oxidised state (ie functionally important) and to a major kinetic folding intermediate [79]. It is highly significant that the positions of local minima in the energy landscape seem to be dictated by the protein architecture and are therefore fixed for a given protein. Changes to solution conditions, such as the binding of ligands, change in pH, pressure, etc, alter the relative energies of the minima but do not alter their conformations [3]. This observation adds weight to the argument being made here that the alternative states populated at high pressure are functionally relevant. The next section therefore moves on to look at other alternative states seen at high pressure.

2.3 Alternative structures and their functional implications

The chemical shifts of protein NMR signals change as pressure is increased (Figure 3). Often these changes are linear with pressure. This has two origins: compression of the protein (which is roughly linear with pressure, implying that protein compressibility is more or less unaffected by pressure [64]); and pressure-dependent changes in solvation. The two effects are of similar magnitude and direction [11], and are broadly similar in all proteins. That is, most proteins compress to roughly the same extent. This is consistent with the work of Gekko and Hasegawa [80], who list the compressibilities of 25

proteins as measured by sound velocity, which fall into the fairly limited range 8.8 $\pm$ 3.7 x 10^{-12} cm^2 dyn^{-1}. However, some chemical shifts have a curved pressure dependence. Curvature is usually an indication of an alternative state, whose population is increasing as a consequence of the changing experimental conditions. This effect is well established both for temperature [81] and for pressure [11]. The obvious way to analyse curvature is to fit the pressure-dependent shift for each nucleus *i* to a quadratic equation

$$\delta_i = a_i + b_i(p - p^0) + c_i(p - p^0)^2 \quad (4)$$

where *a* is the shift at atmospheric pressure, *b* is the linear coefficient, and *c* is the non-linear coefficient. A detailed analysis of many proteins [11, 78] shows that the b_i cover a similar range in all proteins, whereas the distribution of c_i differs widely between proteins. It is particularly significant that proteins whose function requires them to be rigid (such as the protease inhibitor BPTI or the IgG-binding domain of protein G) have few residues with significant curvatures, while proteins whose function requires extensive dynamics and allosteric change (such as the Ras-like small signalling protein RalGDS and the transporter β-lactoglobulin) have many. The non-linear shifts tend to occur close to cavities containing buried water molecules, reinforcing the idea that the cavities are protein-specific and relate to function [78]: this was observed for BPTI [14], hen lysozyme [13], RalGDS-RBD [48], β-lactoglobulin [82], and protein G B1 domain [15]. For BPTI, lysozyme, protein G and barnase, the cavities (and the curved shifts and large fluctuations) are also close to the active sites, providing a further link to function.

A particularly clear example is provided by ubiquitin. Ubiquitin is attached via its C-terminus to a range of other proteins, stimulating processes including protein degradation, inflammation, and DNA repair. High-pressure NMR studies on ubiquitin showed a number of curved shift dependences, several of which had a sigmoidal pattern [66, 83]. These could not be fitted to equation (4), but suggested a Boltzmann equilibration between at least two states, with a significant change in relative populations between 0.1 and 350 MPa. The shift changes were therefore fitted to a Boltzmann distribution between two native-like states N_1 and N_2

$$\delta = \frac{\delta_{N1} + \delta_{N2} exp\left\{\frac{\Delta G^0 + \Delta V^0 (p - p_0)}{RT}\right\}}{1 + exp\left\{\frac{\Delta G^0 + \Delta V^0 (p - p_0)}{RT}\right\}} \quad (5)$$

resulting in fitted values for ΔG^0 (the free energy difference between N_1 and N_2 at ambient pressure) of 4.2 $\pm$ 0.4 kJ mol^{-1} and ΔV^0 (the volume difference between N_1 and N_2 at ambient pressure) of -24 $\pm$ 2 ml mol^{-1}. The two conformers exchange on a timescale of about 10 μs [31]. This implies that at atmospheric pressure, the population of N_2 is 15%, rising to 78% at 300 MPa. N_1 is the familiar native state: crystallisation or NMR structure calculation at atmospheric pressure produces a single conformation, essentially identical to N_1. The structure of N_2 was determined by NOE-based methods using data collected at 300 MPa [31], and shows differences from N_1 located primarily at the C-terminus and two regions spatially adjacent to it. This structure calculation is likely to be biased because of the significant population of N_1 still present at this pressure. Therefore, a mutation Q41N was identified that stabilises the N_2 conformation, such that at ambient pressure Q41N is 70% in the N_2 conformation, and 98% at 300 MPa [84]. Structure calculation of Q41N at both at 0.1 MPa [84] and at 250 MPa [32] confirms the structural changes observed previously, and indicates a water penetrated into the protein, giving N_2 a smaller partial molar volume than N_1. The N_2 structure is closely similar to the conformation adopted by ubiquitin when it binds to the ubiquitin-activating enzyme E1, suggesting that this conformational change is functionally important [32]. Following identification of the importance of the Q41-I36 hydrogen bond from high-pressure NMR, subsequent investigations confirmed that this hydrogen bond is also important for structural fluctuations at ambient pressure [85]. The same equilibration between N_1 and N_2 is also seen for the ubiquitin-like proteins NEDD8 [67] and SUMO-2 [37], which have similar structure and activation, but quite different

biological function. The free energy difference between the two states is significantly different in the three proteins, providing another example of energy landscapes that have minima in similar places but with different relative energies.

Another interesting example is the R3 domain of talin [86]. This domain is a key part in the establishment of focal adhesion complexes that attach the cell to the extracellular matrix, and their subsequent linking to the actin cytoskeleton. R3 binds to the RAP-1-GTP-interacting adaptor molecule RIAM, and thereby fixes talin to integrin cell surface receptors. Talin binds weakly to actin, but a physical pulling force on actin fibres causes a conformational change to the R3 domain, which opens out R3 and enables it to bind vinculin, thereby greatly strengthening the affinity for actin. The biological function of R3 is therefore to act as a mechanosensitive switch, opening up as a result of shear stress.

NMR spectra of R3 at variable pressure show marked curvature of some chemical shift trajectories (Figure 3), implying a high population of an alternative state. There is also a large linear shift change. It was therefore not possible to fit the shift changes to equation (5), and a more complicated equation was used that includes both linear shift changes and pressure-dependent Boltzmann population changes:

$$\delta = \frac{(\delta_1^0+\Delta\delta_1\{p-p_0\})+(\delta_2^0+\Delta\delta_2\{p-p_0\})exp\left(-\left[\Delta G^0+\Delta V^0\{p-p_0\}\right]/_{RT}\right)}{1+exp\left(-\left[\Delta G^0+\Delta V^0\{p-p_0\}\right]/_{RT}\right)} \quad (6)$$

where the symbols have the meanings defined above; in addition, $\delta_1{}^0$ and $\delta_1{}^0$ are the chemical shifts of the ground and alternative states respectively at ambient pressure, and $\Delta\delta_1$ and $\Delta\delta_2$ are the gradients of the shift changes with pressure. This equation has four parameters per nucleus plus an extra two global parameters (ΔG^0 and ΔV^0) and therefore requires careful fitting. This was done by fitting the two global parameters using a small subset of the most curved nuclei (specifically, those with the largest χ^2 values for fitting to the simple parabola of equation 4), and then fixing the two global parameters and fitting everything else. Despite the remarkably curved pattern for some residues, all nuclei could be fitted well to this equation, resulting in a value of ΔG^0 very close to zero, implying essentially 50% of each of the two conformations at ambient pressure. This is a clear benefit of using equation (6), because the more standard equation (4) does not provide this information. The fitting therefore matches the expected mechanosensitive switch function, because it shows that the N_1/N_2 equilibrium is exactly poised in the middle, and is therefore in exactly the right place to be affected by external factors such as binding partners or shear force. The other big benefit of using equation (6) is that the fitting produces chemical shifts for ^1H_N, ^{15}N, $^{13}C_\alpha$, $^{13}C_\beta$ and $^{13}C'$ for all residues in both the N_1 and N_2 states at atmospheric pressure. This enables standard chemical shift-based structure calculations to be used, in this case TALOS-N [87] together with additional information available from the $^{13}C_\alpha$ and $^{13}C_\beta$ shifts [88]. These calculations showed that the N_1 state has the four-helix bundle structure calculated using standard structure calculations at atmospheric pressure [89], while the N_2 state has helix 1 opened out (Figure 4). This structure is consistent with the biological function of the domain, which requires helix 2 to interact with vinculin, using a face normally buried by contact with helix 1 [86].

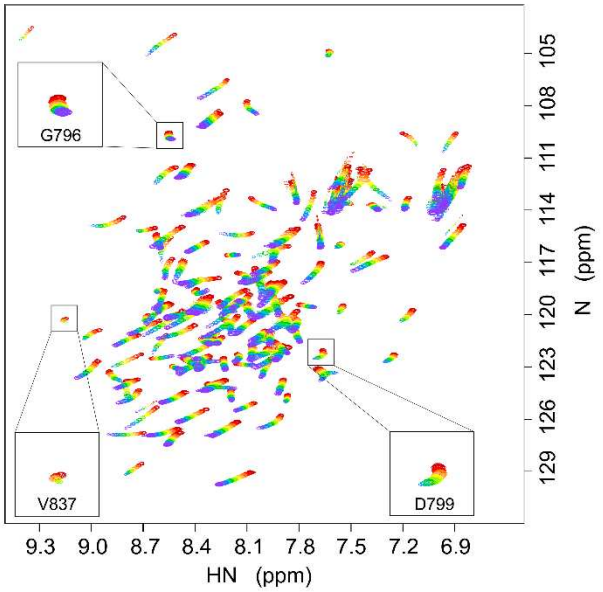


Figure 3. Changes in ^{1}H and ^{15}N chemical shifts in the HSQC spectrum of the R3 domain of talin. Pressures range from 0.1 MPa (red) to 250 MPa (violet). Three residues with unusually highly curved shift changes are highlighted. Adapted from Baxter *et al*., *Structure* (2017) *25*:1856-1866, with permission.

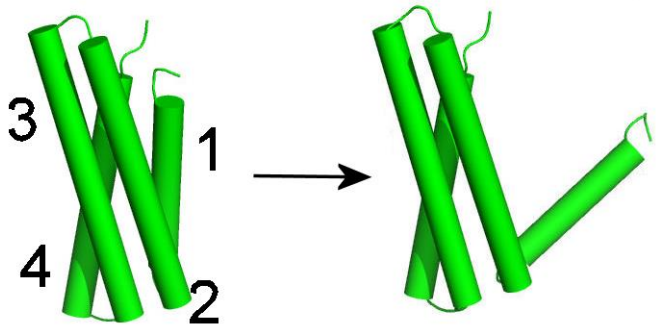


Figure 4. Structural change in the R3 domain of talin as measured using high pressure NMR. Left is the closed (low pressure) form, with the helices labelled. Right is the high pressure form, in which helix 1 is opened out. Adapted from Baxter *et al*., *Structure* (2017) *25*:1856-1866, with permission.

Thus for both ubiquitin and talin R3 domain, there is unambiguous evidence to show that the pressure-stabilised alternative state is functional. The same can be said for dihydrofolate reductase (DHFR) and prion protein. *E. coli* DHFR shows peak splitting for several resonances at high pressure, indicating the pressure-induced appearance of an alternative conformation which has not been characterised in detail but matches the structure change expected on going from the occluded to open states, necessary for binding of NADPH [75, 90]. The human and Syrian hamster prion proteins have almost identical native structures, but do not seed cross-infectivity. High-pressure NMR experiments showed that both proteins have alternative states present that are stabilised by pressure. They both have native-like states N_1 and N_2 (with N_2 about 22% populated in both) as well as higher energy states I_1 and I_2, the latter of which is populated to only 0.05% in human prion protein. It was suggested that the I_2 states, which are different for the two species, are the most likely intermediates on going between PrP^{C} to PrP^{Sc}, with a good match to known hotspots for prion disease [91]. Finally, we should mention a cavity mutant of T4 lysozyme, L99A. This is a well-studied protein, and the large cavity is

known to become gradually hydrated as the pressure is increased. It undergoes a transition to an alternative state at high pressure. Both states were characterised using methyl group dynamics, showing that conformational fluctuations are located around the cavity, with the fluctuations being smaller in the high-pressure state. The high-pressure state appears to have the Phe114 sidechain inserted into the cavity, a conformation seen transiently at atmospheric pressure [92]. Thus in this example (of an artificial site-directed mutant), the high-pressure state is not functionally important, although it is demonstrated to be a state populated transiently at ambient pressure. However, for the other examples discussed here, the excited state is often as functionally important as the ground state, if not more so.

A few studies have been reported of the effect of pressure on NMR spectra of intrinsically disordered proteins (IDPs) [93-95]. Two of the three studies reported non-linear pressure-dependent chemical shift changes, which the authors interpreted as indicating a change in the conformational ensemble with pressure. In both cases, the change was proposed to be from a β-sheet towards a polyproline-II helix. In one case, α-synuclein [95], this was supported by changes in *J* coupling constants. It may therefore be possible to investigate partial or residual structure in IDPs using high pressure NMR.

2.4 Fluctuations at elevated pressure

The fluctuations undergone by proteins are important. Pressure can provide useful information, not easily attainable from other methods.

The fluctuations characterised using high pressure are volume fluctuations. These have been studied since Wagner's measurement of the activation volume for ring flips in BPTI [26], and in many different measurements since [12]. ^{15}N relaxation measurements provide information mainly on rapid fluctuations (ps/ns), and show that fluctuations on this timescale are affected little by pressure [36, 37, 95]. However, larger fluctuations are slowed dramatically by high pressure. We have already seen that protein folding is often more than ten times slower at high pressure. Ring flips are also slowed by between 10 and 100-fold at high pressure [36]. These conclusions are in agreement with results from quasielastic neutron scattering and molecular dynamics calculations [96, 97].

A useful source of information on fluctuations comes from measurements of amide proton exchange rates. Under EX2 conditions, these are directly related to the probability of local opening. The effect of pressure on exchange rates has been measured [33-35, 39], and should provide information on the activation volume for the local unfolding process. However, they have proved difficult to interpret, with adjacent sites having very different apparent activation volumes, and no correlation for example with changes in entropy or enthalpy. A recent publication [98] has shown that amide proton exchange requires simultaneous binding of at least two water molecules to the amide group, and it may be that modelling this will make more sense of the data.

One way to obtain volume fluctuations is through equation (3), which presents the thermodynamic connection between compressibility and volume fluctuation. Compressibility is essentially the amount of compression at each site in the protein, for which chemical shift changes in HSQC spectra are a good proxy. Although equation (3) is valid only for the whole system and not for individual parts of it, it appears to hold for local fluctuations as well as global fluctuations (discussed in [99]), possibly because the fluctuations are at high frequency and thermodynamically almost uncoupled from each other. As discussed in [11], linear pressure-dependent chemical shift changes are broadly similar across a range of different proteins, implying that the average compressibility of proteins is fairly constant: the data quoted above from Gekko and Hasegawa [80] also support this idea. However local compressibilites can be very variable, as shown by the linear shift coefficients, and in particular by the non-linear coefficients presented in [11]. The detailed structure changes presented for BPTI [14], lysozyme [13], protein G [15] and barnase [99] at high pressure also show that local

compressibilities can be very variable, with some being negative (ie local expansion with pressure). The fluctuations are largest close to buried water molecules, further highlighting the importance of buried waters, and suggesting that a good proxy for volume fluctuations could be the magnitude of the non-linear shift term (*c* in equation (4)). In barnase, it was concluded that the fluctuations occur on a timescale of ns-μs, a difficult timescale to measure experimentally by other methods [99]. In free barnase, the overall volume fluctuation is around 60 $Å^3$: roughly the same volume as the activation volume for flipping a restricted aromatic ring, so a very large fluctuation if concentrated in one place. An interesting observation made by Gekko using ultrasound [100], and later confirmed by NMR [99], is that addition of ligands can cause major changes in local compressibility. Gekko pointed out that addition of ligands to DHFR changed the compressibility by between -19% (with addition of reaction products) and +15% (with addition of reactants), while addition of inhibitory ligands to lysozyme progressively decreased the compressibility as the size and potency of the ligands increased: GlcNAc -17%, $GlcNAc_2$ -27% and $GlcNAc_3$ -73% [101]. Wilton et al saw a reduction in compressibility of barnase by 70% on adding the inhibitor d(CGAC) [99]. Measurements of local compressibility have also been made by methyl group relaxation under pressure, and showed that local compressibility varies widely in different regions of ubiquitin and decreases at higher pressure; in detail the local compressibilities follow no obvious pattern [102]. We therefore conclude that high pressure (and probably something as simple as the non-linear shift coefficients) is a good way to measure volume fluctuations.

Protein fluctuations increase the entropy of the system. It should therefore be true that if for example the fluctuations decrease on addition of a ligand, then the entropy change is less favourable and the ligand should bind more weakly [103]. Fluctuations could thus be used to understand the energetics of ligand binding. The experimental measurement of conformational entropy is not easy, and a leading method currently is the order parameters of methyl groups, which appears to correlate well with entropy [104]. It does indeed appear that conformational entropy has a major role in ligand binding affinity [105]. It would clearly be worth investigating non-linear shift coefficients as an alternative measure, in particular because they provide a measure of local fluctuation at almost every residue, as compared to methyl order parameters, which only sample dynamics where there are methyl groups.

2.5 Dynamic allostery?

Over the last few years it has become clear that allosteric effects can be communicated not just by the traditional static structure mechanisms, which rely on some kind of mechanical coupling between sites, but also by dynamic effects. As commented as far back as 1984, 'proteins may have evolved to take functional advantage of thermal fluctuations' [106]. In order to have coupling, it is merely necessary that the effect of ligand binding at one site be communicated to another site. This communication can be (thermo)dynamic rather than structural: binding of a ligand may for example change the dynamics in one domain, which alters the interface between that domain and its neighbour, and thereby passes on allosteric information [107]. In this context, we note a recent paper which specifically linked volume fluctuations to allosteric behaviour [108]. High pressure NMR is a good way of investigating this kind of behaviour, and is an exciting opportunity. A particularly good example comes in the study of kinases. Kinase function is tightly regulated by allostery, and NMR studies of kinases are difficult because typically a large fraction of signals are unobservable due to intermediate rate conformational effects. One region often missing from NMR spectra of kinases is the catalytic or DFG loop (so called because of the amino acid sequence) which is highly conserved and is in the hinge region between the two domains of the kinase, close to the activation loop. Crystallographic studies have shown that this loop can exist either in the DFG-in conformation or the DFG-out conformation, the latter of which is incapable of binding ATP and is inactive. Different ligands can stabilise one or the other form. Nielsen et al [109] studied the kinase P38α at high pressure. Application of pressure to

the apoprotein results in appearance of signals from the DFG loop, presumably as a result of slowed conformational dynamics. It also gave rise to chemical shift changes suggesting that the conformation of the loop changes to a DFG-out state. Conversely, applying pressure to a complex with the ligand BIRB796, which is in a DFG-out conformation, alters the conformation to something resembling the apo state. It therefore appears that pressure alters the height of regions of the energy landscape in a way that mimics the addition of allosteric ligands. The authors comment that 'pressure selects excited-state conformations of biological relevance' [109].

There have been other observations of allosteric effects linked to high pressure [110]. Kalbitzer has worked extensively on signalling proteins related to Ras, and also on high pressure, and it is therefore not surprising that he has observed population shifts among different states using high pressure [111]. Ras has to cycle through different conformations as part of its function. The active form of Ras is bound to GTP, and the inactive form is bound to GDP. Release of GDP is induced by binding of the GDP-bound form to a guanine nucleotide exchange factor (GEF), while hydrolysis of GTP to GDP is catalysed by binding of Ras-GTP to a GTPase activating protein (GAP). An analysis suggests that there should therefore be at least 8 forms present at significant population. In the presence of GTP, the four predominant forms were identified using a combination of ^{31}P NMR [76] and ^{1}H, ^{15}N HSQC [111] at variable pressure (Figure 5). Three out of the four can be made the major species in solution depending on the pressure.

The significance of this observation is that the different conformations of Ras are all potential drug targets, because stabilisation of Ras in one form has important effects on Ras-dependent signalling pathways. The same is of course true for many other proteins that display allosteric behaviour, and indeed high pressure has been suggested as a way of identifying drug targets for several of these [62, 111-113].

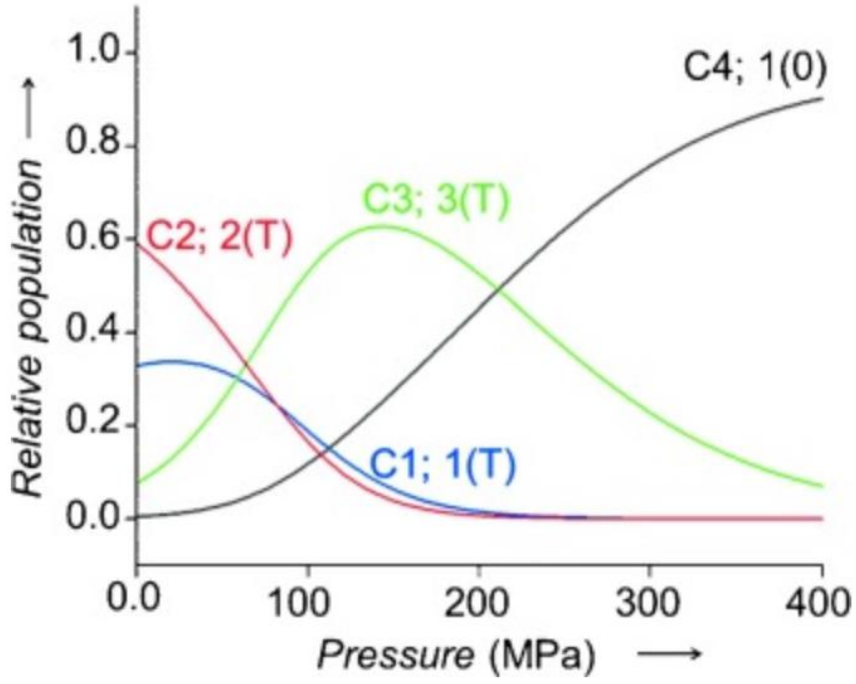


Figure 5. The conformational states of Ras accessible by use of high pressure. C1 is the conformation of the form bound to GTP and guanine nucleotide exchange factor (GEF). C2 is the conformation bound to GTP and the effector. C3 is the conformation bound to GTP and the GTPase activating protein (GAP). C4 is the conformation bound to GEF but no nucleotide cofactor. These are 4 of the ca. 8 states

accessed by Ras during its activity cycle. Adapted from Kalbitzer *et al* (2013) *Angew. Chem. Int. Edn. 52*:14242-14246, with permission.

3. Conclusions

Experimental observations have confirmed the general correctness of the energy landscape model, and shown that proteins usually populate the major ground state conformation to at least 90% under working conditions. However, they clearly also populate other conformations. Enzymes minimally have closed and opened conformations, and usually several more when bound to specific substrates or combinations of substrates. A good example is provided by dihydrofolate reductase [114]. It binds two reactants, and its catalytic cycle requires it to cycle round at least five different complexes. For each of these complexes, NMR measurements have shown that it adopts a different conformation, but is capable at each stage of sampling at least two more, these being the conformations required for the previous and next stages in the catalytic cycle. The binding of its different ligands at each stage thus alters the energy landscape to favour the appropriate conformations. Similarly, signalling proteins have several conformations, and switch between them stimulated by the binding of ligands or allosteric effectors. It is worth repeating that it appears that the binding of ligands alters the *energies* of the different local minima, but does not seem to alter their *conformations,* which are presumably dictated by the protein architecture, the geometries of mutually consistent sets of hydrogen bonds, and so on. A major challenge in protein science is to identify these conformations, and work out their energies, and the energy barriers between them, in order to understand the kinetics of conformational exchange.

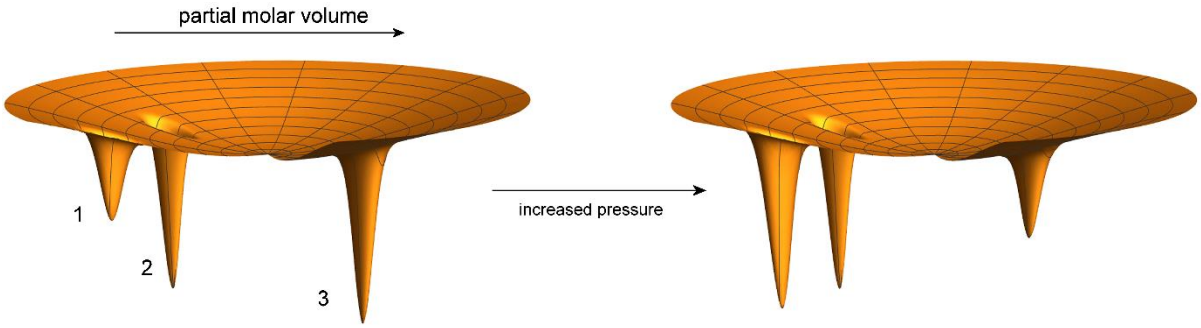


Figure 6. Hydrostatic pressure biases the energy landscape according to the partial molar volume of each protein conformation. State 3 is the native state, and has a larger partial molar volume than the excited states 1 and 2. State 1 is more activated/partially unfolded than state 2, and consequently has the smallest partial molar volume. High pressure therefore stabilises 1 and destabilises 3. Figure generated using a *Mathematica*™ script written by T. Oas (Duke University).

High-pressure NMR can be useful in this enterprise. It also biases the energy landscape, in a predictable way, by disfavouring the ground state and favouring excited states to different degrees depending on a quantifiable extensive physical property, their partial molar volume (Figure 6). In doing so it can alter the populations of different states very greatly, without adding much extra energy to the system, and thus make previously unobservable conformations observable, and sometimes even the major species present. This makes it easier to characterise and sometimes even determine structures of minor (but functionally important) conformers. In general it also slows down the exchange between different conformers, further helping to identify the different conformers. This

could be of particular benefit in allosteric systems, and help in the design of drugs to stabilise specific conformations. Pressure-dependent chemical shifts may also be a useful measure of conformational entropy. With the recent availability of a simple and reliable commercial system, there would seem to be a bright future for high-pressure NMR.

References

[1] P.W. Bridgman, The coagulation of albumen by pressure, J Biol Chem 19 (1914) 511-512.
[2] C.A. Royer, Revisiting volume changes in pressure-induced protein unfolding, Biochim. Biophys. Acta: Protein Structure Mol. Enzymol. 1595 (2002) 201-209.
[3] M.P. Williamson, How Proteins Work, Garland Science, Place Published, 2011.
[4] R.W. Peterson, N.V. Nucci, A.J. Wand, Modification of encapsulation pressure of reverse micelles in liquid ethane, J. Magn. Reson. 212 (2011) 229-233.
[5] C.R. Chen, G.I. Makhatadze, Molecular determinant of the effects of hydrostatic pressure on protein folding stability, Nature Comms 8 (2017) 14561.
[6] O. Carugo, Statistical survey of the buried waters in the Protein Data Bank, Amino Acids 48 (2016) 193-202.
[7] Y.O. Kamatari, L.J. Smith, C.M. Dobson, K. Akasaka, Cavity hydration as a gateway to unfolding: An NMR study of hen lysozyme at high pressure and low temperature, Biophys Chem 156 (2011) 24-30.
[8] M.A. Williams, J.M. Goodfellow, J.M. Thornton, Buried waters and internal cavities in monomeric proteins, Prot Sci 3 (1994) 1224-1235.
[9] S. Fischer, C.S. Verma, Binding of buried structural water increases the flexibility of proteins, Proc. Natl Acad. Sci. USA 96 (1999) 9613-9615.
[10] S. Fischer, J.C. Smith, C.S. Verma, Dissecting the vibrational entropy change on protein/ligand binding: Burial of a water molecule in bovine pancreatic trypsin inhibitor, J. Phys. Chem. B 105 (2001) 8050-8055.
[11] R. Kitahara, K. Hata, H. Li, M.P. Williamson, K. Akasaka, Pressure-induced chemical shifts as probes for conformational fluctuations in proteins, Progr. Nucl. Magn. Reson. Spectrosc. 71 (2013) 35-58.
[12] M.P. Williamson, Pressure-dependent conformation and fluctuation in folded protein molecules, in: K. Akasaka, H. Matsuki (Eds.) High pressure bioscience, Springer, Place Published, 2015, pp. 109-127.
[13] M. Refaee, T. Tezuka, K. Akasaka, M.P. Williamson, Pressure-dependent changes in the solution structure of hen egg-white lysozyme, J. Mol. Biol. 327 (2003) 857-865.
[14] M.P. Williamson, K. Akasaka, M. Refaee, The solution structure of bovine pancreatic trypsin inhibitor at high pressure, Protein Sci. 12 (2003) 1971-1979.
[15] D.J. Wilton, R.B. Tunnicliffe, Y.O. Kamatari, K. Akasaka, M.P. Williamson, Pressure-induced changes in the solution structure of the GB1 domain of protein G, Proteins: Struct. Funct. Bioinf. 71 (2008) 1432-1440.
[16] A. Cooper, Thermodynamic fluctuations in protein molecules, Proc. Natl. Acad. Sci. USA 73 (1976) 2740-2741.
[17] R. Fourme, E. Girard, K. Akasaka, High-pressure macromolecular crystallography and NMR: status, achievements and prospects, Curr. Opin. Struct. Biol. 22 (2012) 636-642.
[18] K. Akasaka, Probing conformational fluctuation of proteins by pressure perturbation, Chem. Rev. 106 (2006) 1814-1835.
[19] J. Jonas, P. Koziol, X. Peng, C. Reiner, D.M. Campbell, High-resoution NMR spectroscopy at high pressures, J. Magn. Reson. Ser. B 102 (1993) 299-309.
[20] J. Jonas, High-resolution nuclear magnetic resonance studies of proteins, Biochim. Biophys. Acta 1595 (2002) 145-159.

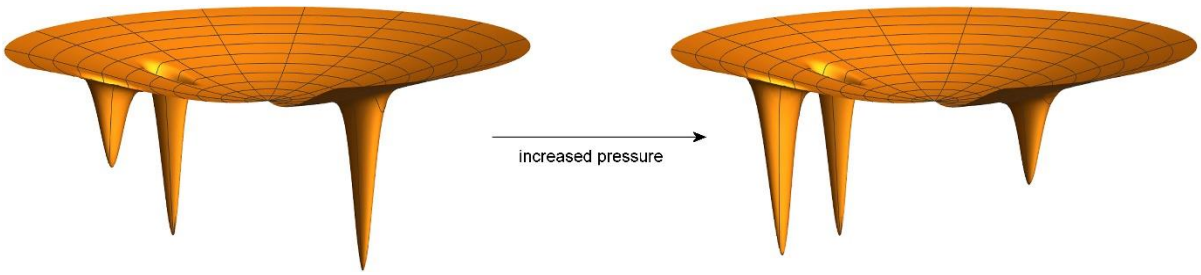
[21] C.A. Royer, A.P. Hinck, S.N. Loh, K.E. Prehoda, X.D. Peng, J. Jonas, J.L. Markley, Effects of amino acid substitutions on the pressure denaturation of Staphylococcal nuclease as monitored by fluorescence and nuclear magnetic resonance spectroscopy, Biochemistry 32 (1993) 5222-5232.
[22] T. Meier, S. Khandarkhaeva, S. Petitgirard, T. Körber, A. Lauerer, E. Rössler, L. Dubrovinsky, NMR at pressures up to 90 GPa, J. Magn. Reson. 292 (2018) 44-47.
[23] H. Yamada, Pressure-resisting glass cell for high pressure, high resolution NMR measurement, Rev Sci Instrum 45 (1974) 640-642.
[24] H. Yamada, High-resolution NMR measurement under high pressure and pressure dependence of proton chemical shifts, Chem Letts (1972) 747-750.
[25] T. Yamaguchi, H. Yamada, K. Akasaka, Thermodynamics of unfolding of Ribonuclease A under high pressure: A study by proton NMR, J Mol Biol 250 (1995) 689-694.
[26] G. Wagner, Activation volumes for the rotational motion of interior aromatic rings in globular proteins determined by high-resolution ^{1}H NMR at variable pressure, FEBS Lett. 112 (1980) 280-284.
[27] I. Morishima, M. Hara, High-pressure nuclear magnetic resonance studies of hemoproteins. Pressure-induced structural changes in the heme environments of ferric low-spin metmyoglobin complexes, Biochemistry 22 (1983) 4102-4107.
[28] J. Roche, M. Dellarole, J.A. Caro, D.R. Norberto, A.E. Garcia, B. Garcia-Moreno, C. Roumestand, C.A. Royer, Effect of internal cavities on folding rates and routes revealed by real-time pressure-jump NMR spectroscopy, J Am Chem Soc 135 (2013) 14610-14618.
[29] J.A. Caro, A.J. Wand, Practical aspects of high-pressure NMR spectroscopy and its applications in protein biophysics and structural biology, Methods (San Diego, Calif.) (2018) in press.
[30] M.R. Arnold, H.R. Kalbitzer, W. Kremer, High-sensitivity sapphire cells for high pressure NMR spectroscopy on proteins, J. Magn. Reson. 161 (2003) 127-131.
[31] R. Kitahara, S. Yokoyama, K. Akasaka, NMR snapshots of a fluctuating protein structure: Ubiquitin at 30 bar-3 kbar, J. Mol. Biol. 347 (2005) 277-285.
[32] S. Kitazawa, T. Kameda, A. Kumo, M. Yagi-Utsumi, N.J. Baxter, K. Kato, M.P. Williamson, R. Kitahara, Close identity between alternatively folded state N_2 of ubiquitin and the conformation of the protein bound to the ubiquitin-activating enzyme, Biochemistry 53 (2014) 447-449.
[33] E.J. Fuentes, A.J. Wand, Local stability and dynamics of apocytochrome b_{562} examined by the dependence of hydrogen exchange on hydrostatic pressure, Biochemistry 37 (1998) 9877-9883.
[34] T.K. Hitchens, R.G. Bryant, Pressure dependence of amide hydrogen-deuterium exchange rates for individual sites in T4 lysozyme, Biochemistry 37 (1998) 5878-5887.
[35] M.E. Dixon, T.K. Hitchens, R.G. Bryant, Comparisons of pressure and temperature activation parameters for amide hydrogen exchange in T4 lysozyme, Biochemistry 39 (2000) 248-254.
[36] H. Li, K. Akasaka, Conformational fluctuations of proteins revealed by variable pressure NMR, Biochim Biophys Acta: Proteins Proteomics 1764 (2006) 331-345.
[37] R. Kitahara, C. Zhao, K. Saito, S. Koshiba, M. Ioune, T. Kigawa, S. Yokoyama, K. Akasaka, Basic folded and low-populated locally disordered conformers of SUMO-2 characterized by NMR spectroscopy at varying pressures, Biochemistry 47 (2008) 30-39.
[38] D.M. Korzhnev, I. Bezsonova, F. Evanics, N. Taulier, Z. Zhou, Y.W. Bai, T.V. Chalikian, R.S. Prosser, L.E. Kay, Probing the transition state ensemble of a protein folding reaction by pressure-dependent NMR relaxation dispersion, J. Am. Chem. Soc. 128 (2006) 5262-5269.
[39] L. Nisius, S. Grzesiek, Key stabilizing elements of protein structure identified through pressure and temperature perturbation of its hydrogen bond network, Nature Chem 4 (2012) 711-717.
[40] H. Li, H. Yamada, K. Akasaka, A.M. Gronenborn, Pressure alters electronic orbital overlap in hydrogen bonds, J. Biomol. NMR 18 (2000) 207-216.
[41] J. Roche, J.M. Louis, A. Bax, R.B. Best, Pressure-induced structural transition of mature HIV-1 protease from a combined NMR/MD simulation approach, Proteins:Struct Funct Bioinf 83 (2015) 2117-2123.

[42] Y. Zhang, S. Kitazawa, I. Peran, N. Stenzoski, S.A. McCallum, D.P. Raleigh, C.A. Royer, High pressure ZZ-exchange NMR reveals key features of protein folding transition states, J Am Chem Soc 138 (2016) 15260-15266.
[43] M. Karl, M. Spoerner, P. Thuy-Vy, S.P. Narayanan, W. Kremer, H.R. Kalbitzer, Pressure response of ^{31}P chemical shifts of adenine nucleotides, Biophys. Chem. 231 (2017) 50-54.
[44] M. Spoerner, M. Karl, P. Lopes, M. Hoering, K. Loeffel, A. Nuehs, J. Adelsberger, W. Kremer, H.R. Kalbitzer, High pressure ^{31}P NMR spectroscopy on guanine nucleotides, J Biomol NMR 67 (2017) 1-13.
[45] J.L. Silva, D. Foguel, C.A. Royer, Pressure provides new insights into protein folding, dynamics and structure, Trends Biochem Sci 26 (2001) 612-618.
[46] G. Pappenberger, C. Saudan, M. Becker, A.E. Merbach, T. Kiefhaber, Denaturant-induced movement of the transition state of protein folding revealed by high-pressure stopped-flow measurements, Proc Natl Acad Sci USA 97 (2000) 17-22.
[47] S. Neumaier, M. Büttner, A. Bachmann, T. Kiefhaber, Transition state and ground state properties of the helix-coil transition in peptides deduced from high-pressure studies, Proc Natl Acad Sci USA 110 (2013) 20988-20993.
[48] K. Inoue, H. Yamada, K. Akasaka, C. Hermann, W. Kremer, T. Maurer, R. Doker, H.R. Kalbitzer, Pressure-induced local unfolding of the Ras binding domain of RalGDS, Nature Struct Biology 7 (2000) 547-550.
[49] R. Kitahara, H. Yamada, K. Akasaka, P.E. Wright, High pressure NMR reveals that apomyoglobin is an equilibrium mixture from the native to the unfolded, J Mol Biol 320 (2002) 311-319.
[50] K. Akasaka, R. Kitahara, Y.O. Kamatari, Exploring the folding energy landscape with pressure, Arch Biochem Biophys 531 (2013) 110-115.
[51] J. Jonas, A. Jonas, High-pressure NMR spectroscopy of proteins and membranes, Ann Rev Biophys Biomol Struct 23 (1994) 287-318.
[52] D. Nash, B.S. Lee, J. Jonas, Hydrogen-exchange kinetics in the cold denatured state of ribonuclease A, Biochim Biophys Acta: Protein Struct Mol Enzymol 1297 (1996) 40-48.
[53] M.J. Fossat, T.P. Dao, K. Jenkins, M. Dellarole, Y. Yang, S.A. McCallum, A.E. Garcia, D. Barrick, C. Roumestand, C.A. Royer, High-resolution mapping of a repeat protein folding free energy landscape, Biophys J 111 (2016) 2368-2376.
[54] G. Weber, H.G. Drickamer, The effect of high pressure upon proteins and other biomolecules, Quart Rev Biophys 16 (1983) 89-112.
[55] F. Persson, B. Halle, Transient access to the protein interior: Simulation versus NMR, J Am Chem Soc 135 (2013) 8735-8748.
[56] H. Yamada, T. Nagae, N. Watanabe, High-pressure protein crystallography of hen egg-white lysozyme, Acta Cryst D 71 (2015) 742-753.
[57] V.V. Mozhaev, K. Heremans, J. Frank, P. Masson, C. Balny, High pressure effects on protein structure and function, Proteins: Struct Funct Genet 24 (1996) 81-91.
[58] J. Roche, J.A. Caro, D.R. Norberto, P. Barthe, C. Roumestand, J.L. Schlessman, A.E. Garcia, B. Garcia-Moreno E, C.A. Royer, Cavities determine the pressure unfolding of proteins, Proc. Natl. Acad. Sci. USA 109 (2012) 6945-6950.
[59] M.D. Collins, C.U. Kim, S.M. Gruner, High-pressure protein crystallography and NMR to explore protein conformations, Ann Rev Biophys 40 (2011) 81-98.
[60] G.A.P. de Oliveira, M.d.A. Marques, C. Cruzeiro-Silva, Y. Cordeiro, C. Schuabb, A.H. Moraes, R. Winter, H. Oschkinat, D. Foguel, M.S. Freitas, J.L. Silva, Structural basis for the dissociation of α-synuclein fibrils triggered by pressure perturbation of the hydrophobic core, Sci Rep 6 (2016) 37990.
[61] Y.O. Kamatari, R. Kitahara, H. Yamada, S. Yokoyama, K. Akasaka, High-pressure NMR spectroscopy for characterizing folding intermediates and denatured states of proteins, Methods 34 (2004) 133-143.
[62] N. Kachel, W. Kremer, R. Zahn, H.R. Kalbitzer, Observation of intermediate states of the human prion protein by high pressure NMR spectroscopy, BMC Struct Biol 6 (2006) 16.

[63] N.V. Nucci, B. Fuglestad, E.A. Athanasoula, A.J. Wand, Role of cavities and hydration in the pressure unfolding of T_4 lysozyme, Proc Natl Acad Sci USA 111 (2014) 13846-13851.
[64] K. Heremans, L. Smeller, Protein structure and dynamics at high pressure, Biochim Biophys Acta Protein Struct Mol Enzymol 1386 (1998) 353-370.
[65] C. Charlier, T.R. Alderson, J.M. Courtney, J. Ying, P. Anfinrud, A. Bax, Study of protein folding under native conditions by rapidly switching the hydrostatic pressure inside an NMR sample cell, Proc. Natl. Acad. Sci. USA 115 (2018) E4169-E4178.
[66] R. Kitahara, K. Akasaka, Close identity of a pressure-stabilized intermediate with a kinetic intermediate in protein folding, Proc. Natl Acad. Sci. USA 100 (2003) 3167-3172.
[67] R. Kitahara, Y. Yamaguchi, E. Sakata, T. Kasuya, K. Tanaka, K. Kato, S. Yokoyama, K. Akasaka, Evolutionally conserved intermediates between ubiquitin and NEDD8, J. Mol. Biol. 363 (2006) 395-404.
[68] I. Bezsonova, D.M. Korzhnev, R.S. Prosser, J.D. Forman-Kay, L.E. Kay, Hydration and packing along the folding pathway of SH3 domains by pressure-dependent NMR, Biochemistry 45 (2006) 4711-4719.
[69] J. Roche, C.A. Royer, C. Roumestand, Monitoring protein folding through high pressure NMR spectroscopy, Progr Nucl Magn Reson Spectrosc 102 (2017) 15-31.
[70] C. Charlier, J.M. Courtney, T.R. Alderson, P. Anfinrud, A. Bax, Monitoring ^{15}N chemical shifts during protein folding by pressure-jump NMR, J. Am. Chem. Soc. 140 (2018) 8096-8099.
[71] S.D. Samarasinghe, D.M. Campbell, A. Jonas, J. Jonas, High-resolution NMR study of the pressure-induced unfolding of lysozyme, Biochemistry 31 (1992) 7773-7778.
[72] D.P. Nash, J. Jonas, Structure of pressure-assisted cold denatured lysozyme and comparison with lysozyme folding intermediates, Biochemistry 36 (1997) 14375-14383.
[73] J. Zhang, X.D. Peng, A. Jonas, J. Jonas, NMR study of the cold, heat, and pressure unfolding of Ribonuclease A, Biochemistry 34 (1995) 8631-8641.
[74] R. Kitahara, C. Roumestand, H. Yamada, J.L. Saldana, K. Akasaka, C.A. Royer, Pressure-jump fluorescence and $^{15}N/^{1}H$ 2D NMR studies of the unfolding of the beta-barrel protein, $P13^{MTCP1}$, Biophys J 82 (2002) 296A-296A.
[75] K. Akasaka, Highly fluctuating protein structures revealed by variable-pressure nuclear magnetic resonance, Biochemistry 42 (2003) 10875-10885.
[76] H.R. Kalbitzer, M. Spoerner, P. Ganser, C. Hozsa, W. Kremer, Fundamental link between folding states and functional states of proteins, J Am Chem Soc 131 (2009) 16714-16719.
[77] A. Gershenson, L.M. Gierasch, A. Pastore, S.E. Radford, Energy landscapes of functional proteins are inherently risky, Nature Chem Biol 10 (2014) 884-891.
[78] K. Akasaka, H. Li, Low-lying excited states of proteins revealed from nonlinear pressure shifts in ^{1}H and ^{15}N NMR, Biochemistry 40 (2001) 8665-8671.
[79] M.P. Williamson, Many residues in cytochrome *c* populate alternative states under equilibrium conditions, Proteins 53 (2003) 731-739.
[80] K. Gekko, Y. Hasegawa, Compressibility-structure relationship of globular proteins, Biochemistry 25 (1986) 6563-6571.
[81] N.J. Baxter, L.L.P. Hosszu, J.P. Waltho, M.P. Williamson, Characterisation of low free-energy excited states of folded proteins, J. Mol. Biol. 284 (1998) 1625-1639.
[82] K. Kuwata, H. Li, H. Yamada, C.A. Batt, Y. Goto, K. Akasaka, High pressure NMR reveals a variety of fluctuating conformers in β-lactoglobulin, J Mol Biol 305 (2001) 1073-1083.
[83] R. Kitahara, H. Yamada, K. Akasaka, Two folded conformers of ubiquitin revealed by high-pressure NMR, Biochemistry 40 (2001) 13556-13563.
[84] S. Kitazawa, T. Kameda, M. Yagi-Utsumi, K. Sugase, N.J. Baxter, K. Kato, M.P. Williamson, R. Kitahara, Solution structure of the Q41N variant of ubiquitin as a model for the alternatively folded N_2 state of ubiquitin, Biochemistry 52 (2013) 1874-1885.
[85] S. Kitazawa, M. Yagi-Utsumi, K. Kato, R. Kitahara, Interactions controlling the slow dynamic conformational motions of ubiquitin, Molecules 22 (2017) 1414.

[86] N.J. Baxter, T. Zacharchenko, I.L. Barsukov, M.P. Williamson, Pressure-dependent chemical shifts in the R3 domain of talin show that it is thermodynamically poised for binding to either vinculin or RIAM, Structure 25 (2017) 1856-1866.
[87] Y. Shen, A. Bax, Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks, J. Biomol. NMR 56 (2013) 227-241.
[88] M. Iwadate, T. Asakura, M.P. Williamson, Cα and Cβ carbon-13 chemical shifts in proteins from an empirical database, J. Biomol. NMR 13 (1999) 199-211.
[89] B.T. Goult, T. Zacharchenko, N. Bate, R. Tsang, F. Hey, A.R. Gingras, P.R. Elliott, G.C.K. Roberts, C. Ballestrem, D.R. Critchley, I.L. Barsukov, RIAM and vinculin binding to talin are mutually exclusive and regulate adhesion assembly and turnover, J. Biol. Chem. 288 (2013) 8238-8249.
[90] R. Kitahara, S. Sareth, H. Yamada, E. Ohmae, K. Gekko, K. Akasaka, High pressure NMR reveals active-site hinge motion of folate-bound *Escherichia coli* dihydrofolate reductase, Biochemistry 39 (2000) 12789-12795.
[91] W. Kremer, N. Kachel, K. Kuwata, K. Akasaka, H.R. Kalbitzer, Species-specific differences in the intermediate states of human and Syrian hamster prion protein detected by high pressure NMR spectroscopy, J Biol Chem 282 (2007) 22689-22698.
[92] A. Maeno, D. Sindhikara, F. Hirata, R. Otten, F.W. Dahlquist, S. Yokoyama, K. Akasaka, F.A.A. Mulder, R. Kitahara, Cavity as a source of conformational fluctuation and high-energy state: High-pressure NMR study of a cavity-enlarged mutant of T4 lysozyme, Biophys J 108 (2015) 133-145.
[93] D.J. Rosenman, N. Clemente, M. Ali, A.E. Garcia, C. Wang, High pressure NMR reveals conformational perturbations by disease-causing mutations in amyloid beta-peptide, Chem. Commun. 54 (2018) 4609-4612.
[94] M.B. Erlach, J. Koehler, E. Crusca, Jr., C.E. Munte, M. Kainosho, W. Kremer, H.R. Kalbitzer, Pressure dependence of side chain ^{13}C chemical shifts in model peptides Ac-Gly-Gly-Xxx-Ala-NH_2, J. Biomol. NMR 69 (2017) 53-67.
[95] J. Roche, J. Ying, A.S. Maltsev, A. Bax, Impact of hydrostatic pressure on an intrinsically disordered protein: A high-pressure NMR study of α-synuclein, Chembiochem 14 (2013) 1754-1761.
[96] V. Calandrini, V. Hamon, K. Hinsen, P. Calligari, M.C. Bellissent-Funel, G.R. Kneller, Relaxation dynamics of lysozyme in solution under pressure: Combining molecular dynamics simulations and quasielastic neutron scattering, Chem Phys 345 (2008) 289-297.
[97] L. Meinhold, J.C. Smith, A. Kitao, A.H. Zewail, Picosecond fluctuating protein energy landscape mapped by pressure-temperature molecular dynamics simulation, Proc Natl Acad Sci USA 104 (2007) 17261-17265.
[98] F. Persson, B. Halle, How amide hydrogens exchange in native proteins, Proc Natl Acad Sci USA 112 (2015) 10383-10388.
[99] D.J. Wilton, R. Kitahara, K. Akasaka, M.J. Pandya, M.P. Williamson, Pressure-dependent structure changes in barnase reveal changes in dynamics on ligand binding, Biophys. J. 97 (2009) 1482-1490.
[100] T. Kamiyama, K. Gekko, Effect of ligand binding on the flexibility of dihydrofolate reductase as revealed by compressibility, Biochim. Biophys. Acta 1478 (2000) 257-266.
[101] K. Gekko, K. Yamagami, Compressibility and volume changes of lysozyme due to inhibitor binding, Chem. Lett. (1998) 839-840.
[102] Y. Fu, V. Kasinath, V.R. Moorman, N.V. Nucci, V.J. Hilser, A.J. Wand, Coupled motion in proteins revealed by pressure perturbation, J Am Chem Soc 134 (2012) 8543-8550.
[103] A.J. Wand, The dark energy of proteins comes to light: conformational entropy and its role in protein function revealed by NMR relaxation, Curr. Opin. Struct. Biol. 23 (2013) 75-81.
[104] J.A. Caro, K.W. Harpole, V. Kasinath, J. Lim, J. Granja, K.G. Valentine, K.A. Sharp, A.J. Wand, Entropy in molecular recognition by proteins, Proc Natl Acad Sci USA 114 (2017) 6563-6568.
[105] C. Diehl, O. Engstrom, T. Delaine, M. Hakansson, S. Genheden, K. Modig, H. Leffler, U. Ryde, U.J. Nilsson, M. Akke, Protein flexibility and conformational entropy in ligand design targeting the carbohydrate recognition domain of Galectin-3, J Am Chem Soc 132 (2010) 14577-14589.

[106] A. Cooper, D.T.F. Dryden, Allostery without conformational change: A plausible model, Eur Biophys J Biophys Letts 11 (1984) 103-109.
[107] V.J. Hilser, E.B. Thompson, Intrinsic disorder as a mechanism to optimize allosteric coupling in proteins, Proc Natl Acad Sci USA 104 (2007) 8311-8315.
[108] A.B. Law, P.J. Sapienza, J. Zhang, X. Zuo, C.M. Petit, Native state volume fluctuations in proteins as a mechanism for dynamic allostery, J Am Chem Soc 139 (2017) 3599-3602.
[109] G. Nielsen, H.R.A. Jonker, N. Vajpai, S. Grzesiek, H. Schwalbe, Kinase in motion: Insights into the dynamic nature of p38α by high-pressure NMR spectroscopic studies, Chembiochem 14 (2013) 1799-1806.
[110] K. Inoue, T. Maurer, H. Yamada, C. Herrmann, G. Horn, H.R. Kalbitzer, K. Akasaka, High-pressure NMR study of the complex of a GTPase Rap1A with its effector RalGDS: A conformational switch in RalGDS revealed from non-linear pressure shifts, FEBS Lett. 506 (2001) 180-184.
[111] H.R. Kalbitzer, I.C. Rosnizeck, C.E. Munte, S.P. Narayanan, V. Kropf, M. Spoerner, Intrinsic allosteric inhibition of signaling proteins by targeting rare interaction states detected by high-pressure NMR spectroscopy, Angew Chem Int Ed 52 (2013) 14242-14246.
[112] C.E. Munte, M.B. Erlach, W. Kremer, J. Koehler, H.R. Kalbitzer, Distinct conformational states of the Alzheimer β-amyloid peptide can be detected by high-pressure NMR spectroscopy, Angew Chem Int Ed 52 (2013) 8943-8947.
[113] M.B. Erlach, H.R. Kalbitzer, R. Winter, W. Kremer, Conformational substates of amyloidogenic hIAPP revealed by high pressure NMR spectroscopy, Chemistryselect 1 (2016) 3239-3243.
[114] D.D. Boehr, D. McElheny, H.J. Dyson, P.E. Wright, The dynamic energy landscape of dihydrofolate reductase catalysis, Science 313 (2006) 1638-1642.



Graphic abstract