Use of High Pressure NMR Spectroscopy to Rapidly Identify Proteins with Internal Ligand-Binding Voids

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Classification: Biological Sciences / Biophysics and Computational Biology

Keywords: high pressure NMR, ligand binding, protein cavities

Abstract

Small molecule binding within internal cavities provides a way to control protein function and structure, as exhibited in numerous natural and artificial settings. Unfortunately, most ways to identify suitable cavities require high-resolution structures *a priori* and may miss potential cryptic sites. Here we address this limitation via high-pressure solution NMR spectroscopy, taking advantage of the distinctive nonlinear pressure-induced chemical shift changes observed in proteins containing internal cavities and voids. We developed a method to rapidly characterize such nonlinearity among backbone ^1H and ^{15}N amide signals without needing to have sequence-specific chemical shift assignments, taking advantage of routinely available ^{15}N -labeled samples, instrumentation, and 2D $^1\text{H}/^{15}\text{N}$ HSQC experiments. From such data, we find a strong correlation in the site-to-site variability in such nonlinearity with the total void volume within proteins, providing insights useful for prioritizing domains for ligand binding and indicating mode-of-action among such protein/ligand systems. We suggest that this approach provides a rapid and useful way to rapidly assess otherwise hidden dynamic architectures of protein that reflect fundamental properties associated with ligand binding and control.

19 **Significance Statement**

20 Many proteins can be regulated by internally binding small molecule ligands, but
 21 it is often not clear *a priori* which proteins are controllable in such a way. Here we
 22 describe a rapid method to address this challenge, using solution NMR spectroscopy to
 23 monitor the response of proteins to the application of high pressure. While the locations
 24 of NMR signals from most proteins respond to high pressure with linear chemical shift
 25 changes, proteins containing internal cavities that can bind small molecule ligands
 26 respond with easily identified non-linear changes. We demonstrate this approach on
 27 several proteins and protein/ligand complexes, suggesting that it has general utility.

Introduction

Small molecule cofactors and ligands play critical roles in controlling protein structure and function, the understanding of which often gives insight both into natural and artificial modes of regulation. Of particular interest are identifying sites where the irregular structures of proteins give rise to cavities, voids and other features which can serve as internal sites for such compounds to bind (**Fig. 1**) and allosterically control protein structure and function (1, 6-9). The traditional method to identify such locations – high resolution X-ray crystallography, ideally with better than 2 Å resolution to aid the identification of internally bound waters – can be powerful, particularly when combined with computational analyses for cavity identification (5, 10-16) or experimentally solving multiple structures of proteins soaked with different organic solvents or small molecule fragments (17, 18). Accordingly, this approach relies on having well-diffracting crystals, which are not always available for all systems and can be time-consuming to produce even when successful.

As an alternative method to experimentally determine which proteins might contain internal cavities suitable for ligand binding, we explored the potential for using high pressure solution NMR to do so. We thought this approach might be useful given the need for proteins to undergo dynamic changes to allow for ligand binding within pre-existing internal cavities and voids (**Fig. 1**). While such changes may occur rarely at ambient pressure, elevated pressure will easily increase the equilibrium populations of the less populated, low-volume conformers associated with hydration of cavities and voids (20). Such low-lying excited state conformers (N'), if present, will usually equilibrate with the ground state folded conformer (N) rapidly on the NMR time scale

($\tau \ll \text{ms}$), giving rise to averaged single peaks in multidimensional spectra which exhibit non-linear chemical shift changes as pressure affects the $N \rightleftharpoons N'$ equilibrium (21). Based on prior work by several groups (21-24), we anticipated that this effect might be easily detected by solution NMR at pressures in the 1000-2000 bar range, below the pressures which typically partially or completely unfold proteins (21, 25, 26).

To evaluate this approach, we examined the effects of high hydrostatic pressure on the NMR chemical shifts of a collection of protein domains and protein/ligand

complexes. Many of these proteins are members of the Per-ARNT-Sim (PAS) family of ligand-controlled protein/protein interaction domains, which often internally bind different small molecule cofactors to sensitize them to environmental factors like O₂, light, and xenobiotics (27, 28). Changes to the occupancies or configurations of these cofactors trigger conformational changes in the surrounding protein, regulating the activity of various effector domains in natural and engineered proteins (28, 29). While a number of high-resolution structures of apo- and ligand-bound PAS domains have been solved, these models and predictions which can be made from them can provide insights into only a small fraction of the many thousands of “orphan” PAS domains without known ligands. Complementing these proteins, we added additional proteins and protein/ligand complexes from a wide range of domain types – including some which are known to bind ligands internally, some not – to establish the generality of this approach for quickly probing protein structure and function.

Here we test the ability of high-pressure NMR to rapidly identify void-containing proteins, with three key advances. First, by analyzing pressure titration data from over 40 proteins and protein/ligand complexes, we show that an easily accessible metric – how differently sites within a protein respond to increasing pressure, as assessed by the diversity of non-linear chemical shift perturbations observed in a simple titration without requiring site-specific assignments – correlates well with the void volume within a protein. We find that this metric is robust enough to predict total void volume on its own, allowing the prioritization of potential ligand-binding capability amongst several targets. Second, we demonstrate that internal ligand binding within a protein can reduce this heterogeneity, quickly providing information on ligand binding and, in certain cases,

mode of action. Finally, we illustrate how this method can also be used to rapidly assess the impact of point mutations/repacking, such as those used to fill or generate cavities to facilitate artificial control, on the prevention or enabling of water entry. Taken together, our data show the general utility of these rapidly acquired, easily analyzed data to provide this important biophysical characterization of new proteins.

Results

Evaluation of non-linear NMR chemical shift responses to high pressure: We began by examining the pressure dependence of NMR signals from a set of well-characterized proteins using the workflow in **Fig. 2**. For each U-¹⁵N labeled protein sample, we acquired ¹H/¹⁵N HSQC spectra at increasingly higher pressures from 20-2500 bar. After each individual dataset was acquired at high pressure, we lowered the pressure to 20 bar and acquired a spectrum to confirm the reversibility of conformational changes; after any sign of irreversible changes in peak intensity or location, the pressure series was stopped. These series were typically composed of 21 spectra each taking 60 min apiece, for a total of approximately 21 hr. Post-processing, peaks were picked and the pressure dependence of changes of their ¹H and ¹⁵N chemical shifts were independently fit to a second-order polynomial equation:

$$\delta_i = a_i + b_i p + c_i p^2 \text{ (Eq. 1)}$$

As established by Akasaka and co-workers (21, 25), the linear (b_i) and nonlinear (c_i) coefficients of these pressure responses reflect different properties of each protein. To evaluate various analyses of these data, we examined an initial group of nine proteins (“test set”) with high resolution structures with total void volumes ranging from approximately 1500-8500 Å³ as assessed by ProteinVolume (5). This approach provides a sum of the volumes of a wide array of packing defects, cavities, etc. regardless of their size or distribution within a protein structure by simply calculating the difference between the solvent-accessible volume of a protein and the volume occupied by protein atoms (**Fig. 1B**). Pressure titration data were additionally recorded from more than 30 additional proteins and protein ligand complexes, with varying degrees of structural information to build the “complete set” of data for subsequent analyses.

As an initial analysis, we examined the absolute values of the two chemical shift coefficients ($|b_i|$, $|c_i|$), separately averaged over all backbone amide protons and nitrogens (typically 25-125) within each protein. From these analyses, we confirmed that the averaged ¹H and ¹⁵N $|b_i|$ values were fairly uniform across proteins with less than two-fold variation, while the corresponding $|c_i|$ values varied over 4-10 fold ranges (**Fig. S1**). These data expand the original data sets of Akasaka and Li (25) from seven proteins and complexes to over 40 total, add an independent measurement and analysis of the GB1 protein to assess the impact of different equipment, labs, and software for data acquisition and analyses (e.g. 750 MHz in Kobe University by K.A.; 700-800 MHz in New York by K.H.G.), and support the original interpretations that the linear $|b_i|$ component is relatively fixed among systems while $|c_i|$ depends on a protein-specific feature (25).

Further investigating these pressure-dependent chemical shift changes, we noted a trend towards proteins with larger total void volumes having both higher c_i values (**Fig. S1**) and a greater range of individual residue-specific c_i parameters than proteins with smaller voids, both of which we thought might reflect a more heterogeneous structural response. While the larger c_i values are captured by the previously-described average $|c_i|$ parameter (25), we examined several ways to quantitate the heterogeneity in c_i values, settling on a combination of histograms and the standard deviation of the c_i ($\text{stdev}(c_i)$). While the correlation between average $|c_i|$ and $\text{stdev}(c_i)$ values for amide ^1H and ^{15}N shifts (**Fig. S2**) suggest similarities between the two metrics, we opted to proceed using $\text{stdev}(c_i)$ to take advantage of the larger range of values provided by dropping the absolute value operation. We also observed a high correlation between the $\text{stdev}(c_i)$ values of ^1H and ^{15}N (**Fig. S3**), suggesting that non-linear shift changes of either nucleus report on pressure-induced changes despite differences in the structural factors which influence them (30). Our subsequent analyses utilized $\text{stdev}(c_i)$ of ^{15}N chemical shift changes ($= \text{stdev}(c_i[^{15}\text{N}])$), which are thought to be most strongly influenced by changes in backbone torsion angles (25, 30).

To examine the linkage of $\text{stdev}(c_i)$ to total void volume, we measured the nonlinear components ($c_i[^{15}\text{N}]$) of a test set of nine proteins (with one, GB1, repeated in duplicate) with known structures and a range of total void volumes (**Fig. 3**). Histograms of $\text{stdev}(c_i)$ parameters exhibited the previously-mentioned variability, with proteins having larger total void volumes typically having broader distributions than those with smaller total void volumes (**Fig 3 inset**). We interpreted this trend as stemming from voids enabling proteins to increasingly shift from the native N conformation to a second

149 folded N' conformation under pressure, reflected in the non-linear chemical shift
 150 responses as the N' is progressively populated. Quantitating the breadth of these
 151 distributions by $\text{stdev}(c_i [^{15}\text{N}])$ and plotting these versus total void volume showed a
 152 linear correlation coefficient (r^2) of 0.929, supporting the linkage between pressure-
 153 induced chemical shift nonlinearities to identify cavities. Of note, the proteins with a
 154 $\text{stdev}(c_i [^{15}\text{N}])$ above 50 (2×10^{-10} ppm/bar²) are all known to bind other proteins or

small molecules, suggesting an arbitrary value which could be useful to prioritize for ligand-screening.

We note that the primary outlier of the observed linear correlation, hen egg white lysozyme, is quite thermostable with a T_m of almost 75°C (31), likely hampering its transition into an excited state under pressure unless its intrinsic stability of its basic folded state is substantially lowered (e.g. by cooling close to the cold denaturation temperature (32)). In addition, we cannot rule out potential contributions from lysozyme being an enzyme instead of a signal transduction component, which may contribute a different ability to adopt alternative conformations reflected in pressure-induced non-linear chemical shift changes. This remains to be seen in future studies.

Use of pressure NMR to reveal ligand binding mode of action – Well-defined cases:

Our correlation between increased heterogeneity of pressure-induced chemical shift changes and void size makes a strong prediction that this route should provide a rapid way to assess ligand binding: ligands which bind within the protein and reduce total void volume should decrease the stdev(c) value of spectra recorded on the receptor. To test this prediction, we evaluated how ligand binding affects the PAS-B domains of the human HIF-2 α and ARNT proteins, both of which are involved in the human hypoxia response (33) and contain internal cavities known to bind artificial small molecule ligands (1, 3, 6).

Prior studies of the HIF-2 α PAS-B domain by our group and others (1, 6, 34, 35) have shown that it contains a single internal water-filled 290 Å³ cavity with no obvious access to external solvent. However, a variety of screening efforts – from NMR-based

178 fragment binding screens to high throughput screens of HIF-2 α /ARNT disruption (1, 2,
179 6, 35) or protein stability (35) – have identified a wide range of small molecules which
180 bind into this cavity with nano- to micromolar affinities. High-resolution X-ray structures
181 of these complexes show that the ligands displace water and reduce HIF-2 activity by
182 impacting HIF-2 α /ARNT interactions (36-38). To accommodate these binding events
183 within a solvent-inaccessible cavity, the HIF-2 α PAS-B protein must dynamically
184 fluctuate to allow for a conformation which allows the entry of small molecules into the
185 interior (39). Our pressure NMR analysis confirmed this hypothesis, showing a very
186 broad distribution of $c_i(^{15}\text{N})$ responses with a correspondingly large $\text{stdev}(c_i[^{15}\text{N}])$ of 85
187 $\times (2 \times 10^{-10} \text{ ppm/bar}^2)$ (**Figs. 4A and S4**). We anticipated that repeating these
188 measurements in the presence of two nanomolar-affinity compounds (**2** (1) and **37** (2))
189 would show smaller non-linear chemical shift changes than the apo protein given the
190 smaller total void volume and expected reduced flexibility of the protein/ligand
191 complexes. Our data supported this, as we observed decreases in $\text{stdev}(c_i[^{15}\text{N}])$ from
192 $85 \times (2 \times 10^{-10} \text{ ppm/bar}^2)$ for the apo protein to 64 (**2**-bound) and 53 (**37**-bound) $\times (2 \times 10^{-10}$
193 $\text{ppm/bar}^2)$, respectively. Notably, these decreases do not solely correlate with loss of
194 void volume in some cases: measurements conducted on HIF-2 α D1, a
195 computationally-repacked variant with five point mutations which reduce the cavity
196 volume to 77 $\text{\AA}^3$ (40) while retaining function, shows an *increase* in $\text{stdev}(c_i[^{15}\text{N}])$ up to

197 131 x (2x10⁻¹⁰ ppm/bar²) (**Fig. 4A**). We speculate that differences between some
198 aspect of this engineered protein, perhaps in the dynamics or thermodynamic stability,
199 and natural proteins contribute to non-linear responses to pressure.

We used the same approach with the ARNT PAS-B domain, which has the same tertiary structure as HIF-2 α PAS-B but contains smaller internal cavities which total 150 Å³ in volume. We have previously used NMR-based fragment screening, isothermal titration calorimetry (ITC) and microscale thermophoresis (MST) to identify several compounds which bind ARNT PAS-B with micromolar affinities; two of these, **KG-548** and **KG-655**, are further known to disrupt ARNT PAS-B interactions with coactivator proteins (3). However, the binding modes of these compounds remain unclear without co-complex structures solved by NMR or X-ray crystallography. By acquiring pressure NMR data, we confirmed that ARNT PAS-B retains a flexibly-accessible interior cavity with a $\text{stdev}(c_i[^{15}\text{N}])$ of $84 \times (2 \times 10^{-10} \text{ ppm/bar}^2)$ (**Figs. 4B and S5**). Repeating these in the presence of the **KG-655** ligand, we observed a drop of $\text{stdev}(c_i[^{15}\text{N}])$ to $51 \times (2 \times 10^{-10} \text{ ppm/bar}^2)$, consistent with an interior binding location for this small ligand. However, similar studies with the larger **KG-548** ligand show a $\text{stdev}(c_i[^{15}\text{N}])$ comparable to the apo protein, with an observed value of $96 \times (2 \times 10^{-10} \text{ ppm/bar}^2)$ that suggest that this compound binds in a different mode than **KG-655**, potentially outside the cavity.

Use of pressure NMR to reveal ligand binding – Poorly-defined cases: As a next demonstration of this approach, we examined its utility for PAS domains outside the hypoxia response and with less well understood regulation. We considered two such targets, the first of which is the N-terminal PAS domain of human PAS kinase (PASK PAS-A), a PAS domain-regulated serine/threonine kinase conserved among eukaryotes (4, 41). This domain has a canonical PAS structure, with a five-stranded β -sheet flanked to one side by several α helices (27, 42). While only a small surface groove of

73 Å³ could be identified near the F/G loop in the representative member of the solution structure ensemble, an NMR-based fragment screen of 750 compounds identified several small molecules that bound with micromolar affinity to PASK PAS-A (4). Chemical shift perturbations suggest that these compounds bind within the domain interior, analogous to the subsequently-discovered HIF-2α and ARNT PAS-B binding compounds, but the lack of a PASK PAS-A/ligand complex structure leaves this as an open issue. To address this, we used high-pressure NMR to examine the response of PASK PAS-A in its apo form and when saturated with two compounds with K_D =13-24 μM affinities (**KG-535**, **KG-571** = compounds **1** and **2** in (4)). For the apo- protein, we observed a stdev(c_i [¹⁵N]) of 45 x (2x10⁻¹⁰ ppm/bar²); from the correlation identified in our test set (**Fig. 3**), this value predicts that PASK PAS-A contains a total void volume slightly smaller than the 3345 Å³ calculated from the representative member of the solution structure ensemble (**Figs. 5A and S6**). Upon addition of the **KG-535** and **KG-571** ligands, we observed smaller stdev(c_i [¹⁵N]) values for both ligand-bound forms (35 and 28 x (2x10⁻¹⁰ ppm/bar²) for the **KG-535** and **KG-571**-bound forms, respectively; **Fig. 5A**). These data suggest that both compounds bind within the void volume of PASK PAS-A, with **KG-571** having a greater effect on the domain adopting alternative folded conformations.

We completed our analyses of natural proteins by examining a prokaryotic PAS domain from a novel histidine kinase from *Rhizobium etli* (43), chosen because of a). homology with the light-regulated PAS-histidine kinase EL346 (44, 45) and b). the widespread usage of small molecule ligands to control the structure and function of PAS-containing proteins (including many histidine kinases (28)). While no experimental

structures are available of this domain, which we designate RE137, we hypothesized that the structure may contain a cavity that can accommodate a small molecule given precedence from other PAS domains (28). Pressure NMR analyses of the apo protein support this possibility, returning a stdev(c_i[¹⁵N]) value of 53 x (2x10⁻¹⁰ ppm/bar²) (**Figs. 5B and S7**), suggesting the presence of a 2800 Å³ total void volume from the correlation seen in **Fig. 3**. To provide an initial assessment of small molecule binding,

we used an R_2 -filtered ^{19}F NMR assay to search for binders of RE137 within a 100-member library of fluorinated compounds (46). For this study, we used two of those compounds **15** and **47**; pressure analyses of RE137 saturated with each compound exhibited decreases of 43 and 39% with $\text{stdev}(c_i[^{15}\text{N}])$ values of 30 and 32 x (2×10^{-10} ppm/bar²) for the **15**- and **47**-bound forms, respectively. We interpret these data to indicate that RE137 can bind both compounds in such a way as to reduce the total void volume of the protein.

Use of pressure NMR to probe artificially-designed proteins: As a final demonstration of the utility of this method, we examined its utility in investigating the flexibility of an artificially-designed protein, CA01 (47). With advances in protein engineering enabling the development of artificial ligand-binding biosensor proteins (48), we sought to probe whether a protein such as CA01 – which contains a completely-enclosed 75 Å³ cavity within approximately 3500 Å³ of total void volume – might show similar pressure-dependent non-linear chemical shift changes as natively-evolved counterparts. From our correlation in **Fig. 3** we expected to see a $\text{stdev}(c_i[^{15}\text{N}])$ value of approximately 75 (2×10^{-10} ppm/bar²) from a natural protein with void volume of this size, but we instead observed a substantially smaller value of 38 x (2×10^{-10} ppm/bar²) (**Fig. 6**). As CA01 is extremely stable to thermal and chemical denaturation, requiring 5 M guanidinium hydrochloride to exhibit a complete thermal melt with a 75°C T_m (47), we view this observation supports our prior findings with lysozyme suggesting thermostable proteins being less able to adopt alternative conformations (**Fig. 3**).

Discussion

In the present work, we successfully used non-linear pressure-induced chemical shift effects at backbone amide ¹H and ¹⁵N nuclei to rapidly assess whether proteins contain substantial total void volumes within them and if these can bind small molecule ligands. A particular strength of our implementation is its simplicity, as 1). the necessary U-¹⁵N labeled samples are routinely available from *E. coli* or *in vitro* expression systems, 2). the NMR data needed are easily obtained from conventional ¹H/¹⁵N HSQC experiments recorded at variable pressures using commercially-available pressure sources, and 3). straightforward data analyses are done by simply tracking peak locations without requiring chemical shift assignments, eliminating one of the more time-consuming steps of NMR analyses.

Mechanistically, we attribute the non-linear chemical shift changes with increasing pressure to site-specific conformational changes of the protein from the native folded structural ensemble (N) to the low-lying excited state ensemble (N') with smaller volume (17). Our specific choice of backbone amide ^1H and ^{15}N chemical shifts give us complementary views of these transitions (**Fig. S3**), as ^1H chemical shifts are dominated by hydrogen bonding to these atoms, while ^{15}N chemical shifts are influenced by a broader set of factors, including changes in hydrogen bonding (to the both the adjacent ^1H and carbonyl CO groups), local backbone dihedrals and sidechain conformations (49). Of note, non-linear pressure-dependent chemical shift changes at these sites are much smaller in peptides or intrinsically disordered proteins (50). Indeed, non-linear pressure-dependent amide ^1H and ^{15}N chemical shift changes have long been recognized as reflecting idiosyncratic differences among proteins (51). Prior work by one of our groups (K.A.) suggested that this property depends on the density of cavities within the protein (25); here we both expand the number of proteins examined from that work and suggest that contributions likely arise from small and large voids distributed throughout the protein which collectively give rise to the total void volumes calculated by the program ProteinVolume (5) given the excellent correlation we present in **Fig. 3**. We posit that such an approach reveals aspects that will be correlated to the ability of proteins to be similarly affected by allosteric regulators and other ligands, linking these non-linear chemical shift effects to the potential for small molecule binding and regulation.

In addition to getting information on the total void volume within a given protein, the strengths of this analysis include the ability to identify ligands which bind to a given

target and (de)stabilize one conformation in a way that may provide allosteric switching. As such, this provides trivially-accessible mode-of-action information that is often challenging to get otherwise without resources such as compounds of known binding location for competition studies or cavity-filling point mutants. Our comparison of two cavity-perturbing effects for HIF-2 α PAS-B – small molecule binding and cavity-filling redesign – produced opposite effects, with the small molecules stabilizing the native fold and mutations destabilizing in our pressure NMR analyses. We underscore that these effects occur despite minor changes in ground state structure among structures of the apo, ligand-bound and redesigned proteins (1, 6, 40). As such, pressure-dependent chemical shift effects give us insight into details of the packing and dynamics of proteins more simply than most other methods can provide. Finally, our comparison of the ARNT PAS-B binding compounds **KG-548** and **KG-655** provide an outstanding example of two molecules known to bind by NMR chemical shift perturbations, but with limited binding site information previously available. With high pressure NMR, we predict different binding modes between the two ligands, with **KG-655** binding primarily to an internal site while **KG-548** binds externally.

We emphasize that our approach requires very little preliminary information on the target, needing neither structures nor site-specific chemical shift assignments, as demonstrated by the hPASK PAS-A and RE137 cases. In both cases, the $\text{stdev}(c_i[^{15}\text{N}])$ values of the apo proteins exceed the value of approximately 50 (2×10^{-10} ppm/bar²) value we suggest as indicating potential ligand binding, and in both cases, we were able to quickly identify compounds which bound to these proteins with micromolar affinities from small libraries using either protein- or ligand-detected NMR methods. Our high

pressure NMR results quickly add important information, showing that these compounds bind in such a way as to reduce the flexibility of the receptors, probably through binding internal cavities.

Finally, while our demonstrations have focused on relatively small ~15 kDa ligand binding domains, we note that several routes can expand the reach of this approach to larger and more complex systems by reducing spectral complexity. Options include biochemical approaches to generate proteins with simplified ^{15}N -labeling patterns, as provided by amino-acid type selective or sortase/intein-based domain-specific techniques, to $^1\text{H}/^{15}\text{N}$ TROSY or HNCQ-type pulse sequences which can provide improved resolution. Additional options may be provided by the improved NMR signal properties of $^1\text{H}/^{13}\text{C}$ methyl signals for similar pressure-sensitive studies, particularly given the placement of these residues near internal cavities.

Materials and Methods

Proteins were expressed in *E. coli* with uniform ^{15}N labeling, purified using combination of Ni(II) affinity and gel filtration chromatography before being exchanged into a barostatic Tris:phosphate buffer mix which limits pH changes during pressurization (52) and concentrated to 100-700 μM for NMR spectroscopy. $^1\text{H}/^{15}\text{N}$ HSQC spectra were acquired at increasing pressures from 20-2500 bar, interleaving each high pressure spectrum with a low pressure (20 bar) spectrum to confirm that changes in peak locations and intensities were reversible. All NMR data were processed with NMRFX (One Moon Scientific) (53, 54) and analyzed with NMRViewJ (One Moon Scientific) (53, 55). After individually processing each spectrum, we picked

peaks and tracked their changes in chemical shifts as a function of pressure. Separately handling movement in the ^1H and ^{15}N dimensions, we fit these trends to a second-order polynomial equation (**Eq. 1, Fig. 2**). Protein volume analyses of single isolated cavities utilized *cavfinder* (1), while measurements of total void volumes utilized *ProteinVolume* (5). Additional detailed procedures are found in **SI Materials and Methods**.

Acknowledgements

The authors gratefully acknowledge useful discussions and software contributions from Bruce Johnson (ASRC), assistance in sample preparation from Dong Lee and Andrew Palacios (ASRC), and comments on manuscript by Xingjian Xu (ASRC) and Hua Li (Chinese Academy of Sciences, Shanghai). $\text{U-}^{15}\text{N}$ samples of FABP proteins were generously provided by May Poh Lai and Ruth Stark (CCNY); plasmids of Brd proteins were generously provided by Elena Rusinova and Ming-Ming Zhou (Mt. Sinai Medical Center) while plasmids of CA01 were generously shared by T.M. Jacobs and Brian Kuhlman (UNC Chapel Hill). This work was supported by NSF grant MCB-1818148 (K.H.G.) and NIH grant R01 GM106239 (K.H.G.), as well as a postdoctoral fellowship to D.G. (number B3X from the Fonds de Recherche Québec – Nature et Technologie (FRQNT)).

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