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

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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 ^{1}H 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}H/^{15}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.

Significance Statement

Many proteins can be regulated by internally binding small molecule ligands, but it is often not clear *a priori* which proteins are controllable in such a way. Here we describe a rapid method to address this challenge, using solution NMR spectroscopy to monitor the response of proteins to the application of high pressure. While the locations of NMR signals from most proteins respond to high pressure with linear chemical shift changes, proteins containing internal cavities that can bind small molecule ligands respond with easily identified non-linear changes. We demonstrate this approach on 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, 10-12). 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, 13-20) or experimentally solving multiple structures of proteins soaked with different organic solvents or small molecule fragments (21, 22). 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 (24). 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

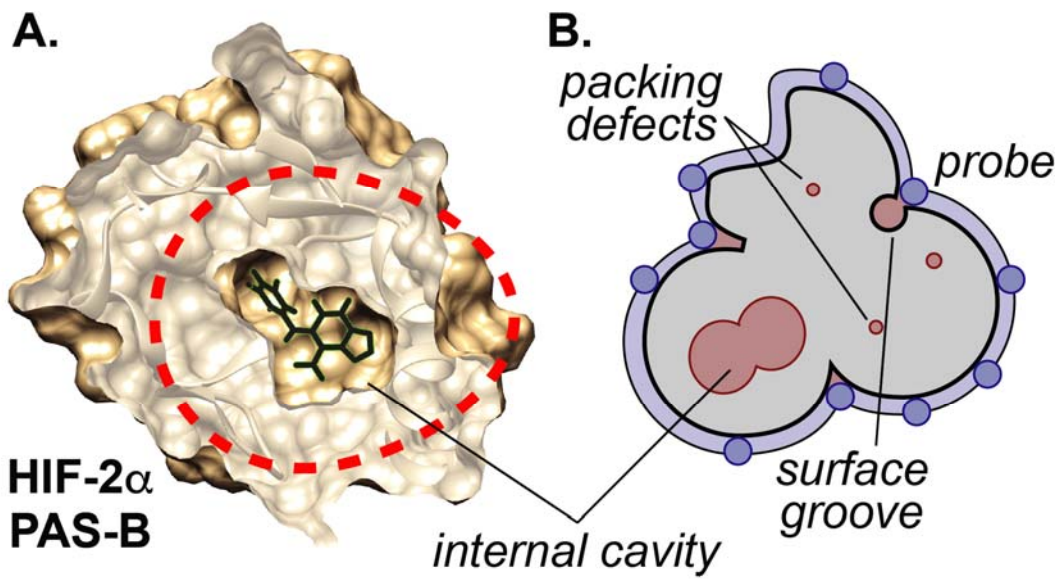

Fig. 1: Example of internal ligand binding cavity and computational analysis. A. Example of protein/ligand complex utilizing an internal cavity (HIF-2α PAS-B, PDB: 3f1o (6)) that is sequestered over 6 Å from solvent. In the apo- form of this protein (not shown), a pre-formed 290 $Å^3$ cavity with 8 crystallographically-ordered water molecules is present at this site. **B.** Schematic definitions of cavities, voids, and algorithm used by *ProteinVolume* (5) as used for Fig. 3 and onwards in the manuscript. We refer to *cavities* as internal openings larger than a single water molecule (V > 30 $Å^3$ (23)), while *voids* are more generally non-protein filled spaces that include cavities along with other types of packing defects distributed throughout and around a protein. With a protein structure available (gray), the *total void volume* can be straightforwardly calculated by the difference of the solvent-excluded volume of a protein (generated by rolling probes over the molecular surface, typically with water-sized radii) and the volume taken by protein atoms = volume under blue surface minus gray volume (5).

(τ<<ms), giving rise to averaged single peaks in multidimensional spectra which exhibit non-linear chemical shift changes as pressure affects the N ⇌ N' equilibrium (25). Based on prior work by several groups (25-28), 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 (25, 29, 30).

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 O_2, light, and xenobiotics (31, 32). 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 (32, 33). 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-^{15}N labeled protein sample, we acquired ^{1}H/^{15}N 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 ^{1}H and ^{15}N chemical shifts were independently fit to a second-order polynomial equation:

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

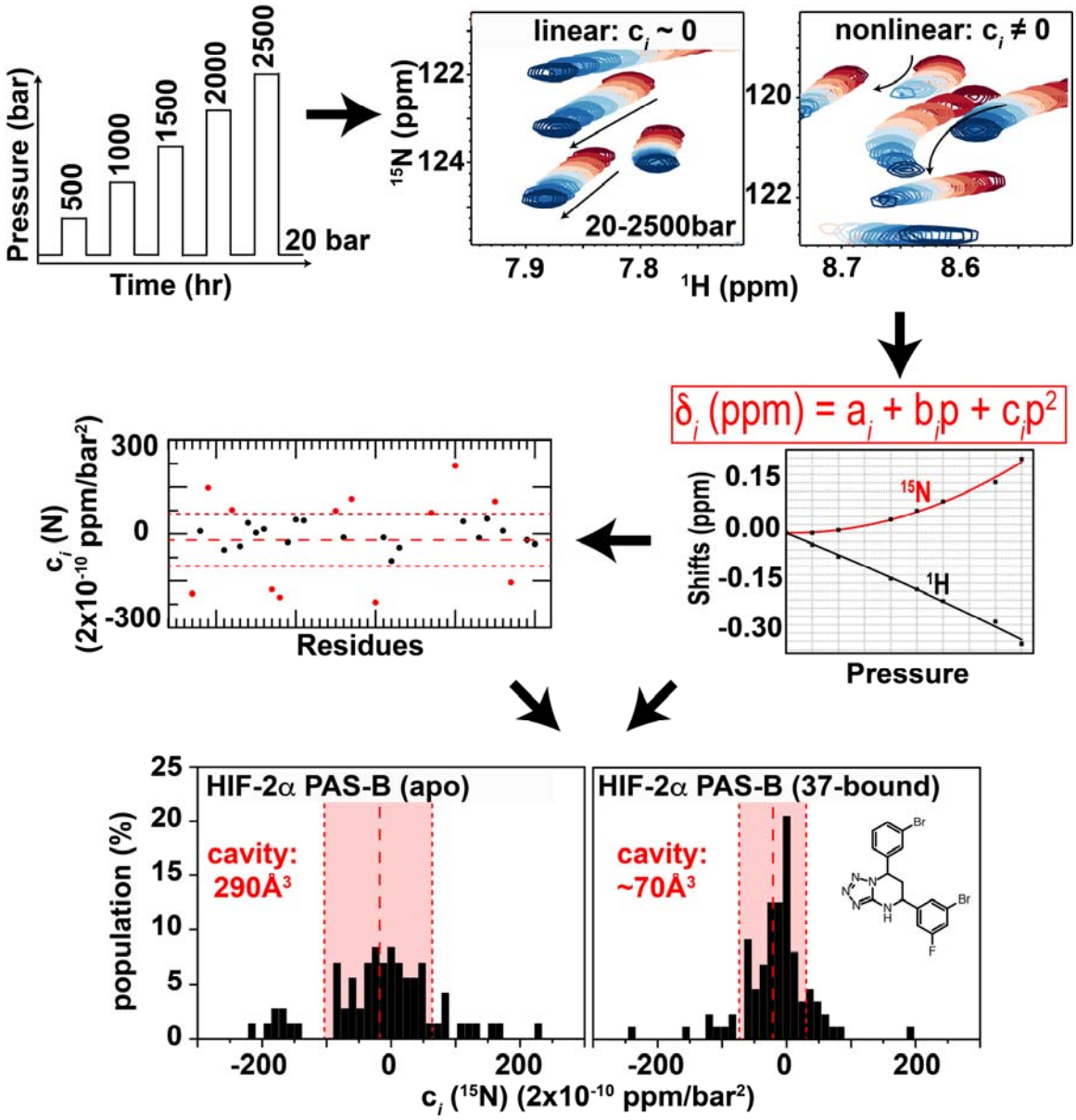

Fig. 2: Workflow of pressure NMR analyses. For each protein analyzed, ^{1}H/^{15}N HSQC spectra were acquired at increasing pressures from 20-2500 bar, interleaving additional spectra at 20 bar between those at higher pressure to assess protein reversibility. Following processing, peaks were picked and chemical shifts monitored as a function of increasing pressure. Independently treating ^{1}H and ^{15}N movements, the pressure-dependent chemical shift changes of each peak are fit to the second order polynomial indicated to obtain linear (b_i) and nonlinear (c_i) coefficients. Subsequent analyses of the nonlinear (c_i) coefficients include either plotting c_i values as a function of residue number (for proteins with backbone chemical shift assignments) to identify regions with likely pressure-dependent conformational changes or to simply generate histograms of c_i values to give a quick initial characterization of likely ability to adopt multiple folded conformations.

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As established by Akasaka and co-workers (25, 29), 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 $Å^3$ 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 (a complete list of all analyzed proteins and complexes is provided in **Table S1**).

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 ^{1}H and ^{15}N $|b_i|$ 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 (29) 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 (GB1: 750 MHz in Kobe University by K.A.; GB1' 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 (29).

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 heterogenous structural response. While the larger c_i values are captured by the previously-described average $|c_i|$ parameter (29), 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 (stdev(c_i)). While the correlation between average $|c_i|$ and stdev(c_i) values for amide ^{1}H and ^{15}N shifts (**Fig. S2**) suggest similarities between the two metrics, we opted to proceed using 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 stdev(c_i) values of ^{1}H 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 (34). Our subsequent analyses utilized stdev(c_i) of ^{15}N chemical shift changes (= stdev(c_i [^{15}N])), which are thought to be most strongly influenced by changes in backbone torsion angles (29, 34).

To examine the linkage of stdev(c_i) to total void volume, we measured the nonlinear components (c_i [^{15}N]) of a test set of nine proteins (with one, GB1, duplicated as noted above) with known structures and a range of total void volumes (**Fig. 3**). Histograms of stdev(c_i) parameters exhibited the previously mentioned variability, with proteins having larger total void volumes typically having broader distributions than

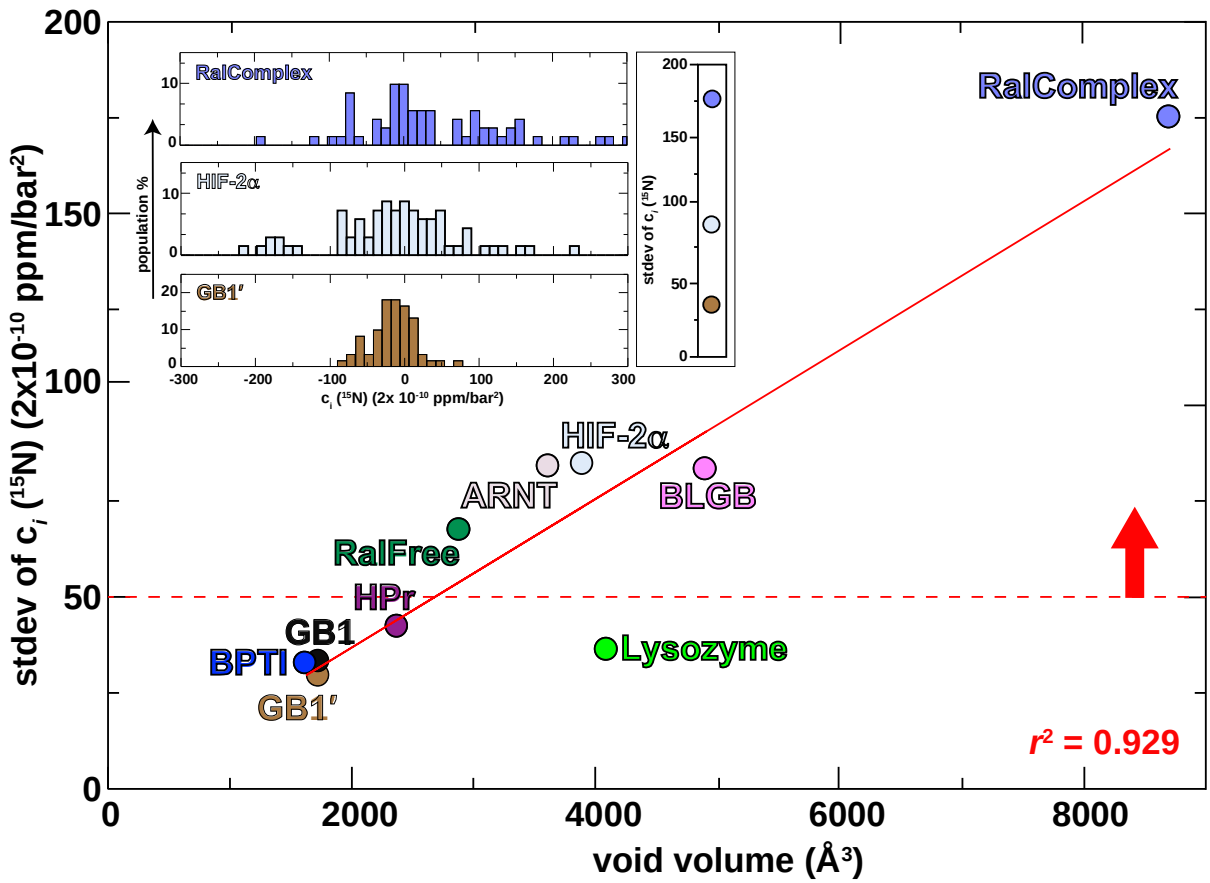

Fig. 3: **Increasing diversity of non-linear pressure dependent chemical shift changes correlates with increased void volume**. Total void volumes calculated by ProteinVolume (5) for each protein are presented on the x-axis; the y-axis plots the standard deviation of the non-linear c_i parameters for the amide ^{15}N chemical shifts, using data from 25-125 crosspeaks for each protein. Note that the GB1 protein was analyzed from independently-produced and acquired data from two labs, as noted in the text. The linear regression (red line, y= –1.564+0.0194x, omitting the lysozyme point) shows a correlation coefficient (r^2) of 0.929. The red dashed line indicates an arbitrary value of approximately 50 (x2x10^{-10}) ppm/bar^2 as a recommended minimum value for potential small molecule binding cavities, as noted in the text. Inset: histograms of measured ^{15}N c_i parameters for three selected proteins (using the same color scheme as main figure; histograms for all ten proteins provided in **Fig. S8**).

those with smaller total void volumes (**Figs. 3 inset** and **S8**). We interpreted this trend as stemming from voids enabling proteins to increasingly shift from the native N conformation to a second folded N' conformation under pressure, reflected in the non-linear chemical shift responses as the N' is progressively populated. Quantitating the

breadth of these distributions by stdev(c_i [^{15}N]) and plotting these versus total void volume showed a linear correlation coefficient (r^2) of 0.929, supporting the linkage between pressure-induced chemical shift nonlinearities to identify cavities. Of note, the proteins with a stdev(c_i [^{15}N]) above 50 (x $2x10^{-10}$ ppm/bar^2) 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 thermostable with a T_m of almost 75°C (35), 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 (36)). In addition, we cannot rule out potential contributions from lysozyme being an enzyme instead of a signal transduction component, which may contribute to a different ability to adopt alternative conformations reflected in pressure-induced non-linear chemical shift changes. This remains to be explored more broadly 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_i) 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-2α and ARNT proteins, both of which are involved in the human hypoxia

response (37) and contain internal cavities known to bind artificial small molecule ligands (1, 3, 6).

Prior studies of the HIF-2α PAS-B domain from us and others (1, 6, 38, 39) have shown that it contains an internal water-filled 290 $Å^3$ cavity with no obvious access to external solvent. However, a variety of screening efforts – from NMR-based fragment binding screens to high throughput screens of HIF-2α/ARNT disruption (1, 2, 6, 39) or protein stability (39) – have identified a wide range of small molecules which bind into this cavity with nano- to micromolar affinities. High-resolution structures of these complexes show that the ligands displace water and reduce HIF-2 activity by impacting HIF-2α/ARNT interactions (40-42). To accommodate these binding events within a solvent-inaccessible cavity, the HIF-2α PAS-B protein must dynamically fluctuate to allow the entry of small molecules into the interior (43). Our pressure NMR analysis confirmed this hypothesis, showing a very broad distribution of c_i (^{15}N) responses with a correspondingly large stdev(c_i [^{15}N]) of 85 x ($2x10^{-10}$ ppm/bar^2) (**Figs. 4A and S4**). We anticipated that repeating these measurements in the presence of two nanomolar-affinity compounds (2 (1) and 37 (2)) would show smaller non-linear chemical shift changes than the apo protein given the smaller total void volume and expected reduced flexibility of the protein/ligand complexes. Our data supported this, as we observed decreases in stdev(c_i [^{15}N]) from 85 x ($2x10^{-10}$ ppm/bar^2) for the apo protein to 64 (2-bound) and 53 (37-bound) x ($2x10^{-10}$ ppm/bar^2), respectively. Notably, these decreases do not solely correlate with loss of void volume in some cases: measurements conducted on HIF-2α D1, a computationally-repacked variant with five point mutations which reduce the cavity volume to 77 $Å^3$ (44) while retaining function, shows an

increase in stdev(c_i [^{15}N]) up to 131 x (2x10^{-10} ppm/bar^2) (**Fig. 4A**). We speculate that some dynamic or thermodynamic stability aspect of this engineered protein differs markedly natural proteins, contribute to its unusual pressure response.

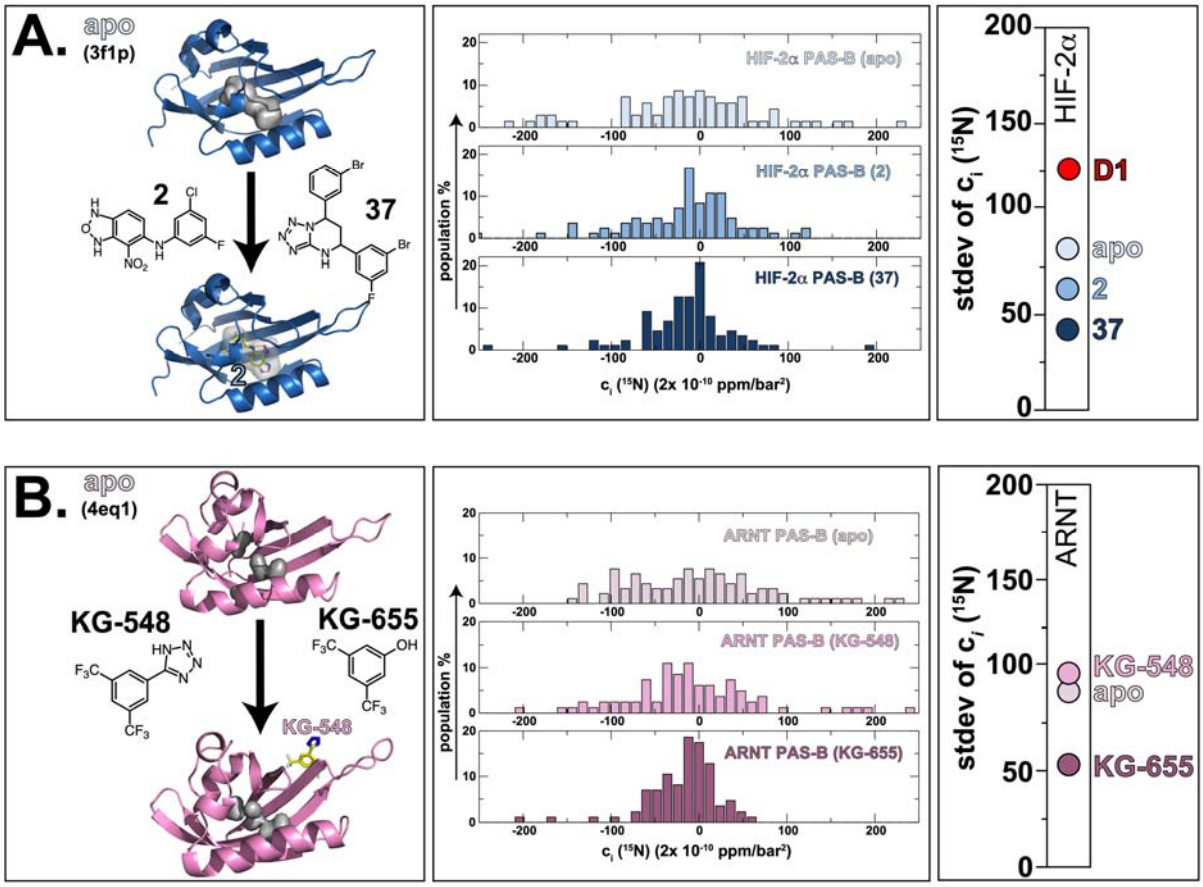

Fig. 4: High pressure NMR can provide mode-of-action information on ligand binding, even without structural information. (Left panel) **A**. Ribbon diagrams of HIF-2α PAS-B in its apo (PDB: 3f1p) and holo forms (complexed with 2, PDB: 4ghi), highlighting the location of the internal ligand binding cavity. Structures of two high-affinity inhibitors, 2 and 37 (1, 2) are also shown. **B**. Ribbon diagram of ARNT PAS-B (apo PDB: 3f1p; KG-548 complex PDB: 8ga4) with the location of internal cavities, along with the structures of two moderate affinity binders, KG-548 and KG-655 (3). No structural details were available for a compound-bound form at the outset of this work. (Central panel) Histograms of the ^{15}N c_i coefficients measured on apo- and holo forms of HIF-2α and ARNT PAS-B domains using the approach diagrammed in **Fig. 2**. (Right panel) Comparisons of the stdev(c_i [^{15}N]) values of HIF-2α and ARNT PAS-B apo and ligand-bound samples, showing substantial effects upon binding small molecules (or point mutations, in the case of HIF-2α PAS-B "D1" variant) into the internal cavities.

We used the same approach with the ARNT PAS-B domain, which has the same fold as HIF-2α PAS-B but contains smaller internal cavities totalling 150 $Å^3$ 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 remained unclear without NMR or X-ray co-complex structures available at the outset of this work.

To gain more insight on how KG-548 and KG-655 bound ARNT PAS-B, we used our pressure NMR analysis, confirming that ARNT PAS-B retains a flexibly-accessible interior cavity with a stdev(c_i [^{15}N]) of 84 x ($2x10^{-10}$ ppm/bar^2) (**Figs. 4B and S5**). Repeating these in the presence of the KG-655 ligand, we observed a drop of a stdev(c_i [^{15}N]) to 51 x ($2x10^{-10}$ ppm/bar^2), consistent with an interior binding location for this small ligand analogous to the HIF-2α PAS-B examples above. However, similar studies with the larger KG-548 ligand show a stdev(c_i [^{15}N]) comparable to the apo protein, with an observed value of 96 x ($2x10^{-10}$ ppm/bar^2) that suggest that this compound binds in a different mode than KG-655, potentially outside the cavity. We subsequently verified this external binding mode for KG-548 by determining the X-ray crystal structure of ARNT PAS-B with KG-548 soaked into the crystal (**Table S2**). The resulting 1.97 Å model (**Fig. S9**) clearly demonstrated that KG-548 bound across the outside of the PAS β-sheet, which we subsequently validated in solution (Xu *et al*., manuscript under review).

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, 45). This domain has a canonical PAS structure, with a five-stranded β-sheet flanked to one side by several α helices (31, 46). While only a small surface groove of 73 $Å^3$ 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 [^{15}N]) of 45 x ($2x10^{-10}$ ppm/bar^2); 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 $Å^3$ 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 [^{15}N]) values for both ligand-bound forms (35 and 28 x ($2x10^{-10}$ ppm/bar^2) 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 (7, 47)*, chosen because of a). homology with the light-regulated PAS-histidine kinase EL346 (48, 49) and b). the widespread usage of small molecule ligands to control the structure and function of

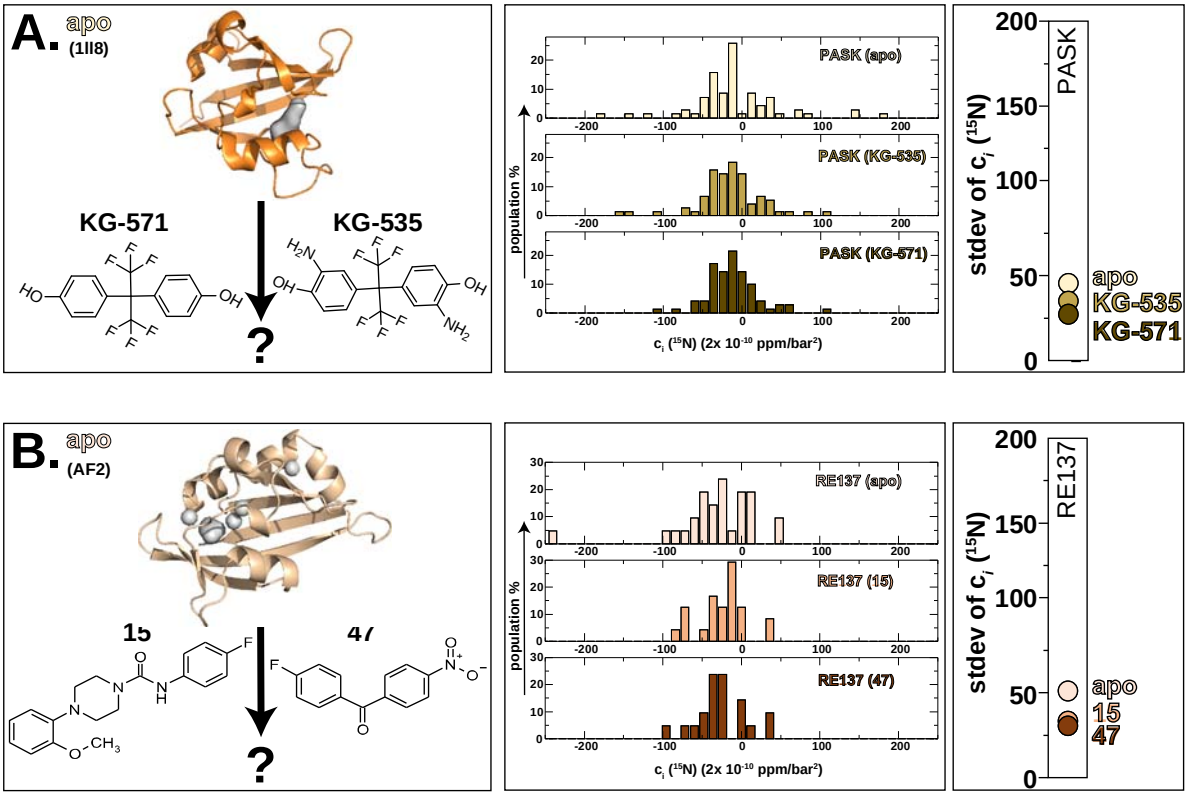

Fig. 5: High pressure NMR can provide ligand binding information even in the absence of experimental structure. (Left panel) **A.** Three-dimensional structure of PASK PAS-A (PDB: 1ll8) and structures of two binding compounds, KG-535 and KG-571 (4). No high-resolution structures of protein/ligand complexes are available. **B.** No experimental structural information is available for the RE137 PAS domain (7) (either apo or bound forms), structure shown here was generated using AlphaFold2 (AF2) (8, 9). Structures of two binding compounds identified by ^{19}F ligand-detected NMR screening, 15 and 47. (Central panel) Histograms of the ^{15}N c_i coefficients measured on apo- and holo forms of PASK and RE137 PAS domains using the approach diagrammed in **Fig. 2**. (Right panel) Comparisons of the stdev(c_i [^{15}N]) values of PASK and RE137 apo- and ligand-bound samples.

PAS-containing proteins (including many histidine kinases (32)). 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 (32). Pressure NMR analyses of the apo protein support this possibility, returning a stdev(c_i [^{15}N]) value of 53 x (2x10^{-10} ppm/bar^2) (**Figs. 5B and S7**), suggesting the presence of a 2800 Å^3 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 (50). 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 stdev(c_i [^{15}N]) values of 30 and 32 x (2x10^{-10} ppm/bar^2) 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.

<u>*Use of pressure NMR to probe artificially-designed proteins:*</u> As a final demonstration of the utility of this method, we examined its utility in investigating the flexibility of an artificially-designed protein, CA01 (51). With advances in protein engineering enabling the development of artificial ligand-binding biosensor proteins (52), we sought to probe whether a protein such as CA01 – which contains a completely-enclosed 75 Å^3 cavity within approximately 3500 Å^3 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 stdev(c_i [^{15}N]) value of approximately 75

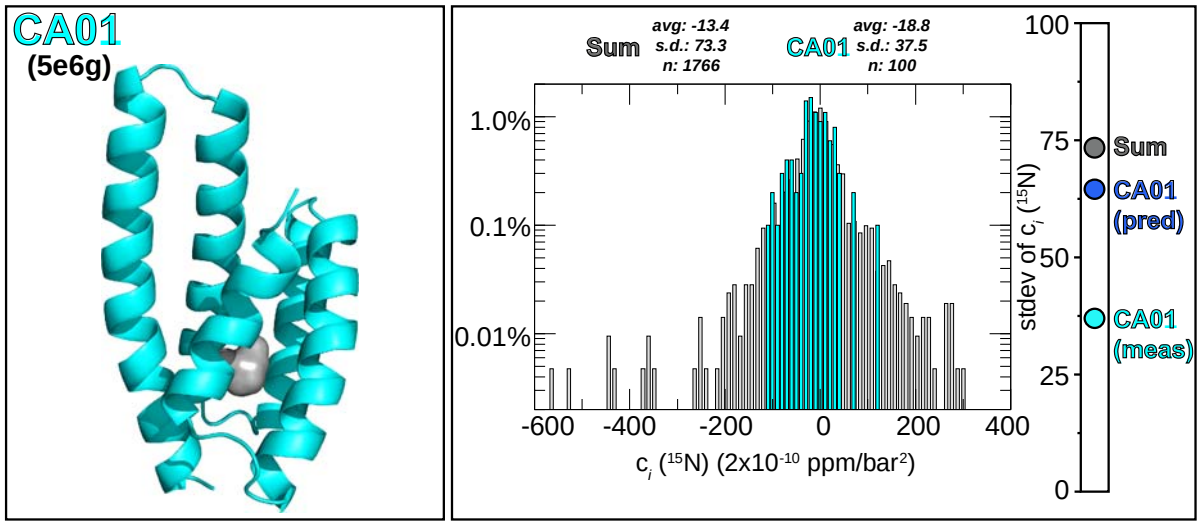

Fig. 6: High pressure NMR of a thermostable designed protein with an internal cavity gives smaller non-linear pressure dependent chemical shift changes than anticipated from void volume. (Left panel) Three-dimensional structure of CA01 (PDB: 5e6g), highlighting the location of a 75 Å^3 internal cavity within a total void volume of 3500 Å^3. (Right panel) Histogram of the ^{15}N c_i coefficients measured on CA01 (aqua) and 22 apo proteins listed in **Fig. S1** (grey) using the approach diagrammed in **Fig. 2**. Bar on right indicates stdev(c_i [^{15}N]) measured for CA01 (aqua) and 22 apo proteins (grey), along with the value predicted for CA01 from the correlation in **Fig. 3** (blue).

(2x10^{-10} ppm/bar^2) from a natural protein with void volume of this size, but we instead observed a substantially smaller value of 38 x (2x10^{-10} ppm/bar^2) (**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 (51), 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 ^{1}H and ^{15}N 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-^{15}N labeled samples are routinely available from *E. coli* or *in vitro* expression systems, 2). the NMR data needed are easily obtained from conventional ^{1}H/^{15}N 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 a smaller volume (17). Our specific choice of backbone amide ^{1}H and ^{15}N chemical shifts give us complementary views of these transitions (**Fig. S3**), as ^{1}H 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 both the adjacent ^{1}H and carbonyl CO groups), local backbone dihedrals and sidechain conformations (53). Of note, non-linear pressure-dependent chemical shift changes at these sites are much smaller in peptides or intrinsically disordered proteins (54). Indeed, non-linear pressure-dependent amide ^{1}H and ^{15}N chemical shift changes have long been recognized as reflecting idiosyncratic differences among proteins (55). Prior work by one of our groups (K.A.) suggested that this property depends on the density of cavities within the protein (29); 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-2α 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, 44). 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 provided an outstanding example of the power of the pressure NMR method to quickly and accurately provide mode-of-action binding site information even with limited structural data available. In these cases, high pressure NMR – using rapidly-acquired spectra without needing any chemical shift assignments – indicated different binding modes between the two

ligands, with KG-655 binding primarily to an internal site while KG-548 bound externally. These findings were subsequently validated both by a ARNT PAS-B:KG-548 crystal structure (**Fig. S9**) and accompanying solution biophysical investigation (Xu *et al.*, manuscript under review).

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 stdev(c_i [^{15}N]) values of the apo proteins exceed the value of approximately 50 ($2x10^{-10}$ ppm/bar^2) 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}H/^{15}N TROSY or HNCO-type pulse sequences which can provide improved resolution. Additional options may be provided by the improved NMR signal properties of ^{1}H/^{13}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 a 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 (56) and concentrated to 100-700 µM for NMR spectroscopy. ^{1}H/^{15}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) (57, 58) and analyzed with NMRViewJ (One Moon Scientific) (57, 59). 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 ^{1}H 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**.

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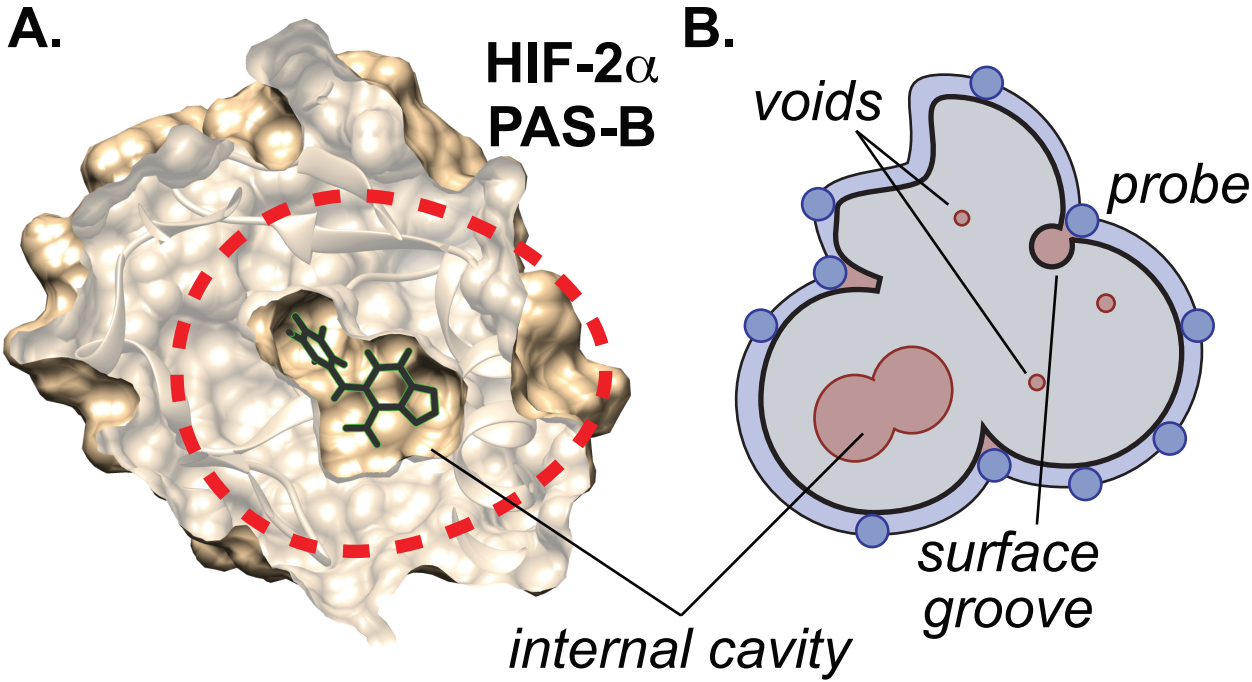
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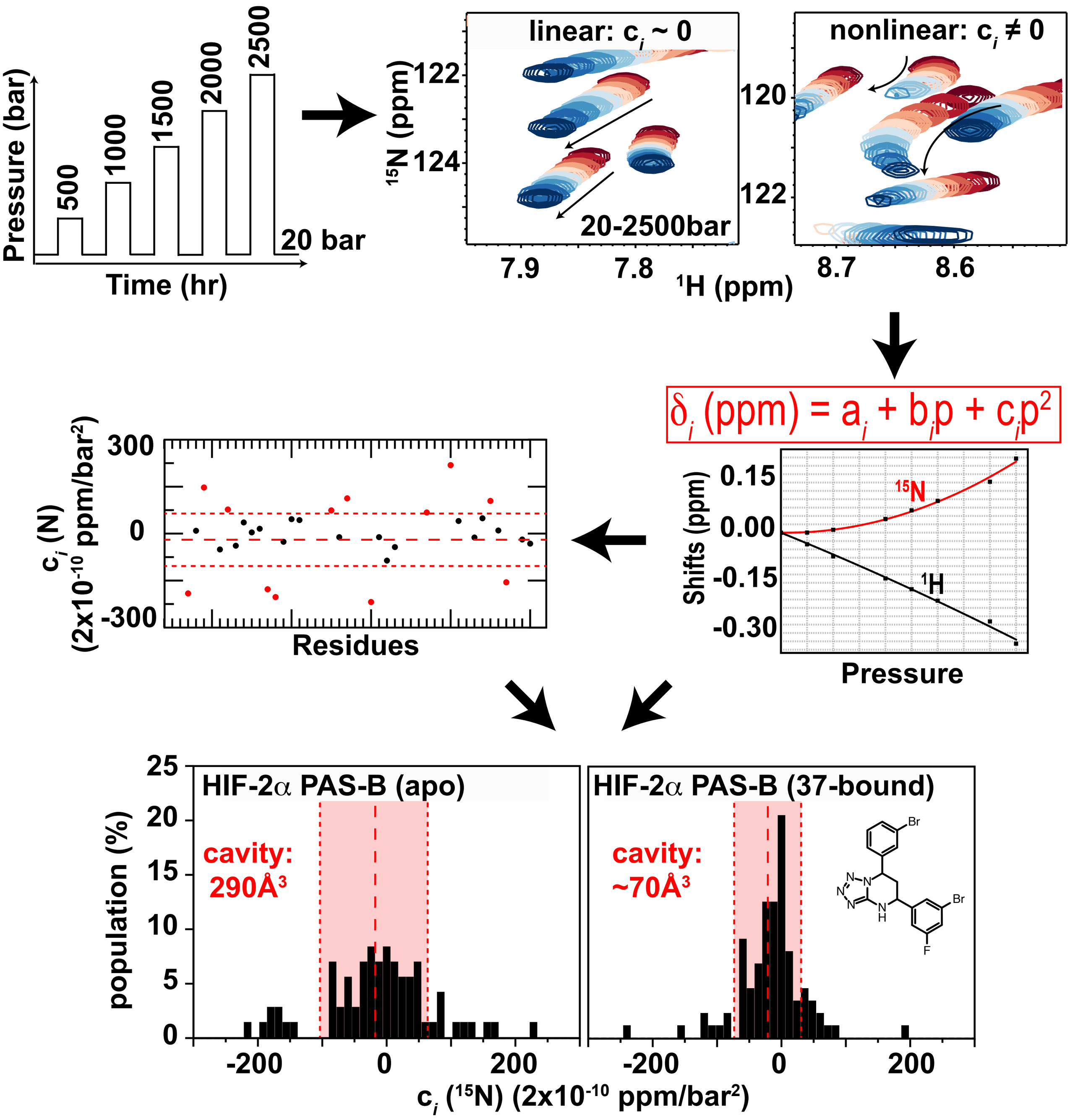
PAS-B
B.
voids
probe
surface
groove
internal cavity


Pressure (bar)
500
1000
1500
2000
2500
20 bar
Time (hr)
linear: c_i ~ 0
nonlinear: c_i ≠ 0
^{15}N (ppm)
122
124
120
122
20-2500bar
7.9
7.8
^{1}H (ppm)
8.7
8.6
δ_i (ppm) = a_i + b_ip + c_ip^2
Shifts (ppm)
0.15
0.00
-0.15
-0.30
^{15}N
^{1}H
Pressure
c_i (N) (2x10^{-10} ppm/bar^2)
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0
-300
Residues
HIF-2α PAS-B (apo)
cavity: 290Å^3
HIF-2α PAS-B (37-bound)
cavity: ~70Å^3
Br
Br
F
N
N
N
N
H
population (%)
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20
15
10
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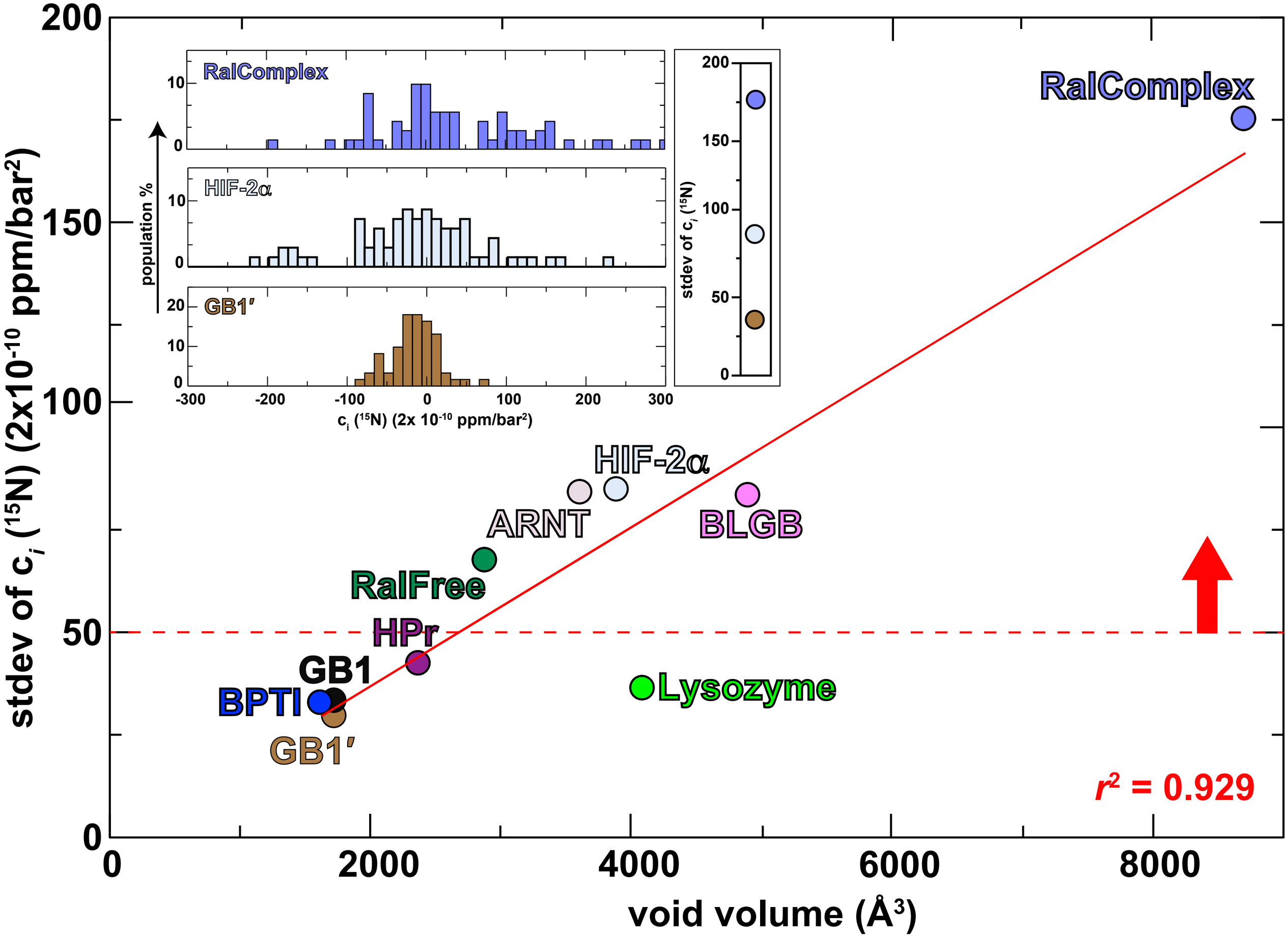
200
c_i (^{15}N) (2x10^{-10} ppm/bar^2)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stdev of c_i (^{15}N)
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HIF-2α
ARNT
BLGB
RalFree
HPr
GB1
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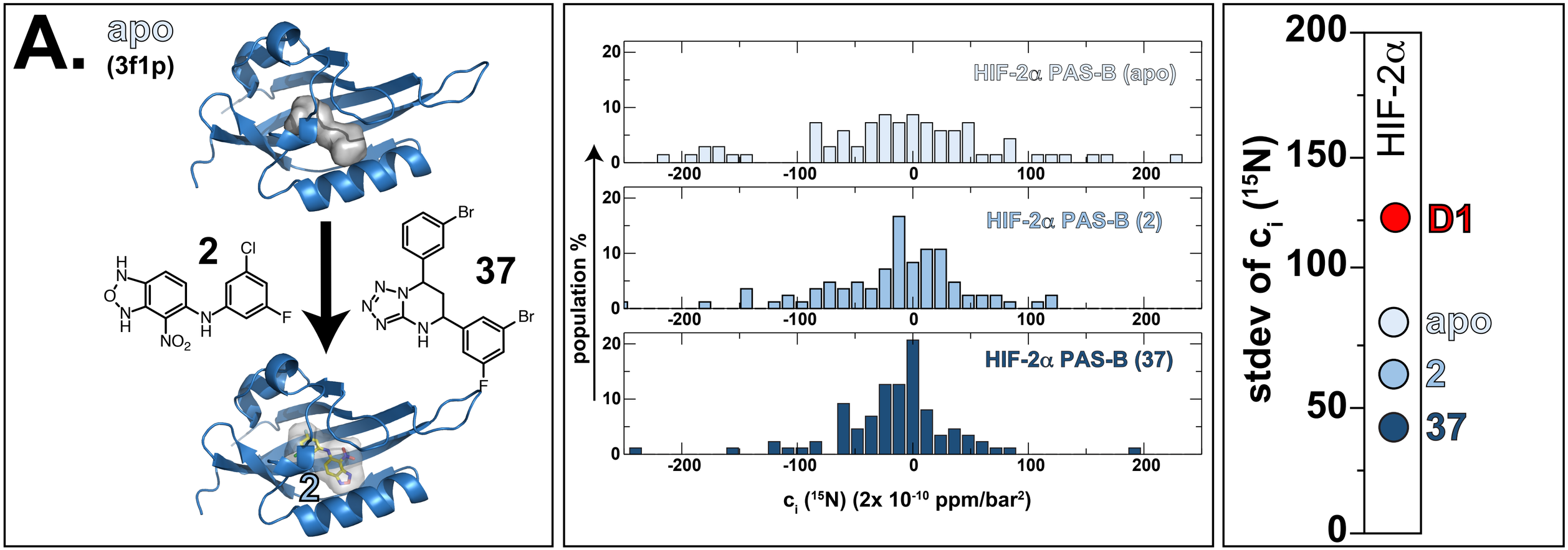
void volume (Å^3)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population %
c_i (^{15}N) (2x 10^{-10} ppm/bar^2)
stdev of c_i (^{15}N)
HIF-2α
D1
apo
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37
B.
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KG-548
KG-655
ARNT PAS-B (apo)
ARNT PAS-B (KG-548)
ARNT PAS-B (KG-655)
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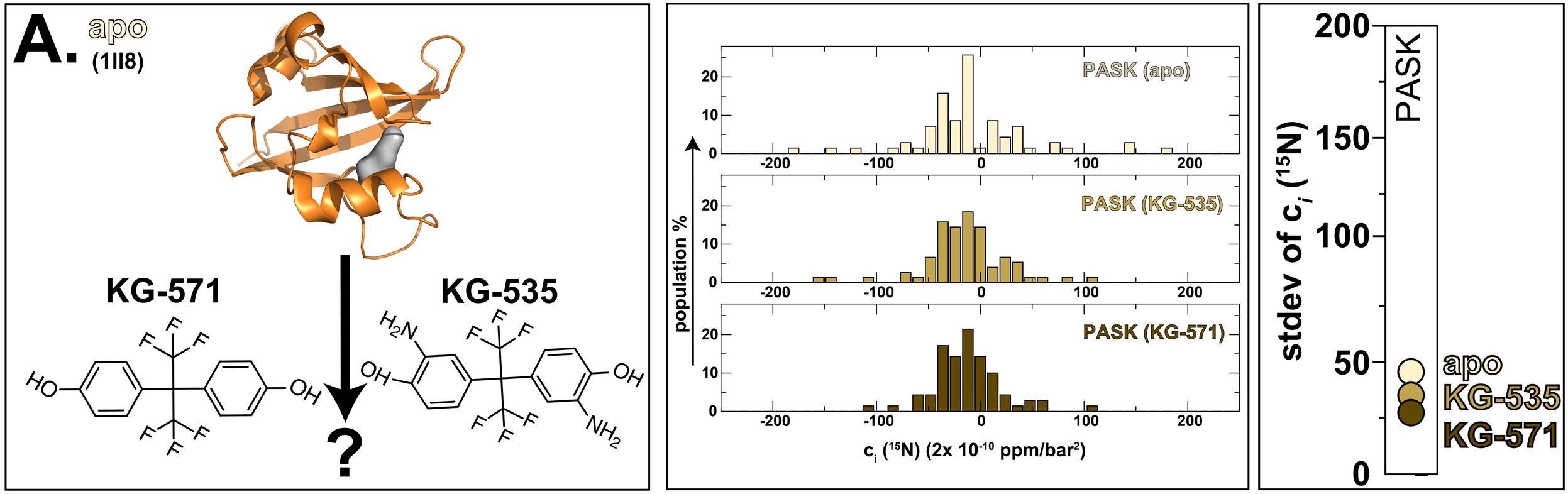
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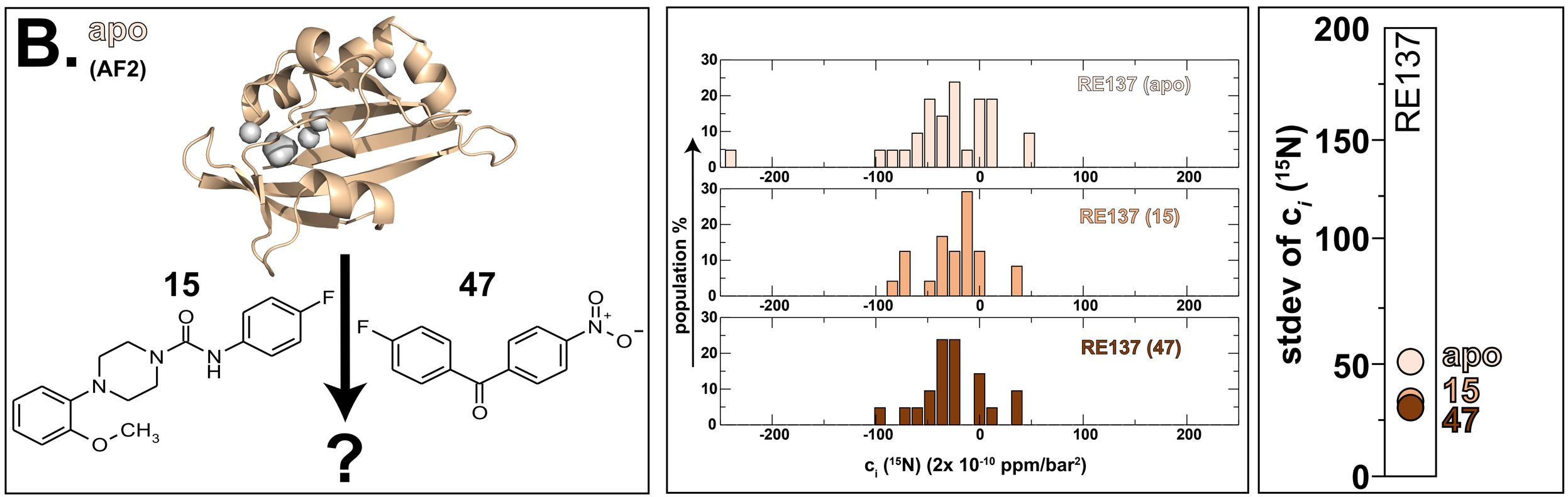
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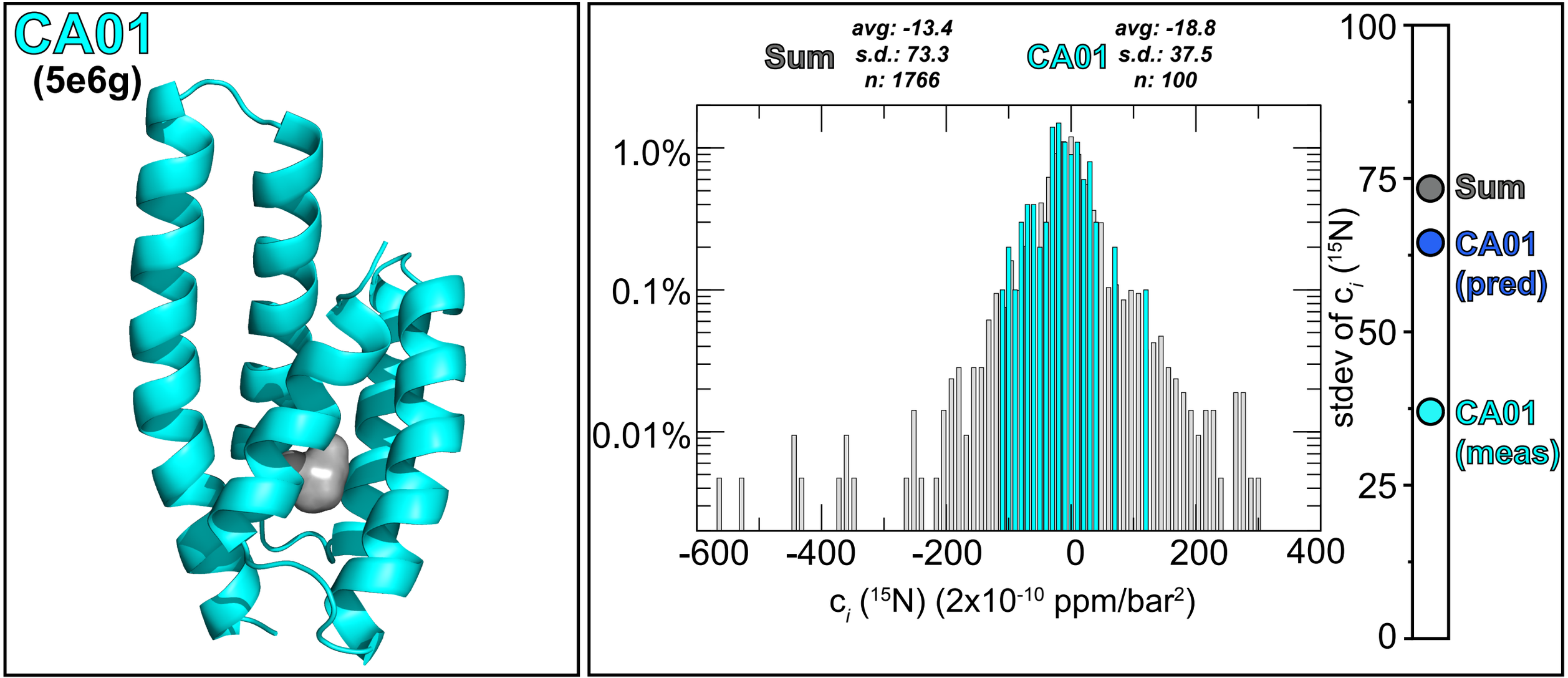
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CA01
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s.d.: 37.5
n: 100
1.0%
0.1%
0.01%
-600
-400
-200
0
200
400
c_i (^{15}N) (2x10^{-10} ppm/bar^2)
stdev of c_i (^{15}N)
100
75
50
25
0
Sum
CA01
(pred)
CA01
(meas)