

# Volume and Compressibility Differences Between Protein Conformations

## Revealed by High-Pressure NMR

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## ABSTRACT

Proteins often interconvert between different conformations in ways critical to their function. While manipulating such equilibria for biophysical study is often challenging, the application of pressure is a potential route to achieve such control by favoring the population of lower volume states. Here, we use this feature to study the interconversion of ARNT PAS-B Y456T, which undergoes a dramatic +3 slip in beta-strand register as it switches between two stably-folded conformations. Using high pressure biomolecular NMR approaches, we obtained the first quantitative data testing two key hypotheses of this process: the slipped conformation is both smaller and less compressible than the wildtype equivalent, and the interconversion proceeds through a chiefly-unfolded intermediate state. Data collected in steady state pressure and time-resolved pressure-jump modes, including observed pressure-dependent changes in the populations of the two conformers and increased rate of interconversion between conformers, support both hypotheses. Our work exemplifies how these approaches, which can be generally applied to protein conformational switches, can provide unique information that is not easily accessible through other techniques.

## 32    **STATEMENT OF SIGNIFICANCE**

33            Proteins often interconvert between conformations via processes that can be difficult to  
34    characterize due to the low populations and short lifetimes of intermediate and end states. This  
35    can be addressed with high-pressure perturbation stabilizing conformations with smaller system  
36    volumes, as elegantly applied in high pressure NMR studies of protein folding. Here we  
37    demonstrate comparable utility for probing the thermodynamics and kinetics of a protein  
38    interconverting between two stably-folded conformations. Combining steady-state and time-  
39    resolved pressure NMR, we measured volumes and compressibilities of ground and intermediate  
40    states which are otherwise challenging to access, letting us test a proposed interconversion  
41    mechanism. These data establish both fundamental parameters of the process and guide artificial  
42    control via ligand binding.

## INTRODUCTION

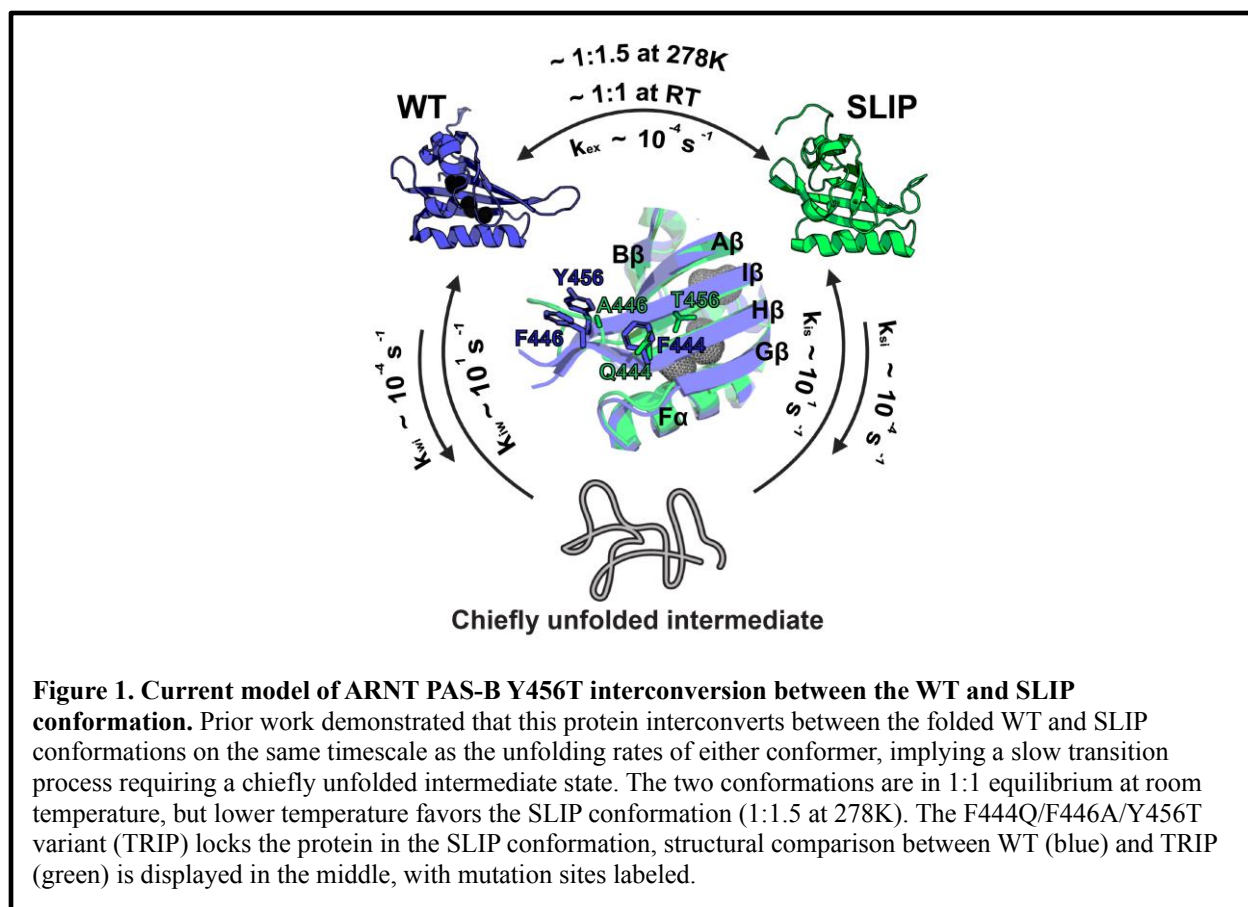
The energy landscape theory of folding states that proteins sample many conformations before reaching the native state (1, 2). Although the native conformation is usually the lowest free energy state under physiological conditions, proteins can be trapped in stably-folded local minima that are markedly different from the native conformation (3-5). Transitions between conformations, which can be spontaneous or triggered by changes in environmental conditions (e.g. pH, temperature, light) or ligand binding, are often critical for shifting proteins between functionally inactive and active forms (3-7). Such shifts can range from simple local rearrangements to ‘metamorphic’ proteins, which reversibly adopt different stable folds in different environmental conditions (8-10). However, identifying and characterizing alternative conformational states of proteins can be challenging, as they are often high-energy states and sparsely populated (11, 12). Several techniques currently allow quantitative characterization of conformational sub-states despite challenges introduced by low population, particularly solution nuclear magnetic resonance (NMR) techniques such as relaxation dispersion experiments (11-14).

To aid in characterizing such excited states, it is routine for one to manipulate their populations by adding small molecule ligands or changing experimental conditions, such as pH or temperature (11, 15-17). Increasingly, pressure has also been utilized to control conformational equilibria, aided by the introduction of commercially-built pumps and sample cells compatible with NMR and other biophysical instrumentation (18-20). Pressure directly affects such equilibria by acting on differences in the partial volumes and compressibilities of different conformers, with increasing pressure favoring those with lower volumes (21). Given that unfolded proteins are generally smaller than their folded forms, pressure studies have been

particularly useful at investigating protein unfolding reactions, which typically occur above 2000 bar under native conditions (20-25). However, the application of sub-unfolding pressures can cause proteins to shift populations among multiple folded states (18, 26-28), providing an easy way to manipulate protein conformational equilibria to facilitate their study.

These advantages of pressure-NMR led us to examine its applicability to characterize a protein domain which slowly exchanges between two folded states via an unusual  $\beta$ -strand slip (29, 30). This system is derived from the human ARNT (Aryl hydrocarbon Receptor Nuclear Translocator) protein, a bHLH-PAS (basic helix-loop-helix / Per-ARNT-Sim) transcription factor which binds a variety of partners via its two PAS domains, PAS-A and PAS-B (31-35). PAS domains adopt a mixed  $\alpha/\beta$  fold, with helical and sheet layers often encapsulating internal cavities between them (31, 32, 34). In many cases, these internal cavities provide binding sites for natural or artificial regulatory molecules (36). For ARNT PAS-B, 105 Å<sup>3</sup> of interconnected internal cavities are seen in the crystal structure, suggesting that small molecules may bind there and control the role of ARNT in several signaling pathways (29, 32, 37, 38).

We have previously reported that the ARNT PAS-B  $\beta$ -sheet can surprisingly adopt an alternatively-folded conformation in certain settings. For example, a Y456T point mutation at a site preceding the final  $\beta$ -strand enables the domain to adopt a new conformation that coexists in a 1:1 equilibrium with the WT fold at room temperature (29, 30) (**Fig. 1**). Additional mutations to nearby residues (F444Q/F446A/Y456T, hereafter called the TRIP variant) stabilized this alternative conformation, letting us characterize the new structure and show that it differs from the WT conformation by a three residue slip of central I $\beta$ -strand of the domain's five-stranded  $\beta$ -sheet. This slip inverts the topology of the I $\beta$ -strand and isomerizes the neighboring N448-P449



peptide bond, collectively abolishing the domain's ability to interact with some other binding partners (29). Notably, the threonine side chain introduced by the Y456T mutation fills an internal cavity (29, 30), leading us to suspect that interconversion process could be manipulated by pressure perturbations (18, 39-42) to obtain thermodynamic and kinetic information complementary to our prior structural studies.

Here we report results from high-pressure solution NMR studies of ARNT PAS-B Y456T, letting us for the first time obtain quantitative measurements of several thermodynamic and kinetic parameters of the WT:SLIP interconversion. We found that the WT conformation is not only larger in volume than SLIP, but also more compressible as perhaps due to the cavities unique to the WT conformation (29, 32). Additionally, we tested a hypothesis that WT:SLIP interconversion proceeds through a chiefly unfolded intermediate state, previously suggested

from the similarities of interconversion and unfolding rates (30). We found that pressure increases the rate of interconversion, letting us measure both the activation volume and compressibility (30), both validating this model and allowing us to quantitate how unfolded the transition state is. Finally, we demonstrated that both residue-specific compressibility and pressure-dependent chemical shift changes can predict whether residues are near cavities, providing insights into locations of such cavities within the protein. Taken together, our approach exemplifies the ability of high-pressure NMR to enable thermodynamic and kinetic analyses of protein conformational transitions that are otherwise inaccessible.

## **MATERIALS AND METHODS**

### **Protein Purification and Expression**

His<sub>6</sub>-tagged human ARNT PAS-B (residues 356-470) WT and mutants (Y456T and F444Q/F446A/Y456T) were uniformly labeled with <sup>13</sup>C and <sup>15</sup>N and overexpressed in BL21 (DE3) E. coli. Isotopically-labeled proteins were obtained by growing cells in M9 media supplemented with 3 g/L U-<sup>13</sup>C<sub>6</sub> glucose and 1 g/L <sup>15</sup>NH<sub>4</sub>Cl as the carbon and nitrogen sources, respectively. Cells were incubated at 37 °C until an OD<sub>600</sub> of 0.6-0.8. Protein expression was induced with 0.5 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated overnight (~16-18 hr) at 18 °C. Cells were harvested next day, cell pellets were resuspended in 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM imidazole, 1 mM DTT buffer containing 1 mM PMSF, lysed with sonication, and centrifuged. Supernatant was incubated with 5 mL of Ni<sup>2+</sup> Sepharose<sup>TM</sup> High Performance beads and eluted with 500 mM imidazole buffer. His<sub>6</sub>-Tagged ARNT PAS-B proteins were exchanged into a low imidazole buffer (8 mM imidazole, 50 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM DTT) and incubated overnight with His<sub>6</sub>-tobacco etch virus (TEV) protease for tag cleavage. The freed His<sub>6</sub>-tag and His<sub>6</sub>-TEV were removed by a second round of

Ni<sup>2+</sup> column purification. ARNT PAS-B proteins were further purified by passing through a Superdex 75 size exclusion column and exchanged into baroresistant buffer (44.7 mM Tris pH 7.0, 5.3 mM phosphate, 17 mM NaCl, 1 mM DTT) (43) and concentrated with an Amicon stirred ultrafiltration unit with a 3 kDa filter to 320  $\mu$ M. Samples were flash frozen with liquid nitrogen and were stored at -80 °C for later use.

### **Pressure-Jump NMR Experiments**

Solution NMR experiments were performed on a Bruker Avance III HD 700 MHz NMR spectrometer equipped with a 5-mm QCI-F cryoprobe. NMR samples were prepared by mixing 80% of the purified protein samples with 20% D<sub>2</sub>O yielding a final concentration of around 250  $\mu$ M. Samples were loaded into a commercially-available pressure resistant NMR cell (Daedalus Innovations, Aston, PA, USA), with pressure between 20 bar to 2500 bar applied through a Xtreme-60 Syringe Pump from the same vendor. The baseline pressure was set to 20 bar as it is a low pressure that can be stably maintained by the pump. We performed two types of pressure-jump experiments, direct pressure-jump experiments where pressure was increased directly from 20 bar to the destination pressure of up to 2500 bar, and cumulative step pressure-jump experiments with constant intervals, where pressure was increased incrementally from baseline pressure to the final pressure of 2500 bar. To set up direct pressure-jump experiments, samples were thawed and stored on ice, and first equilibrated at 20 bar and 278.1 K for 1 hr and raised to a higher pressure (between 125 bar to 1500 bar) and equilibrated at that pressure. As identified in preliminary experiments, the duration of equilibration process varies depending on the pressure: interconversion of the two populations at 20 bar takes around 12 hours to complete, while it occurs more quickly as pressure increases (30). Equilibration time under pressure was therefore set to 12 hr at 125 bar, and gradually reduced to 5 hr for pressures between 500 to 1000 bar, and

145 finally 4 hr for pressures beyond 1000 bar. During the equilibration, a series of 1D  $^{13}\text{C}$  edited  $^1\text{H}$   
146 NMR experiments were recorded to detect the rate of interconversion, with each spectrum  
147 requiring ca. 4.25 min to acquire. The system was relaxed for 12.5 hr at 20 bar post  
148 equilibrations at different pressures, 1D  $^{13}\text{C}$ -edited  $^1\text{H}$  NMR experiments were also recorded  
149 during relaxation. For step pressure-jump experiments, samples were first equilibrated at 20 bar  
150 and 278.1K, then pressurized and equilibrated in steps of 250 bar, until reaching 2500 bar.  
151 Equilibration time was set to 9 hr at 250 bar, 6 hr at 500 bar, 5 hr for 750 and 1000 bar, and 4 hr  
152 for 1250, 1500, 1750, 2000, and 2250 bar. Equilibration time was extended to 6 hr at 2500 bar to  
153 monitor any unfolding effects at the highest pressure safely accessible with our instrumentation.  
154 1D NMR spectra were acquired as described earlier.  $^{15}\text{N}/^1\text{H}$  and  $^{13}\text{C}/^1\text{H}$  HSQC spectra were also  
155 acquired at system equilibrium at 20, 250, 500, 750, 1000, 1250, 1500, and 2500 bar.  $^{15}\text{N}/^1\text{H}$   
156 HSQC experiments were acquired with 16 scans and FIDs of 2048x256 complex points.  $^{13}\text{C}/^1\text{H}$   
157 HSQC experiments were acquired with 8 scans and FID of 2048x256 complex points. All NMR  
158 spectra were processed and analyzed with NMRFX Analyst and NMRViewJ (44, 45). Chemical  
159 shift assignments from previous NMR assignments of ARNT PAS-B WT and  
160 F444Q/F446A/Y456T mutant were used for all analyses (29, 31).

161         Unfolding measurements of ARNT PAS-B WT and the F444Q/F446A/Y456T variant  
162 under pressures accessible by our apparatus (20-2500 bar) was achieved by the addition of urea  
163 (up to 3.0 M).  $^{15}\text{N}/^1\text{H}$  HSQC spectra were acquired between 20 and 2500 bar with increments of  
164 250 bar. Peak assignments from previous work were obtained (29, 31), and transferred to the  
165 acquired spectra following urea titration experiments between 0.5 and 3.0 M.

## NMR Data Analysis

In the 1D  $^{13}\text{C}$ -edited  $^1\text{H}$  NMR experiments, two peaks corresponding to the L391- $\delta 1$  methyl group of the WT and SLIP conformations were used to determine the populations of each conformational state of the protein in the sample. The relative populations (SLIP/WT) at different pressures were obtained by taking the ratio of the integrals of the two peaks. The equilibration (change in relative population) under different pressures and the rates of interconversion were obtained by plotting the relative population as a function of time.

The equilibrium constant  $K$  between the two conformers of the ARNT PAS-B Y456T is given by the relation

$$K = \frac{[\text{SLIP}]}{[\text{WT}]} = e^{-\Delta G/RT} \quad (1)$$

Since the temperature  $T$  was kept constant during the pressure jump experiments, the free energy  $\Delta G$  scales only with pressure  $p$ . The free energy as a function of pressure can be approximated by a Taylor expansion

$$\Delta G(p) = \Delta G^0 + \Delta V^0(p - p_0) - \frac{1}{2} \times \Delta \beta V^0(p - p_0)^2 + \dots - \frac{\Delta G^n(p_0)}{n!}(p - p_0)^n \quad (2)$$

$\Delta G^0$  is the free energy between the two conformers at ambient pressure,  $\Delta V^0$  is the volume difference between the two conformers, representing the first derivative of free energy with respect to pressure, and compressibility between the two conformations,  $\Delta \beta V^0$ , is the second derivative of free energy, with respect to pressure. The equilibrium constants were plotted as a function of pressure, and fit to the following equation, incorporating up to the third term of the Taylor series (18):

$$K_{eq} = e^{-\left(\Delta G^0 + (p-p_0)\Delta V^0 - 1/2 \times \Delta \beta V^0 (p-p_0)^2\right)/RT} \quad (3)$$

The pressure was referenced to atmospheric pressure,  $p_0=1$  bar.

The observed rate of increase of one conformation (or decrease of the other conformation) as a function of pressure could be fit to a biexponential equation:

$$k_{obs} = k_{sw0} e^{-\left(p\Delta V_{sw}^\ddagger - 1/2 \Delta \beta V_{sw}^\ddagger p^2\right)/RT} + k_{ws0} e^{-\left(p\Delta V_{ws}^\ddagger - 1/2 \Delta \beta V_{ws}^\ddagger p^2\right)/RT} \quad (4)$$

yielding the activation volumes ( $\Delta V_{sw}^\ddagger$  and  $\Delta V_{ws}^\ddagger$ ) and compressibility differences between the two folded conformations (WT and SLIP) to the previously-proposed, chiefly unfolded intermediate state ( $\Delta \beta V_{sw}^\ddagger$  and  $\Delta \beta V_{ws}^\ddagger$ ) (30). Together with the two initial rate constants at ambient pressure ( $k_{sw0}$ , SLIP to WT, and  $k_{ws0}$ , WT to SLIP), there were 6 unknown parameters to be fit. To better approximate the two exponential terms, rates at different pressures were solved numerically using the following differential equation pair:

$$\frac{d[S]}{dt} = -k_{sw}[S] + k_{ws}[W] \quad (5)$$

$$\frac{d[W]}{dt} = k_{sw}[S] - k_{ws}[W] \quad (6)$$

The fittings were done in R, using the package deSolve as the ordinary differential equation solver, and Levenberg-Marquardt Nonlinear Least-Squares Algorithm in minpack.lm for parameter fitting (46). The uncertainty of the fitting was estimated by calculating the 95% confidence intervals from the variance covariance matrix of the fit rate constants. 95% confidence intervals were further verified with a bootstrapping procedure. Random noises with mean of 0 and variance of the mean square error from the model were added to the raw data and

fit repeatedly. The estimated parameters were computed to determine if they fall within the 95% confidence interval. The rates of interconversion at any given pressure ( $k_{sw}$  for SLIP to WT,  $k_{ws}$  for WT to SLIP) were plotted separately as functions of pressure, and fit individually to:

$$k_{sw} = k_{sw0} e^{-\left(p\Delta V_{sw}^{\ddagger} - 1/2\Delta\beta V_{sw}^{\ddagger} p^2\right)/RT} \quad (7)$$

$$k_{ws} = k_{ws0} e^{-\left(p\Delta V_{ws}^{\ddagger} - 1/2\Delta\beta V_{ws}^{\ddagger} p^2\right)/RT} \quad (8)$$

obtaining rates at ambient pressure ( $k_{sw0}$ ,  $k_{ws0}$ ), activation volumes, and activation compressibilities as described earlier.

The temperature dependence of the relaxation rates was calculated as the apparent rates during the 1000 to 20 bar re-equilibration step at temperatures of T = 278.1, 283.1, 288.1, and 291.1 K. The change of relative population ([SLIP]/[WT]) over time was fit to a single exponential with the apparent rate of

$$k_{app} = k_{ws} + k_{sw} \quad (9)$$

An Eyring relationship between rate and temperature was found by plotting  $\ln(k_{app}/T)$  against  $1/T$ . The plot was fit to a linear equation to extract the entropic and enthalpic contributions of activation energy of transition from SLIP to WT during relaxation at 20 bar.

For the pressure-dependent unfolding experiments,  $^{15}\text{N}/^1\text{H}$  HSQC spectra at different pressures were collected, maximum peak intensities in each spectrum were obtained and compared with the peaks corresponding to the same residues collected at different pressures. Overlapping peaks were excluded from the analysis. The intensity averages at different pressures were plotted as a function of pressure, and fit to a two-state unfolding model with XMGrace (47, 48),

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$$\frac{I}{I_0} = \frac{e^{-\left(\Delta G_f^0 + P\Delta V_f\right)/RT}}{1 + e^{-\left(\Delta G_f^0 + P\Delta V_f\right)/RT}} \quad (10)$$

227 where  $I_0$  is the peak intensity at initial (20 bar) pressure, R is gas constant, and T is temperature.

228 The apparent volume difference between the folded and unfolded state ( $\Delta V_f$ ) and the free energy

229 of folding ( $\Delta G_f^0$ ) were extracted. For simplicity, the max and min plateau values were set to 1

230 and 0.

231 For the pressure-jump  $^{15}\text{N}/^1\text{H}$  HSQC experiments, we analyzed the peaks under different

232 pressures and determined the change in chemical shifts and intensities under pressures compared

233 to the reference chemical shifts (positions of peaks at 20 bar). We fit the change in chemical

234 shifts to the following equation to determine the nonlinear coefficients(18, 26, 49):

$$\Delta\delta_i = b_i\Delta p + c_i\Delta p^2 \quad (11)$$

236 Where p is pressure (bars),  $b_i$  (parts per million per bar) and  $c_i$  (parts per million per square bars)

237 are the first and second order pressure dependence coefficients on chemical shifts for the  $i$ th

238 residue. A large  $c_i$  value suggests high nonlinear response to pressure. Fittings were performed

239 with the built-in pressure-analysis function of NMRViewJ (44). Residue-specific nonlinear

240 coefficient differences between the two conformations were calculated by subtracting the

241 absolute values of  $c_i$  corresponding to each conformation:

$$\Delta c_i = |c_{iwt}| - |c_{islp}| \quad (12)$$

243 Values were mapped to the crystal structure of WT ARNT PAS-B (32) and visualized with

244 PyMOL (50).

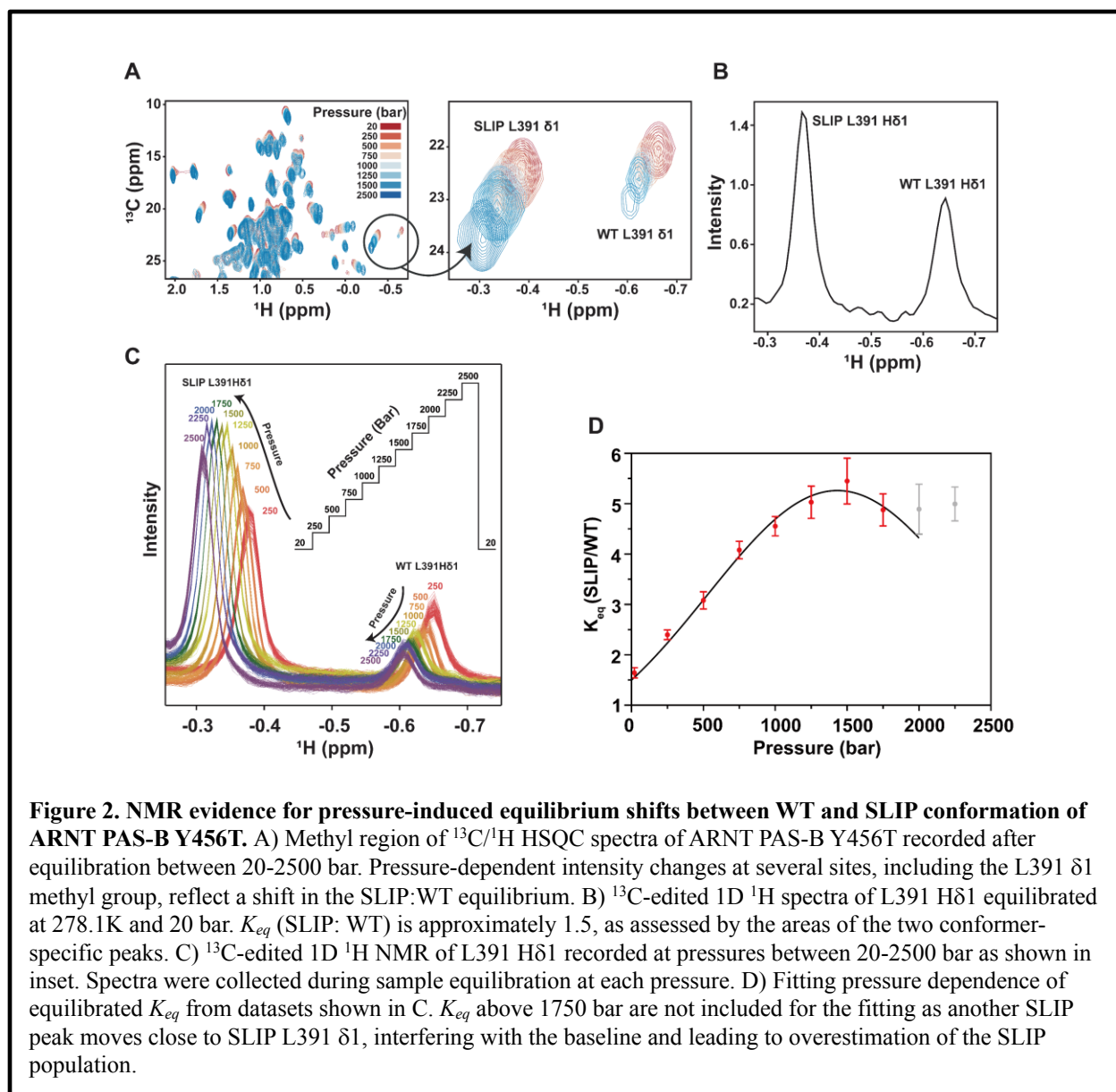
## Void Volume Calculation

Void volumes of ARNT PAS-B WT and SLIP conformations were calculated with the ProteinVolume software package by Chen and Makhataadze (51). Briefly, the total solvent-excluded volume of the protein is calculated by filling the space within the protein's molecular surface with 0.02 Å probes. The van der Waals volume is calculated with the same process but counting only the probes that are within the van der Waals radius of any atoms. The void volume  $V_{\text{void}}$  is given by the difference between the two volumes calculated. Solution NMR structures of WT and SLIP were used for the calculation (29, 31), using the average void volume of all 20 conformers in these ensembles.

## RESULTS AND DISCUSSION

### Equilibrium Analyses: Pressure Dependence of ARNT PAS-B Conformation Equilibrium

We previously determined the solution and crystal structures of WT ARNT PAS-B and the solution structure of the F444Q/F446A/Y456T (TRIP) variant (29, 31, 32). By comparing the average void volumes of the two solution structure ensembles with ProteinVolume (51), we found that the SLIP conformation had approximately 600 Å<sup>3</sup> smaller void volume than WT, leading us to anticipate that the equilibrium between these conformations might be pressure sensitive. To test this possibility, we recorded <sup>15</sup>N/<sup>1</sup>H and <sup>13</sup>C/<sup>1</sup>H HSQC spectra of ARNT PAS-B Y456T at increasing pressures (20, 250, 500, ... up to 2500 bar). Overlaying these spectra (**Fig. 2A, S1**), we noticed that signals from all three (<sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C) nuclei showed pressure-dependent chemical shift and intensity differences between the two conformations, as predicted from the volume differences between them. In general, we observed that increased pressure decreased the intensities of WT signals while concomitantly increasing SLIP signal intensities with good reversibility (**Fig. S2**), confirming the smaller calculated volume of the SLIP conformation (30).



To characterize the thermodynamics and kinetics of the WT:SLIP conformational interconversion, we acquired a series of  $^{13}\text{C}$ -edited 1D  $^1\text{H}$  NMR spectra as the system equilibrated after pressure changes. We have previously shown that the upfield-shifted L391 H $\delta 1$  methyl signal of both WT and SLIP conformations are well resolved in multiple types of NMR spectra, likely due to its location adjacent to a cavity only found in the WT conformation (**Fig. S3**) (30). Using this methyl group as a probe, we determined the equilibrium constant  $K_{eq}$  ( $=[\text{SLIP}]/[\text{WT}]$ ) at different pressures (20 to 2500 bar) by measuring the ratios of the peak

integrals corresponding to these two conformations (**Fig. 2B, C**). Of note, we performed all experiments at 278.1K instead of room temperature to slow down the interconversion process, and thus improving the temporal resolution of the subsequent kinetic analysis.

From these data, we extracted the pressure dependence of  $K_{eq}$ , noting that it monotonically increased until approximately 1500 bar. Beyond 1500 bar, we observed that it remained approximately constant, showing that  $K_{eq}$  does not scale exponentially with pressure and correspondingly that the free energy  $\Delta G$  is not linearly dependent on pressure  $p$  (**Eq. 1** and **Fig. 2D**), necessitating the inclusion of a nonlinear compressibility term for our analyses. Fitting these data to obtain differences in free energy between the two conformations at ambient pressure (referenced to 1 bar,  $\Delta G^0$ ), volume ( $\Delta V^0$ ), and compressibility ( $\Delta\beta V^0$ ) (**Eq. 3**), we found that the SLIP conformation was 40.5 ml/mol smaller than the WT conformation, agreeing reasonably with the ca. 105 Å<sup>3</sup> (= 63 ml/mol) internal cavities unique to the WT structure. We note that these values differ from the ~600 Å<sup>3</sup> void volume difference calculated above (= ~360 ml/mol) from the WT and SLIP structures, as the experimentally-measured value includes contributions from both protein and solvent effects. Additional support for the pressure-driven loss of these cavities came from the measurement that the WT conformation was more compressible than the SLIP by 0.0285 ml/(mol bar). We attribute both the volume and compressibility differences to the collapse of internal cavities and increased packing accompanying the WT to SLIP transition (29, 32).

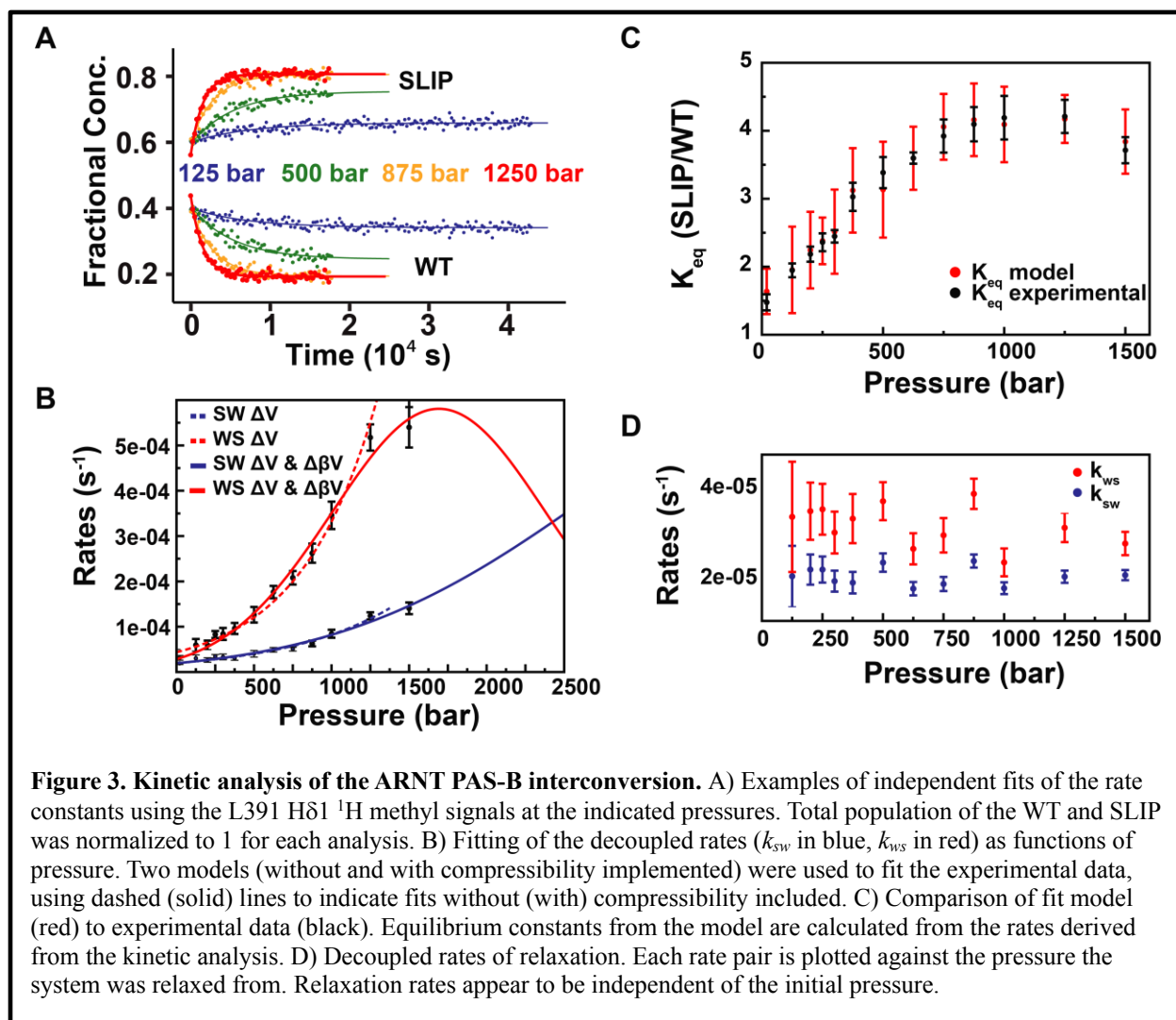
Finally, we compared the pressure-dependent  $K_{eq}$  changes of several other cavity-oriented side chain methyl groups that can be readily identified with <sup>13</sup>C/<sup>1</sup>H HSQC experiments (L391 δ2, L408 δ1, M439 ε, shown in **Fig. S4**), and found that they all exhibit similar behavior as L391 δ1, with negative volume (ranging from -20 to -36 ml/mol) and compressibility (ranging

from -0.012 to -0.025 ml/(mol bar)) changes upon WT to SLIP transition. We note that identifying peak pairs that are not interfered by other peaks at multiple pressure points is challenging, and further studies are warranted to investigate the intrinsic variability of the thermodynamic parameters among different residues.

### **Pressure Jump Kinetic Analyses: WT and SLIP Interconvert by Transitioning into an Intermediate State with Small Volume and Compressibility**

To complement the above equilibrium pressure analyses, we used a pressure jump protocol to obtain complementary kinetics measurements. To do so, we jumped the sample pressure from 20 bar to various higher values, acquiring 1D  $^{13}\text{C}$ -edited  $^1\text{H}$  NMR spectra as the system re-equilibrated and measuring the populations of the two conformations by the area of the respective L391 H $\delta$ 1 signals (**Fig. 3A**). Notably, we conducted these studies with commercially-available instrumentation capable of completing even the largest of these jumps within 30-60 sec, making it possible for us to perform the kinetic analyses due to the slow kinetics of the interconversion process (**Fig. 3A**). Custom-built, NMR-compatible pressure systems have recently been reported which are capable of such jumps approximately 100-fold faster (23, 24).

As an initial control of this approach, we extracted the  $K_{eq}$  values from these direct jump experiments and fit them for the same parameters mentioned in the equilibrium analyses (**Fig. S5**). This yielded a  $\Delta V^0$  of -46.1 ml/mol, and a  $\Delta\beta V^0$  of -0.0433 ml/(mol bar) (**Table 1**), comparable to values we noted above. While direct pressure jumps may induce convergence of  $K_{eq}$  at a slightly lower pressure, as indicated by the bigger difference in compressibilities between the two states, we otherwise believe that these values are similar despite different experimental routes.



320 To analyze the kinetics of the interconversion process, we assumed that a two-state model  
 321 could be applied by our prior observation that the intermediate state of ARNT PAS-B Y456T is  
 322 only transiently visited during interconversion (30). We validated this assumption by establishing  
 323 that the sum population of the two states remained constant at pressures below 2500 bar,  
 324 suggesting the intermediate is not accumulating (**Fig. S6**). In the two-state transition model, the  
 325 apparent exchange rate is the sum of the two rate constants corresponding to the individual  
 326 transitions from SLIP to WT and vice-versa ( $k_{app} = k_{ws} + k_{sw}$ , **Eq. 9**) (52). To obtain the pressure  
 327 dependence on both rates, we initially fit the apparent exchange rate vs. pressure to a

biexponential equation (Eq. 4), to yield the initial rates at 1 bar ( $k_{sw0}$  and  $k_{ws0}$ ), the activation volumes ( $\Delta V_{sw}^\ddagger$  and  $\Delta V_{ws}^\ddagger$ ), and the activation compressibilities ( $\Delta\beta V_{sw}^\ddagger$  and  $\Delta\beta V_{ws}^\ddagger$ ). To avoid needing to simultaneously fit six parameters, we decoupled the two rates by solving them numerically under different pressures as described in Materials and Methods (Eq. 5, 6; Fig. S7, S8). This allowed us to independently fit each rate as a function of pressure with three parameters (Eq. 7, 8; Fig. 3A, B), summarized in Table 1.

**Table 1. Summary of the kinetic and thermodynamic parameters derived from pressure-induced interconversion and unfolding of ARNT PAS-B variants**

| Thermodynamic Analyses               |                       | Kinetic Analyses                             |                       |
|--------------------------------------|-----------------------|----------------------------------------------|-----------------------|
| SLIP – WT, step jumps <sup>a</sup>   |                       | with $\Delta\beta V$                         |                       |
| $\Delta V^0$ (ml/mol)                | $-40.5 \pm 0.47$      | $\Delta V_{sw}^\ddagger$ (ml/mol)            | $-37.6 \pm 3.0$       |
| $\Delta\beta V^0$ (ml/(mol bar))     | $-0.0285 \pm 0.00065$ | $\Delta V_{ws}^\ddagger$ (ml/mol)            | $-82.4 \pm 7.4$       |
| SLIP – WT, direct jumps <sup>b</sup> |                       | $\Delta\beta V_{sw}^\ddagger$ (ml/(mol bar)) | $-0.00875 \pm 0.0034$ |
| $\Delta V^0$ (ml/mol)                | $-46.1 \pm 0.41$      | $\Delta\beta V_{ws}^\ddagger$ (ml/(mol bar)) | $-0.0487 \pm 0.0066$  |
| $\Delta\beta V^0$ (ml/(mol bar))     | $-0.0433 \pm 0.00067$ | without $\Delta\beta V$                      |                       |
| Unfolding (U - F)                    |                       | $\Delta V_{sw}^\ddagger$ (ml/mol)            | $-32.8 \pm 0.62$      |
| WT (ml/mol)                          | $-126.3 \pm 3.1$      | $\Delta V_{ws}^\ddagger$ (ml/mol)            | $-59.0 \pm 1.38$      |
| TRIP (ml/mol)                        | $-83.3 \pm 2.3$       |                                              |                       |

Uncertainties were estimated by bootstrapping. Random noises with mean of 0 and variance of the standard error were added to the raw data and fit repeatedly for  $n \geq 30$  times to determine the 95% confidence interval. <sup>a</sup> step jumps – jumping pressure incrementally with 250 bar steps. <sup>b</sup> direct jumps – jumping pressure directly from 20 bar to various high pressures.

From these analyses, we observed negative activation volumes and compressibilities for both transitions, implying that they proceed through an intermediate state which is both smaller and less compressible than the starting conformations. Moreover, the magnitude of the volume barrier is bigger for the WT to SLIP transition, with a 44.8 ml/mol difference in activation volumes, consistent with the 40.5 ml/mol volume difference obtained from the thermodynamic

analysis. Taking the ratios of the two rate constants ( $k_{ws}/k_{sw}$ ) at different pressures yielded equilibrium constants matching experimental data (**Fig. 3C**). Since both activation volumes were negative, the transition rates were correspondingly accelerated under pressure. Interestingly, the activation volume for the WT to SLIP transition (-82.4 ml/mol, or 137 Å<sup>3</sup>/molecule) is strikingly similar to the cavity size of the WT conformation. The SLIP to WT transition, in contrast, required a much smaller activation volume (**Table 1**). While the pressure-induced volume change of a system is a combined effect from compressing both protein and solvent (20), we found a positive correlation between cavity size and activation volume. From these data, we postulate the transition between the two conformations requires the protein to collapse its internal cavities and voids to reduce its total volume, consistent with substantial unfolding that we quantitate below. This claim is further supported by Roche *et al.*'s (53) recent work highlighting that pressure-driven protein unfolding is a result of cavity elimination.

Additional support for an unfolded transition state is provided by the negative activation compressibilities, as the replacement of interatomic contacts with hydration shells during unfolding will substantially reduce compressibility (54). Notably, as compressibility is small and neglectable at lower pressures (18), we were able to fit the sub-1000 bar data points to a model without compressibility, leading to the same observation that the SLIP conformation is smaller than the WT conformation, albeit to a lesser extent (**Fig. 3B**; **Table 1**).

### **Pressure Induced Interconversion is Reversible and Provides Thermodynamic Information on Transition State**

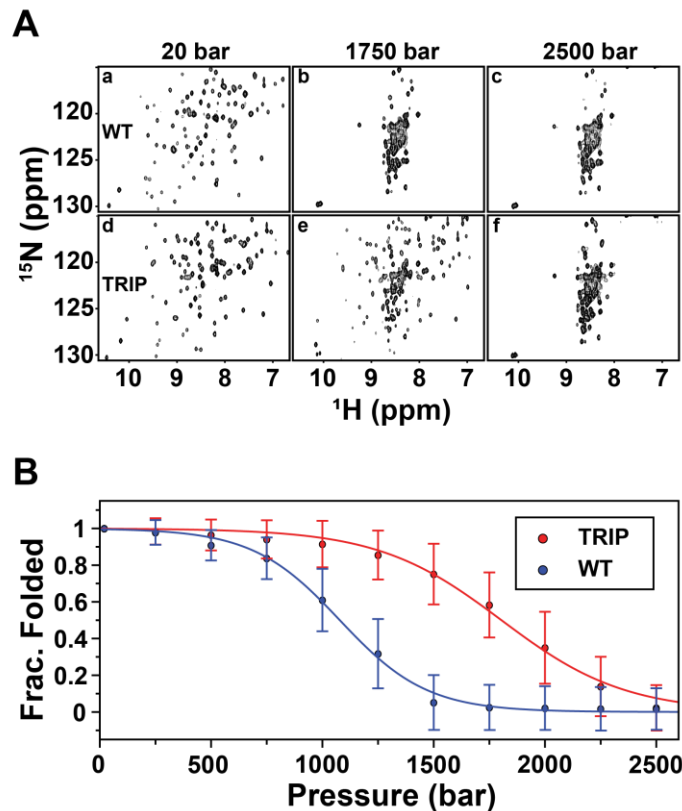
To validate the reversibility of the pressure-jump experiments, we recorded <sup>13</sup>C-edited 1D <sup>1</sup>H NMR spectra as samples were dropped from higher pressures down to 20 bar. From these data, we extracted the two rate constants  $k_{ws}$  and  $k_{sw}$  and plotted them against the pressures prior

to the relaxation (**Fig. 3D**). For all of the pressures we used for extrapolating the rates (from 125 to 1500 bar), the relaxation rates remained the same and the corresponding [SLIP]/[WT] equilibrium constants were the same as we measured in equilibrium studies at 20 bar. These results, together with our comparison of  $^{15}\text{N}/^1\text{H}$  HSQC spectra recorded before and after pressure-jump experiments (**Fig. S2**), provide important experimental controls by establishing that this system is both thermodynamically and kinetically reversible up to at least 1500 bar and that interconversion rates are solely determined by the applied pressure (and not susceptible to hysteresis effects of prior pressure applications).

As an additional verification of the suitability of pressure jumps for kinetic studies of this process, we compared the parameters extracted from these pressure-jumped measurements to those from an Eyring analysis of the rates of interconversion determined after chromatographic isolation of one of the two conformers (30). To do so, we equilibrated the protein at 1000 bar at four different temperatures between 278.1 and 291.1K, then relaxed the system to 20 bar and recorded  $^{13}\text{C}$ -edited 1D  $^1\text{H}$  NMR spectra to extract the apparent exchange rates from SLIP to WT (**Fig. S9**). From Eyring analysis of these data, we extracted entropic (-47.8 cal/(mol degree) = -14.2 kcal/mol at 298K) and enthalpic (7.4 kcal/mol) contributions to the activation energy, which are similar to our previously-reported results with chromatographic perturbations (30). These data both support interconversion via an unfolded transition state, as indicated by the large entropic barrier, and more generally demonstrate that the relaxation process we monitored here after pressure perturbation is the same as we previously observed with chromatographic separation (29, 30).

## Activation Volumes of the ARNT PAS-B Y456T are Comparable to the Unfolding Volumes of the WT and TRIP Variant Detected by Pressure Jump NMR

To calibrate the degree of unfolding involved in the ARNT PAS-B Y456T transition state, we compared the activation volumes of interconversion to the unfolding volumes of the WT and SLIP conformations (as adopted by the WT and TRIP variant). As neither protein substantially unfolded within our 2500 bar experimentally-accessible pressure range, we added 3 M urea to slightly lower the stability of both samples. From  $^{15}\text{N}/^1\text{H}$  HSQC spectra we acquired at different pressures in the presence of this denaturant, it was clear that all the resolved backbone peaks showed pressure-induced reductions in intensity, with no obvious peak broadening (**Fig. S10**). This was accompanied by the appearance of intense peaks with sharp linewidths and poor  $^1\text{H}$  chemical shift dispersion, consistent with pressure-induced protein unfolding that completed at approximately 1750 bar (WT) and 2500 bar (TRIP) (**Fig. 4A, S10**). By fitting the protein unfolding curves for both proteins to a two-state model (**Eq. 10, Fig. 4B**), we extracted two key parameters: the average volume difference between the folded and the unfolded protein ( $\Delta V_f$ ) and the free energy of folding ( $\Delta G_f^0$ ) (20, 55) (**Table 1**). The unfolding volumes indicate a 43.0 ml/mol difference between the two folded structures, and the folding energies predict a 2.7:1 ratio between the SLIP and the WT conformation at ambient pressure, both similar to what we observed for ARNT PAS-B Y456T (40.5 ml/mol and 1.5:1, respectively). The activation volumes of the ARNT PAS-B Y456T, which represent the volume differences between the two folded states to the intermediate state, are smaller but comparable to the unfolding volumes of the WT and TRIP variant (-82.4 ml/mol vs. -126.3 ml/mol for WT, -37.6 ml/mol vs. -83.3 ml/mol for SLIP). These data, coupled with the correlation between the interconversion and

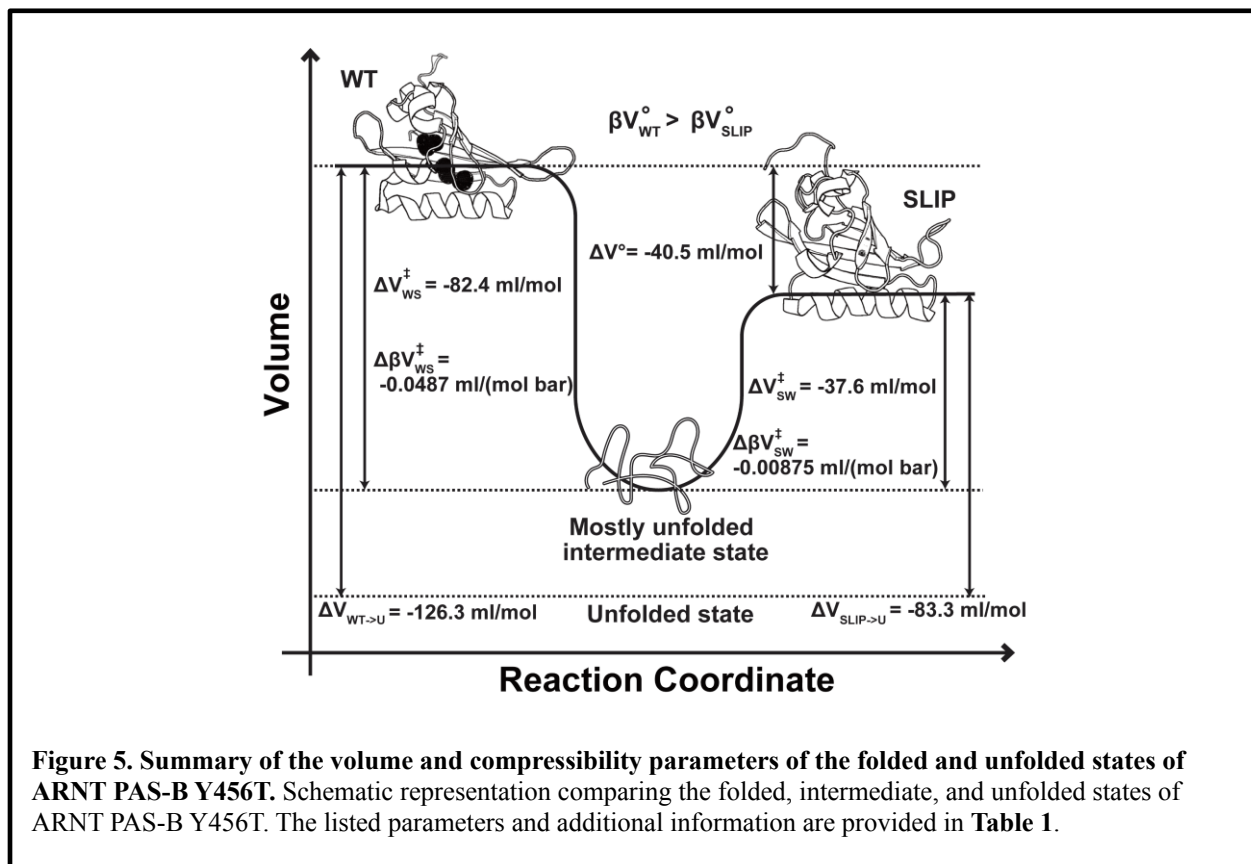


**Figure 4. Unfolding profile of ARNT PAS-B WT and TRIP mutant detected by high-pressure NMR  $^{15}\text{N}/^1\text{H}$  HSQC at 278 K and 3.0 M urea.** A) Example  $^{15}\text{N}/^1\text{H}$ -HSQC spectra of WT and TRIP at different pressures. (a-c) NMR spectra of WT at initial pressure (20 bar), intermediate pressure (1750 bar), and final pressure (2500 bar). (d-f) NMR spectra of TRIP mutant. B) Average unfolding curves for ARNT PAS-B WT and TRIP mutant. Peak intensities are normalized between 0 and 1.

denaturant-free protein unfolding rates we previously reported (30) and the need to significantly remodel at least 15 of 26 inter-strand hydrogen bonds in the beta sheet, lead us to strongly support a model where ARNT PAS-B Y456T largely unfolds as it interconverts between conformations.

We additionally compared the unfolding volumes of WT and TRIP to the unfolding volumes of similarly-sized proteins (10-20 kDa). Indeed, the numbers are in good agreement with several other proteins studied with pressure-dependent unfolding approaches, as

summarized in **Table S1** (42, 53, 56-60). A schematic figure describing the relationships among the two folded conformations, the intermediate state, and the unfolded state is shown in **Fig. 5**.



Lastly, we note that we did not include compressibility changes for the unfolding analysis of ARNT PAS-B WT and TRIP, given that these were based on global averages of signals (> 60 residues) distributed throughout both proteins which we assume to average out contributions from sites near cavities (compressibility-dependent) and those far from cavities (compressibility-independent) (18, 28).

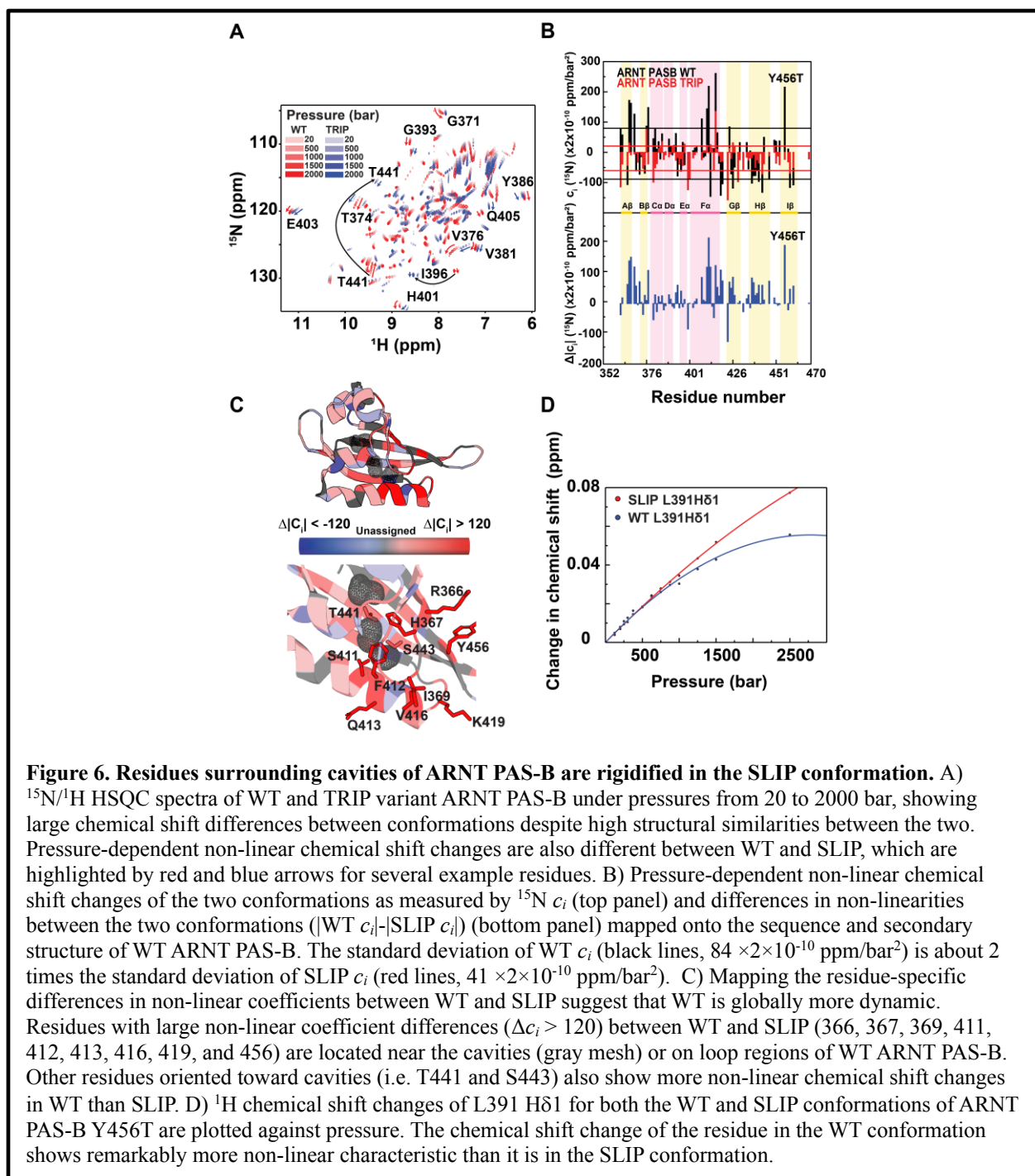
### Pressure-induced Nonlinear Chemical Shift Changes and Residue Compressibilities Predict Cavity Locations

Certain types of pressure-induced NMR chemical shift changes have been related to conformational changes within proteins (26, 27). In particular, non-linear shift changes are

423 hallmarks of such conformational changes, as identified by fitting chemical shifts to pressure  
424 using a linear and a nonlinear term (**Eq. 11**) (18). Of particular interest is the nonlinear  
425 coefficient ( $c_i$ ) of the backbone amide  $^{15}\text{N}$  and  $^1\text{H}$  nuclei, where larger values are particularly  
426 sensitive on the ability of proteins to visit multiple conformational states upon application of  
427 pressure. Such information can be analyzed in bulk, with larger average  $c_i$  values or broader  $c_i$   
428 distributions reflecting the presence of internal cavities or voids which enable protein flexibility  
429 with increased pressure (18, 26, 28, 49). At a residue-specific level, larger  $c_i$  values tend to be  
430 observed from residues located near internal cavities (18, 28).

431         To test if this trend holds for the WT and SLIP conformations of ARNT PAS-B, we  
432 acquired  $^{15}\text{N}/^1\text{H}$  HSQC spectra of both WT and TRIP samples of ARNT PAS-B at pressures up  
433 to 2000 bar (**Fig. 6A**). We fit the pressure-dependent changes in  $^{15}\text{N}$  chemical shifts of  
434 assignable residues to **Eq. 11**, using these to extract  $^{15}\text{N}$   $c_i$  values for both conformations (**Fig.**  
435 **6B**) (29, 31). While the two conformers have similar overall structures, we observed markedly  
436 different distributions of the backbone amide  $^{15}\text{N}$   $c_i$  values. Specifically, we observed a global  
437 reduction of the magnitude of  $c_i$  in the SLIP conformation, supporting the view that it has  
438 reduced internal void volume and flexibility compared to the WT conformation. We calculated  
439 the differences in nonlinear coefficients by subtracting the absolute values of  $c_i$  of the SLIP  
440 conformation from the WT conformation (**Eq. 12**). Residues with the largest  $^{15}\text{N}$   $c_i$  differences  
441 were located near the internal cavities or on loops of the WT structure (**Fig. 6C**), with many  
442 clustered into two regions of the protein (**Fig. 6B**). Intriguingly, the same clusters of residues  
443 have also been associated with the binding of KG-548 to ARNT PAS-B, disrupting interactions  
444 between ARNT PAS-B and the TACC3 co-activator (32). The cavities apparently collapse in the  
445 SLIP conformation due to the +3 shift and inversion of the I $\beta$ -strand, resulting in a more packed

446 local environment, explaining the substantial  $c_i$  reduction of these residues in the SLIP  
 447 conformation. Interestingly, residue Y456, the site of the Y456T point mutation that creates the  
 448 SLIP conformation, has one of the largest  $^{15}\text{N}$   $c_i$  difference between the two conformations.



We suspected that the residue-specific compressibility also depends on whether the residue is near a cavity. As expected, when we fit the pressure dependence of the L391 H $\delta$ 1 peak to **Eq. 11**, we obtained a larger nonlinear coefficient for the WT conformation than the SLIP conformation (**Fig. 6D**), matching the compressibility difference observed between the two states, confirming our speculation. We also compared the pressure-dependent responses of several other methyl peaks corresponding to the WT and SLIP conformations using the  $^{13}\text{C}/^1\text{H}$ -HSQC spectra of ARNT PAS-B Y456T (**Fig. S11**). We found several cavity-oriented methyl groups to have markedly larger  $^1\text{H}$  or  $^{13}\text{C}$   $c_i$  values for the WT conformation (L391  $\delta$ 2, I396  $\delta$ 1, L408  $\delta$ 1 and  $\delta$ 2, and M439  $\epsilon$ ). Correspondingly, the pressure-dependent WT to SLIP transitions were also slowed down at higher pressures as the result of negative compressibility changes, similar to L391  $\delta$ 1 (**Fig. S4**).

Since both nonlinear chemical shift changes and compressibility probe for the volume-dependent local environment a residue resides in, measuring the nonlinear coefficient of chemical shift changes and compressibility can complement each other for more accurate characterization of cavities in proteins (as has been linked mathematically in the past (61)). This is in line with the prior notion that larger compressibility is correlated with larger volume fluctuations, which are generally observed near cavities, where residues are less tightly packed (62). Remarkably, the cavities in the WT ARNT PAS-B were not initially observed in the solution structure (31), showing the potential of pressure-NMR as the means of rapidly identifying smaller cavities from moderate resolution structures where cavities and/or ligand binding pockets are not obvious (31).

## CONCLUSION

The shift of the  $\beta$ -strand register at an interaction interface enabled by a single point mutation is mechanistically intriguing and raises fundamental questions about the relative stabilities of the ground state and energetically close alternate conformers (30, 63, 64). Using 1D and 2D pressure-jump NMR experiments, we examined one such case by elucidating the volume and compressibility differences between two stably folded conformations of ARNT PAS-B which interconvert when enabled by a single point mutation, Y456T. We demonstrated that the wildtype (WT) state has a substantially larger internal volume and compressibility compared to the alternate slip conformation (SLIP), and that these differences can be largely attributed to the cavities unique to the WT state. Furthermore, we show that interconversion between the two states goes through a chiefly-unfolded intermediate state that is smaller in volume and compressibility than both folded conformations. Additionally, we found a possible connection between residue-specific compressibility and NMR nonlinear chemical shift responses to pressure that could help to predict whether residues are located close to cavities. While promising, we emphasize that a more comprehensive analysis is required to validate this hypothesis.

In summary, we have shown how varying pressure can be easily applied to reversibly shift the equilibrium of a protein between two stably folded conformations, letting us quantitatively measure structural and thermodynamic parameters otherwise difficult to access (20). We believe that this approach can be applied to other proteins which undergo large scale conformational changes, particularly with recent instrumentation advances that allow millisecond-timescale pressure jumps (23, 24). We anticipate that such studies will be

particularly useful studying systems where protein flexibility is essential, including enzymatic conformational changes, protein/ligand interactions, and metamorphic systems (9-11).

## **SUPPORTING MATERIAL**

Supplementary Table S1; Figures S1-S11.

## **AUTHOR CONTRIBUTIONS**

X.X, D.G, J.M.A, and K.H.G designed the experiments; X.X, J.M.A, and D.G performed the experiments; X.X and K.H.G. analyzed the data and wrote the manuscript.

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