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Backbone dynamics in an intramolecular prolylpeptide-SH3 complex from the diphtheria toxin repressor, DtxR

Nilakshee Bhattacharya^{1,2,3}, Myunggi Yi^{3,4}, Huan-Xiang Zhou^{3,4,5}, and Timothy M. Logan^{1,2,3,*}

¹ Dept. of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306

² National High Magnetic Field Laboratory, Tallahassee FL 32310

³ Kasha Laboratory, Florida State University, Tallahassee, FL 32306

⁴ Dept. of Physics, Florida State University, Tallahassee, FL 32306

⁵ School of Computational Sciences, Florida State University, Tallahassee, FL 32306

Summary

The diphtheria toxin repressor contains an SH3-like domain that forms an intramolecular complex with a proline-rich (Pr) peptide segment and stabilizes the inactive state of the repressor. Upon activation of DtxR by transition metals, this intramolecular complex must dissociate as the SH3 domain and Pr segment form different interactions in the active repressor. In this study we investigate the dynamics of this intramolecular complex using backbone amide nuclear spin relaxation rates determined using NMR spectroscopy and molecular dynamics trajectories. The SH3 domain in the unbound and bound states showed typical dynamics in that the secondary structures were fairly ordered with high generalized order parameters and low effective correlation times while residues in the loops connecting β -strands exhibited reduced generalized order parameters and required additional motional terms to adequately model the relaxation rates. Residues forming the Pr segment exhibited low order parameters with internal rotational correlation times on the order of 0.6 – 1 ns. Further analysis showed that the SH3 domain was rich in millisecond timescale motions while the Pr segment was rich in motions on the 100s μ s timescale. Molecular dynamics simulations indicated structural rearrangements that may contribute to the observed relaxation rates and, together with the observed relaxation rate data, suggested that the Pr segment exhibits a binding $\leftrightarrow$ unbinding equilibrium. The results of this study provide new insights into the nature of the intramolecular complex and provide a better understanding of the biological role of the SH3 domain in regulating DtxR activity.

Keywords

SH3 domain; DtxR; Lipari Szabo model-free approach; iRED; collective motions

*Corresponding Author: Dr. Timothy M. Logan, Kasha Laboratory, Florida State University, Tallahassee, Florida 32306-4380, USA, Phone: (850)-644-8979; Fax: (850)-644-7244; email: logan@sb.fsu.edu.

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Introduction

The diphtheria toxin repressor (DtxR) proteins function as the primary iron sensors that regulate iron homeostasis, oxidative stress response, and virulence in Gram positive bacteria. First identified in *Corynebacterium diphtheriae* as responsible for the Fe-sensitive expression of the diphtheria toxin^{1, 2}, DtxR homologues have been identified in Gram positive, Gram negative, and archae bacteria. DtxR contains two domains: a large, strongly conserved N-terminal domain that functions in metal binding, dimerization, and DNA binding, and a smaller, less conserved C-terminal domain that structurally resembles eukaryotic SH3 domains^{3–6}. These two domains are connected by a flexible proline-rich (Pr) peptide segment. In the active metal-bound repressor, the Pr segment contacts and stabilizes the N-terminal domain helices that form the dimer interface. In the inactive metal-free repressor, the Pr segment is bound to the SH3 domain, forming an intramolecular complex⁷ (PrSH3) (Figure 1) that resists reactivation of the inactive repressor by stabilizing the monomeric form of the protein. Consequently, a key step in the activation of DtxR by divalent transition metal ions must involve dissociation of the Pr segment from the PrSH3 intramolecular complex. Subsequent binding of the Pr segment to the N domain contributes to the stability of the activated repressor. In this sense, the Pr segment functions as a molecular switch - binding to the SH3 domain represents the “off” state while binding to the N domain represents the “on” state. This switch hypothesis requires that the Pr segment be relatively weakly bound to the SH3 domain to compete with the micromolar metal binding affinity^{8; 9} of the N terminal domain.

A previous study of the PrSH3 intramolecular complex⁷ suggests that the Pr segment is, indeed, weakly bound. Here, we use ¹⁵N-NMR based relaxation measurements and molecular dynamics simulations to probe backbone motions of the PrSH3 intramolecular complex. The PrSH3 construct corresponds to residues D110-L226 of DtxR (Figure 1). Residues P148-L226 form the SH3 domain. Residues R125-L135 interact most strongly with the SH3 domain and represent the intramolecular proline-rich ligand (the Pr segment). Residues D136-A147 form a disordered loop that tethers the Pr segment to the SH3 domain. Residues D110-S124 are referred to as the N-terminal extension. Residues V117-L120 form a single turn α -helix that interacts with $\beta 2'$ in the SH3 domain. Motions in the complex were compared to those in the unbound SH3 domain, which was formed by truncation of the PrSH3 construct, leaving residues D144-L226, of which P148-L226 form the SH3 domain proper (Figure 1).⁷ We find the Pr segment to be particularly rich in motions on multiple NMR-sensitive timescales. In addition, we see Pr segment binding-dependent changes in the SH3 domain backbone dynamics. The nature of the backbone motions would contribute to a weakly stabilized intramolecular complex, consistent with the proposed role for this complex in DtxR.

Results

¹⁵N Relaxation Measurements for SH3

Complete R_1 , R_2 , and NOE data sets were collected at 14.1 and 16.9 T magnetic fields for 73 of the 83 amino acid residues in this construct (Figure 2 and Supplementary Figure 1, respectively). NOE values at 16.9 T showed high variability and, in several cases, exceeded the theoretical maximum for this magnetic field and were not considered further (relaxation rates and heteronuclear NOE values for SH3 have been deposited with the BioMagResBank, accession number 15255). Besides two proline residues (P148 and P160), resonance overlap precluded the interpretation of relaxation data for eight other residues (D144, A146, A156, Q167, D177, E194, N207, and R222). The average relaxation values were $R_1 = 2.18 \pm 0.04 \text{ s}^{-1}$ ($1.93 \pm 0.09 \text{ s}^{-1}$), $R_2 = 7.19 \pm 0.6 \text{ s}^{-1}$ ($8.97 \pm 0.90 \text{ s}^{-1}$) and $\text{NOE} = 0.66 \pm 0.02$ at 14.1 T (16.9T). Higher than average R_1 values were seen for loop regions and for several residues preceding $\alpha 2$, indicating that these regions exhibit nano-second motions. Several residues (I153, D154, L182, A185, D186, I187, I195, and S205) exhibited elevated R_2 values and

increased R_2/R_1 ratios (Figure 2). Most of the residues in the loop preceding $\alpha 2$ (E170-T176 and Q178) showed low NOEs, as did residues preceding $\beta 3$ (D199-H201), indicating higher frequency motions for these residues.

^{15}N Relaxation Measurements for PrSH3

Complete backbone R_1 and R_2 relaxation data were obtained for 92 of the 116 residues in PrSH3 at both fields (Figure 2 and Supplementary Figure 1, respectively). Heteronuclear NOE data were collected at both fields, but the NOE data from the higher field were not used in subsequent analysis (relaxation rates and heteronuclear NOE values for PrSH3 have been deposited with the BioMagResBank, accession number 15254). Relaxation rates for residues in the SH3 domain of PrSH3 were similar to that of SH3, with average values of $R_1 = 1.83 \pm 0.07 \text{ s}^{-1}$ ($1.43 \pm 0.08 \text{ s}^{-1}$), $R_2 = 9.0 \pm 0.7 \text{ s}^{-1}$ ($10.1 \pm 0.9 \text{ s}^{-1}$) and $\text{NOE} = 0.77 \pm 0.05$ at 14.1 T (16.9 T). Several residues (Q181, R188, V196, and D210) exhibited lower NOE values in PrSH3 compared to the same residues in SH3. Interestingly, the relaxation rates for residues preceding the SH3 domain differed from those forming the SH3 domain in PrSH3 (Figure 2). While the average R_1 for residues D110-A147 was approximately the same as that of the SH3 domain, the average R_2 and NOE were substantially lower than for SH3 domain residues.

Concentration effects were investigated by comparing the R_2 values from a $\nu\text{CPMG}=0$ experiment at 1 mM, 0.5 mM, and 0.1 mM at 14.1 T. ANOVA analysis indicated there was no difference between the mean and distribution of R_2 values at these three concentrations. There was a slight difference in R_2 values for residues L125-140 (~10% lower at 0.1 mM versus 1 mM) and 144-161 (~10% higher at the lowest concentration), but these differences were similar in magnitude to the uncertainty in the R_2 measurement at the lowest concentration.

Reduced Spectral Density Mapping

The reduced spectral density functions $J_{\text{eff}}(0)$, $J(\omega_{\text{N}})$, and $J(0.87\omega_{\text{H}})$ were calculated for each residue in SH3 and PrSH3 using relaxation rates and NOE values from 14.1 T (Figure 3 and Supplementary Tables 1 and 2, respectively). Figure 3 compares $J_{\text{eff}}(0)$, $J(\omega_{\text{N}})$, and $J(0.87\omega_{\text{H}})$ for PrSH3 and SH3. $J(\omega_{\text{N}})$ is nearly uniform for all PrSH3 residues, with an average value of ~0.3 ns/rad. $J(\omega_{\text{N}})$ reflects the spectral power for frequencies that contribute significantly to longitudinal relaxation, and consequently shows the same insensitivity to residue position as R_1 . In contrast, $J_{\text{eff}}(0)$ and $J(0.87\omega_{\text{H}})$ indicate clear motional differences between residues of the Pr segment and those of the SH3 domain. Residues preceding P148 show lower $J_{\text{eff}}(0)$ and higher $J(0.87\omega_{\text{H}})$ than residues within the SH3 domain (Figure 3), indicating that slower motions contribute significantly to relaxation in the SH3 domain of PrSH3 while faster motions dominate relaxation for residues of the Pr segment. Within the SH3 domain, residues E170-Q178 and Q181, R188, V196 and D210, which contact the Pr segment, generally showed elevated $J(0.87\omega_{\text{H}})$ and reduced $J_{\text{eff}}(0)$ values compared to the rest of the SH3 domain.

Model-free analysis of backbone motions in SH3

Relaxation rates from two fields and NOE values from one field were used to estimate the diffusion tensor for SH3 using a two-step filtering procedure¹⁰ combined with an additional filtering as described by van Doren¹¹. Residues that satisfied these criteria (33 residues out of 73) were used to characterize the diffusion tensor. The relative moments of inertia for the SH3 construct were calculated to be 1.0:0.85:0.67, suggesting an axial model for the protein. The axial model was chosen over isotropic and anisotropic (or axially asymmetric) models for the SH3 construct based on a comparison of reduced χ^2 and F-statistic values, yielding a $D_{\parallel}/D_{\perp}$ ratio and global rotational correlation time (τ_c) of 1.36 and 4.71 ns, respectively (Table 1).

The internal motion parameters for 73 residues of SH3 were analyzed by Modelfree¹² using relaxation rates from two magnetic fields (14.1 and 16.9 T) and NOE data at 14.1T. SH3 residues were most frequently fitted to model LS-2 (29 of 73 residues; Supplementary Table 3). Thirteen residues were fitted to LS-1, 5 to LS-3, 19 to LS-4, and 4 to LS-5. Three residues (N169, H216, and H219) could not be fitted to any model. Following model selection, Modelfree parameters for SH3 were optimized using an axially symmetrical diffusion tensor, yielding $\tau_c = 4.76$ ns and $D_{\parallel}/D_{\perp} = 1.38$. The results for SH3 are shown in Figure 4. The average generalized order parameter, $\langle S^2 \rangle$, for the backbone was 0.85 ± 0.05 , ignoring residues with high error and unreliable parameters (see below). For the two helical regions, $\langle S^2 \rangle$ was 0.88 ± 0.026 (α_1) and 0.91 ± 0.03 (α_2), respectively, and $\langle S^2 \rangle$ was 0.88 ± 0.026 (β_1), 0.85 ± 0.025 (β_2), 0.86 ± 0.026 (β_2'), 0.86 ± 0.026 (β_3), 0.83 ± 0.025 (β_4) and 0.88 ± 0.025 (β_5) for the five β -strands.

Modelfree analysis for PrSH3

Relaxation rates (14.1 T and 16.9T) and NOE values (14.1T) were used to estimate the diffusion tensor for PrSH3 as outlined above. The relative moments of inertia for the Pr-SH3 construct were 1.0:0.86:0.66. An axially symmetric diffusion model was identified for the PrSH3 construct using the 26 residues that survived the coarse and fine filters. Binding the Pr segment changes the shape of the protein, making it less axially symmetric than the free SH3 domain. Initial estimates for $D_{\parallel}/D_{\perp}$ (1.21) and τ_c (6.5 ns) were obtained (Table 1) and used as input for Modelfree calculations.

Of the 98 PrSH3 residues that were analyzed by Modelfree, 11 were fitted to LS-1, 47 to LS-2, 3 to LS-3, 13 to LS-4, and 20 to LS-5 (Figure 4 and Supplementary Table 4). Four residues could not be fitted to any of the 5 traditional Modelfree models (F128, N142, D144, and N169). A final optimization for all residues assigned motional models yielded $\tau_c = 6.52$ ns and $D_{\parallel}/D_{\perp} = 1.19$. The $\langle S^2 \rangle$ for residues P148-L226 of the PrSH3 intramolecular complex was 0.83 ± 0.06 . $\langle S^2 \rangle$ for the two helical regions in PrSH3 was 0.89 ± 0.03 (α_1) and 0.81 ± 0.04 (α_2) respectively, and, for the five β -strands, was 0.89 ± 0.03 (β_1), 0.80 ± 0.04 (β_2), 0.80 ± 0.04 (β_2'), 0.85 ± 0.10 (β_3), 0.76 ± 0.04 (β_4) and 0.89 ± 0.03 (β_5). The order parameters show β_4 to be the most flexible of the secondary structures, followed by β_2 , β_2' , and α_2 . The majority of residues forming secondary structures have internal correlation times $\tau_e \sim 33$ –160 ps except for residues some of the residues at the ends of the secondary structure elements (K162, E192, and V196) had $\tau_e \sim 1.4$ –2.2 ns (Figure 4). High τ_e values were also obtained for residues I171-F179 and other residues belonging to loops in the SH3 domain. The loop within residues A185-S191 showed $\tau_e \sim 35$ –210 ps, except for R188 ($\tau_e = 1.1$ ns). Residues assigned to LS-3 or LS-4 generally had R_{ex} values in the ~ 1 –7 Hz range (Figure 4). Residue I171 had $R_{ex} = 0.013$ s⁻¹. This residue is assigned to LS-4 but the statistical certainty of this assignment instead of LS-2 is rather low and it may be that neither model adequately describes the motions of this residue. Residues D110-A147, which include the Pr segment (R125-G135) and precede the SH3 domain of PrSH3, were fitted to LS-2 or LS-5, except for K118 and L120, which were fitted to LS-4. The average order parameter for D110-A147 is $\langle S^2 \rangle = 0.31 \pm 0.07$. The internal correlation time for many of these residues was high, with $\tau_e \sim 0.64$ –1.04 ns.

This filtering and model assignment strategy allowed model assignment for nearly all residues in both SH3 and PrSH3. Still, there were several residues for which Modelfree failed to adequately describe the relaxation rates. For example, S158 was consistently assigned to LS-3 in SH3, but gave $S^2 = 1$ with a very small R_{ex} value. In PrSH3, this residue was assigned to LS-2, but was refined to give a large τ_e and associated error. Clearly, neither LS-2 or LS-3 appear to adequately describe the motions in this residue, nor could it be assigned to LS-4 or LS-5, even with the AIC filtering¹³ of the reduced χ^2 values. In addition, A218 and I223 (both assigned to LS-1) also showed $S^2 \rightarrow 1$. S^2 values approaching unity have been observed in

other SH3 domains^{14; 15} and in other proteins.^{11; 16; 17} When this happens, the generalized order parameter loses sensitivity to the observed relaxation rates¹⁸ and becomes less reliable.

Other residues assigned to motional models yielded non-sensible internal motional values. R161, assigned to LS-5 in PrSH3, consistently gave τ_e at the maximal value allowed in the refinement (up to $\tau_e = 10,000$ ps in one refinement). K162, E192, V196, H201, D215, and D216 gave large τ_e values ($\tau_e > \sim 1000$ ps, generally with very large errors) in PrSH3; G208, D212, and D215 exhibited similar behavior in SH3. Motion on this timescale introduces substantial error into the model-free calculation and precludes a quantitative interpretation of the fitted parameters^{19–21}. The axial diffusion tensor used to model the relaxation values can introduce spurious high-frequency motions²². However, the PrSH3 domain, for which most of these residues were identified, was *less* axially symmetric than SH3 and refinement using an isotropic diffusion tensor yielded essentially identical results (not shown). Since many of these residues exhibit internal motions on a timescale approaching that of the global rotational correlation time, it is likely that global and internal motions may not be separable in this protein.

Millisecond Backbone Motions in PrSH3

The preceding spectral density and Modelfree analysis of the relaxation data indicated the presence of slow, millisecond time scale motions for many residues of the SH3 domain in PrSH3, whereas residues of the Pr segment generally showed faster motion. The extent of millisecond motions in PrSH3 was investigated by the difference in transverse relaxation rates, ΔR_2 , determined from CPMG experiments using high and low effective magnetic fields²³, a simplified version of the relaxation dispersion experiment²⁴. Figure 5A plots the ΔR_2 values (14.1 T) for residues in PrSH3 for which this measurement could be made. The data range from a low of -0.53 to a high of 6.07 s^{-1} . The average ΔR_2 value was $2.46 \pm 1.35 \text{ s}^{-1}$; the average was higher for residues of the SH3 domain ($2.89 \pm 1.35 \text{ s}^{-1}$) than for residues preceding the SH3 domain ($1.29 \pm 1.0 \text{ s}^{-1}$). A conservative estimate for the distribution of ΔR_2 values suggests that $\Delta R_2 < 2.18 \text{ s}^{-1}$ or $\Delta R_2 > 2.75 \text{ s}^{-1}$ are significantly different from the mean (2.46 s^{-1}). Using this criterion, residues in most of the secondary structures show millisecond backbone motions. On the other hand, with the exception of L120, residues of the Pr segment and residues E170-F179 and A185-G190 show little motion on this timescale.

Microsecond Backbone Motions in PrSH3

The extent of microsecond motions was investigated by determining the $R_2/R_{1\rho}$ ratio (see BioMagResBank submission for $R_{1\rho}$ values). In the absence of conformational exchange, R_2 and $R_{1\rho}$ should be equal. Therefore, $R_2/R_{1\rho}$ values close to unity indicate an absence of motions in the $100\text{'s } \mu\text{s}$ time scale, whereas values larger than unity indicate the presence of motion on this time scale¹⁴. As shown in Figure 5B, most of the SH3 domain residues show ratios close to unity ($\langle R_2/R_{1\rho} \rangle = 1.01 \pm 0.01$ for SH3 domain residues), indicating little motion on the $\sim 100 \mu\text{s}$ time scale. $R_2/R_{1\rho}$ values > 1 were obtained for several residues (S124, N130, G149, I153, I195, G200, I202, G208, E212, and I223). Residues G200, I202, and G208 either contact or are near residues of the Pr segment, which may explain their elevated $R_2/R_{1\rho}$ values. Finally, the $R_2/R_{1\rho}$ ratio theoretically cannot be less than unity, yet we clearly see $R_2/R_{1\rho} < 1$ for V119, I132, R188, and A218. This may arise from incomplete suppression of conformational exchange at the effective spin-lock field strength used in the $R_{1\rho}$ experiment or from incomplete suppression of CSA – dipole-dipole cross-correlation effects in the R_2 and $R_{1\rho}$ pulse sequences.¹⁴

MD Simulations of PrSH3 and SH3

Molecular dynamics simulations (~ 50 ns) were run for both PrSH3 and SH3. Backbone root-mean-square-deviations of all β -strands were 0.70 and $1.27 \text{ \AA}$, respectively, for the two proteins from their starting NMR structures. Both simulations showed extensive motions of the SH3

domain, including an open-close motion for the residues forming the omega-shaped loop that contains $\alpha 2$ (Q178-D184) and corresponds to the RT loop of eukaryotic SH3 domains. This motion is depicted in Figure 6A, which plots for both proteins the distance between the C_{α} atoms of L182 (in $\alpha 2$) and D210 (in $\beta 4$) along the trajectory. These two residues are located across the top of the Pr segment binding pocket. In the PrSH3 construct the C-terminal end of $\alpha 2$ appeared to be anchored to the Pr segment at the early stages of the trajectory. At about 16 ns the distance between L182 and D210 increased abruptly from 17 to ~ 27 Å and then quickly returned to ~ 17 Å for the remainder of the trajectory. A representative structure of the open form of the PrSH3 construct occurring at 16 ns is shown in Figure 6B. Similar movements of $\alpha 2$ were also observed in the trajectory of the SH3 construct (Figure 6B). The Pr segment of PrSH3 showed extensive flexibility during the 50 ns simulation but remained bound to the SH3 domain.

The trajectories were used to calculate R_1 , R_2 , and NOE25. After scaling the global correlation time by fitting to the 14.1 T experimental data, the calculated relaxation rates and NOE values reproduced the experimental results for residues in the SH3 domain of PrSH3 quite well, although there were exceptions (Figure 7; modeled relaxation rates for the 16.9 T data are shown in Supplementary Figure 2). The modeled R_1 rates captured the elevated R_1 relaxation rates for residues D177 - Q181, but underestimated the rates for L214 - I221 (Figure 7A). The modeled R_2 rates for residues Q173 - T176 were in good agreement with the observed values, but underestimated the observed values for residues I153 - R161, A185 - S191, and G200 - T203 (Figure 7B). NOE values for residues following $\alpha 2$ (A185-S191) were underestimated by the MD results. Model-free analysis required R_{ex} to fit the relaxation data for many of these residues and the MD simulations would not be able to pick up these slower motions. Finally, R_2 and NOE values from the MD trajectory overestimated the experimental values for residues preceding the SH3 domain. However, they could be reconciled with the experimental data by assuming an equilibrium involving bound and unbound forms (see below). R_1 , R_2 , and NOE values calculated from the MD trajectory for SH3 matched the experimental results quite well (not shown).

Discussion

Backbone Dynamics in the Bound and Free SH3 Domain

In the following, we refer to the SH3 construct as the “free” or “unbound” form (grey symbols in Figures 2–5) and the PrSH3 construct as the “bound” form (black symbols). An objective for this research was to determine how backbone dynamics are affected by intramolecular complex formation. In general, our results show that the backbone dynamics of SH3 domain residues is fairly similar in the bound and free forms, but there are some interesting and informative differences.

Residues forming the SH3 domain of the SH3 and PrSH3 constructs show uniform NOE values, with lower values seen for residues in the omega loop (N169-L182) and the $\beta 2'$ - $\beta 3$ loop (R198-I202). Within these two regions, residues from PrSH3 show slightly lower NOE values than residues in SH3. Ligand binding also lowered the NOE value for residues Q173, R188, and D210, suggesting that complex formation increased the high frequency motions in these residues.

The R_1 rates for SH3 and PrSH3 were generally well correlated (squared correlation coefficient to a straight line was 0.85) with the largest differences falling in the omega loop. Specifically, R_1 was consistently lower for residues preceding $\alpha 2$ in SH3 than in PrSH3, suggesting that interactions between these residues and the β -strands present in SH3 may be lost in PrSH3. There are more subtle differences in the R_2 values. As shown in Figure 2B, R_2 for SH3 residues of SH3 are fairly constant, with increases immediately preceding $\alpha 1$, $\alpha 2$, and at the end of $\beta 3$.

SH3 residues in PrSH3 show similar trends, but R_2 values in PrSH3 are also elevated for residues preceding $\beta 1$ and following $\alpha 2$ compared to the SH3 domain. Interestingly, the distribution of R_2 values in PrSH3 differs from that in SH3. R_2 values in SH3 are sharply peaked at the mean with a few extreme values whereas the PrSH3 R_2 values assume a more normal distribution (excess kurtosis value = 3.9 versus 5.0 for PrSH3 and SH3, respectively).

In terms of the Modelfree parameterization of the backbone motions, τ_e partitioned roughly into two groups. For most residues of both SH3 and PrSH3, $\tau_e < 100$ ps, but several residues preceding $\alpha 2$ in the omega loop and for several residues in the loops and turns connecting β -strands in PrSH3 showed $\tau_e \sim 1000$ ps or higher (note the logarithmic ordinate scale in Figure 4A). On the other hand, fewer residues in SH3 show τ_e of this magnitude. Slower motions, as reflected in R_{ex} , are found throughout the backbone of both SH3 and PrSH3. Residues with particularly high R_{ex} values include I153 (preceding $\alpha 1$), L182 and A185-I187 (after $\alpha 2$, respectively). However, R_{ex} terms are required to model the relaxation rates of 24 residues in SH3 compared to only 14 in PrSH3, suggesting that intramolecular complex formation substantially reduces low-frequency motions in the SH3 domain.

Dynamic Behavior of the Pr Segment

Given the extensive ligand-SH3 contacts in the PrSH3 structure⁷ we expected the Pr segment relaxation rates to approximate those of the SH3 domain residues. However, the observed data differed dramatically from this expectation. Specifically, the R_1 (and $J(\omega_N)$) values of the Pr segment are similar to those of the SH3 domain, implying that the backbone of the Pr segment exhibits overall motion on a timescale similar to the backbone residues of the SH3 domain. In contrast, the R_2 ($J_{eff}(0)$) and NOE ($J(0.87\omega_H)$) values differ significantly for the two parts of the PrSH3 construct, suggesting that the Pr segment backbone is nearly as flexible as the backbone of a disordered protein^{17; 26}.

How can we reconcile this paradoxical motional picture? As is well known, R_1 and R_2 exhibit different dependencies on the rotational correlation time, τ_c . R_1 first increases and then decreases with τ_c with a maximum rate that depends on the external magnetic field and $\tau_{eff} = (\tau_e \tau_c) / (\tau_e + \tau_c)$. R_2 approximates R_1 in the extreme narrowing regime but rises approximately linearly as τ_c increases (Figure 8). Proteins typically fall on the high τ_c side of this curve, especially at external magnetic fields above 14.1 T²⁷ and show R_2/R_1 ratios that are much larger than unity. We propose that residues of the SH3 domain of PrSH3 occur on the high τ_c portion of this maximum while the residues of the Pr segment are either closer to the maximum or fall on the low τ_c portion of the curve.

The average spectral density values were used to calculate τ_{eff} for the Pr segment and the SH3 domain separately as²⁸

$$\tau_{eff} = \omega_N^{-1} \left(\frac{\langle J_{eff}(0) \rangle - \langle J(\omega_N) \rangle}{\langle J(\omega_N) \rangle} \right)^{1/2}, \quad [7]$$

yielding $\tau_{eff} \sim 2.2$ ns and $\tau_{eff} \sim 3.8$ ns, respectively, which are to be compared with $\tau_c = 6.5$ ns for PrSH3 calculated from Modelfree. As readily appreciated from Figure 8, changing the τ_c by ± 2 ns from 4.5 ns results in only minor changes in R_1 whereas the same change in τ_c results in substantial changes in R_2 . For residues of the SH3 domain having $\tau_{eff} \sim 3.8$ ns, R_1 and R_2 differ by $\sim 3.1 \text{ s}^{-1}$ (Table 2 and Figure 8), whereas for residues of the Pr segment, with $\tau_{eff} \sim 2.2$ ns, these two values are much closer ($\sim 0.4 \text{ s}^{-1}$), which models the observed differences in R_1 and R_2 for these two regions. The relaxation rates in Table 2 and Figure 8 do not precisely reproduce the experimental values due to the assumptions inherent in calculating the relaxation rates of an isotropically tumbling particle. Introducing axial symmetry in the rotational

diffusion tensor would increase R_1 for prolate ellipses without significantly altering the overall shape or conclusions drawn here; the effect is smaller still for oblate ellipses²⁹. Therefore, these results illustrate the general idea that the Pr segment can exhibit motions with an effective correlation time that differs from the SH3 domain.

Timescales of Motions in the Pr Segment

The PrSH3 relaxation data suggests high flexibility for all residues preceding the SH3 domain; indeed each of these residues is completely exchanged with deuterons within 10 minutes after dissolution in deuterated buffer (not shown). To further define the kinds of motions that may be occurring, we probed the motions of PrSH3 on two different time scales (Figure 5). First, motions on the low millisecond time scale was determined as the difference in R_2 (ΔR_2) values measured in a refocusing pulse-train with $\nu_{\text{CPMG}} \sim 1000$ Hz and $\nu_{\text{CPMG}} \sim 0$ ³⁰. This measurement can be viewed as a limiting case of relaxation dispersion analysis performed using a more extensive range of refocusing fields^{31–34}. In principle, ΔR_2 is similar to the R_{ex} obtained for residues fitted to LS-3 in Modelfree. However, the ΔR_2 measurement has the advantage of being a primary result that does not depend on a specific motional model for fitting, which is the case for R_{ex} . The ΔR_2 values (Figure 5A) show that SH3 domain residues of PrSH3 exhibit extensive motions on the millisecond timescale whereas the Pr segment does not. Comparison of Figure 3A and 5A shows a close correspondence in the absolute values of the exchange rate and in the dependence on residue position along the sequence. For example, residues in $\alpha 1$ require R_{ex} in Modelfree and also show high ΔR_2 values. Similar correspondence is seen for residues in $\alpha 2$ and in the turns between β -strands.

Second, motions on a slightly faster timescale were investigated using the $R_2/R_{1\rho}$ ratio. When R_2 is measured using $\nu_{\text{CPMG}} \sim 1500$ Hz, this ratio is sensitive to motions on the ~ 100 μ s time scale.²³ $R_2/R_{1\rho}$ values near unity indicate little motion on this time scale while values larger than unity more extensive motions. As shown in Figure 5B, the SH3 domain of PrSH3 shows little motion on this timescale, with the exception of a few loops, whereas the entire Pr segment of PrSH3 shows large deviations from unity, indicating the presence of extensive motions on this timescale.

These additional relaxation measurements suggest that SH3 domain residues exhibit a slow “breathing” motion while Pr segment residues undergo more rapid fluctuations. In the following, we use the MD trajectories to speculate on two limiting motions: an equilibrium between bound and flexible unbound states (binding equilibrium) or an ensemble of rapidly interconverting bound states (bound ensemble).

“Binding equilibrium” model

If the Pr segment was sampling both bound and unbound states during the relaxation measurement, we would expect the observed relaxation parameters to reflect a time- or population-weighted average of the relaxation of the two limiting states. As the Pr segment did not dissociate from the binding pocket during the MD simulation of the PrSH3 construct, we assume this represents the hypothetical “100%” bound state (R_2^{bnd}). R_2 for the hypothetical “0%” bound state (R_2^{free}) were obtained from an MD simulation of a peptide corresponding to residues L120-V140, which contains the Pr segment. The global correlation time was rescaled to match with experimental R_2 for ^{15}N -L135 and ^{15}N -L138 measured on the same peptide. We assume a linear change of R_2 between the bound and unbound states and estimate the fraction bound as³⁵

$$f_b = \frac{(R_2^{obs} - R_2^{free})}{(R_2^{bnd} - R_2^{free})} \quad [5]$$

Similar equations can be written for NOE and R_1 , but the relative insensitivity of R_1 to residue position rendered R_1 useless in calculating the fraction bound. Combining the results for R_2 and NOE yielded an average value of ~ 30% for the bound fraction. This number is remarkably similar to the generalized order parameters obtained for residues of the Pr segment assuming $S^2 \rightarrow 0$ and $S^2 \rightarrow 1$ for an unbound and bound Pr segment, respectively. As shown in Figure 7, R_1 , R_2 , and NOE values reconstructed according to eq. [5] for residues L120 to V140 agreed well with experimental results. This low value for the bound fraction may explain the low number and weak intensity of the ligand-protein ^1H - ^1H NOEs measured for the intramolecular complex⁷.

Evidence of the flexible, unbound nature of the Pr segment also comes from the reduced spectral densities. For example, $J(0.87\omega_H)$ is generally higher in unfolded drk than in the folded form26; $J(0.87\omega_H)$ for the Pr segment is roughly five times larger than for residues in the SH3 domain. Similarly, compared to regular secondary structures in eglin c, $J(0)$ was lower by ~ 33% in mobile loops and lower by ~ 66% in the disordered N terminus³⁶; $J_{eff}(0)$ for the Pr segment of PrSH3 is roughly 60% lower than in the SH3 domain. Thus, the reduced spectral densities indicate that the Pr segment is not completely disordered, nor is it as ordered as the SH3 domain.

Motions in the bound state

The MD simulations indicated motions within the SH3 domain that could affect the motions of the bound Pr segment. In particular, the omega loop underwent significant excursions during the simulation, resulting more “open” conformations (Figure 6B). This opening motion occurred in simulations of PrSH3 and SH3, suggesting that it may be intrinsic to this SH3 domain. A flexible omega loop would generate an open binding site and allow the bound Pr segment to adopt an ensemble of bound structures that interconvert on the μs time scale, consistent with the $R_2/R_{1\rho}$ values (Figure 5B).

In summary, the relaxation data and MD simulations suggest that the SH3 domain has an intrinsic flexibility associated with it. In particular, the omega loop appears to undergo “flapping” motions that 15 generate a transiently open binding pocket into which the Pr segment can bind. If flap opening is a rare event, the binding affinity of the Pr segment would be low. Tethering the Pr segment to the SH3 domain would increase the probability that the Pr segment is positioned near the binding pocket, thereby increasing the effective binding affinity^{7; 37}. The relaxation data suggests that ligand binding reduces the long time scale motions of the SH3 domain while increasing faster motions. We can now interpret this in terms of a flexible binding pocket that experiences a restriction in conformational space upon ligand binding, resulting in a redistribution of the motions towards higher-frequency motions.

It is interesting to ask whether the motions of the Pr segment are coupled to that of the SH3 domain or whether they occur independently. According to Brüschweiler,²⁵ correlated motions can be identified from the eigenmodes of the covariance matrix for inter-residue fluctuations along the MD trajectory. The first five eigenmodes are associated with global reorientation and are essentially irrelevant to this discussion. However, we note in passing that the collectivity, κ , which is a measure of the percentage of residue interactions that contribute to a given eigenmode, shows a nearly continuous distribution of eigenvalues for PrSH3 (Figure 9), and the separability index, g , for this trajectory is 2.35. A large separability index indicates complete separation of internal and global motions. By comparison with PrSH3, partially

folded ubiquitin has a separability index of 9.³⁸ This suggests that the internal and external motions of PrSH3 are not completely separable, in agreement with analysis of the NMR-derived relaxation rates. Beyond the global tumbling modes, each of the subsequent eigenmodes can be analyzed for spin interactions that contribute significantly to that mode. For example, the only residues with significant amplitudes for eigenmode 7 are E212, L213, and D215, indicating that this eigenmode represents motion of the loop between $\beta 4$ and $\beta 5$ (Figure 10A, C). In contrast, eigenmode 9 arises from collective motion of residues in the omega loop, the Pr segment and residues forming the other side of the ligand binding pocket (Figure 10B, D). Other eigenmodes also suggest collective motions. For example, eigenmode 8 shows correlated motions involving residues following $\beta 4$ and in $\alpha 1$ that are consistent with the high τ_e values seen for these latter residues in the Modelfree analysis.

Metal Activation of DtxR

Metal activation of the diphtheria toxin repressor induces extensive structural reordering of the repressor. The Pr segment is bound intramolecularly by the SH3 domain in the apo-repressor⁷ whereas it interacts with the helices of the dimer interface in the metal-activated holo-repressor. We showed previously that intramolecular complex formation functions to counteract dimer formation in apoDtxR according to the following minimal scheme:

Here, A represents the monomeric aporepressor, A_2 the dimeric aporepressor and H_2 the dimeric holorepressor. We propose that the $2A \leftrightarrow A_2$ equilibrium reflects the dissociation of the Pr – SH3 intramolecular complex followed by dimerization (apparent $K_D \sim 4 \mu M$ ^{9; 39; 40}) whereas the $A_2 \leftrightarrow H_2$ transition is controlled by metal binding (apparent $K_D \sim 1\text{--}3 \mu M$ ^{9; 39; 40}). In this sense, intramolecular binding and dissociation of the Pr segment functions as a “switch” in DtxR activation. This is reminiscent of regulatory intramolecular complexes found in eukaryotic receptor tyrosine kinases^{41–47} in which a weakly bound, internal proline-rich segment must compete with external proline-rich segments having much higher affinity for the SH3 domain. Linking the internal proline-rich peptide ligand to the SH3 receptor in a single polypeptide chain enhances the binding affinity in a manner that depends on the length of the flexible linker⁴⁸ and minimizes the possibility of homodimerization through the proline-rich peptide segments⁴⁹.

An objective for this study was to further characterize this “switch hypothesis” for the Pr segment in DtxR activation. According to this model, the binding of the Pr segment to the SH3 domain requires delicate balance. An intramolecular binding affinity that is too strong would drive the apo-repressor to the monomeric form and DtxR might then be less sensitive to changes in the intracellular iron concentration. On the other hand, if the Pr segment binding were too weak or non-existent, then metal binding would dominate and the sensor function of DtxR might be compromised. Intermolecular binding of the Pr segment by the DtxR SH3 domain is quite weak ($K_D \sim 750 \mu M$); NMR and MD simulation results reported here suggest that the intramolecularly bound fraction is $\sim 30\%$. It is possible that the dynamic nature of the various species, such as the unbound and bound SH3 domains studied here, facilitate their rapid transition in response to environmental changes.

Given the importance of this intramolecular interaction in regulating the energetics of metal activation in DtxR, it is not surprising that most DtxR homologues exhibit a two-domain structure with homologous SH3 and Pr segment regions. What is surprising, perhaps, is that a few of the known DtxR homologues *do not* have SH3-like domains. Instead, these latter proteins, the manganese-responsive repressors MntR from *Bacillus subtilis* and AntR from *B. anthracis*, contain a single domain with an extended dimer interface^{50; 51} and exhibit metal-

independent dimer formation^{52; 53}. Both MntR and AntR demonstrate apparent metal binding affinity that is lower than the available intracellular “free” Mn(II) concentration so that physiological changes in Mn(II) concentration result in approximately linear changes in repressor activity. On the other hand, modulating the monomer $\leftrightarrow$ dimer equilibrium by intramolecular complex formation in DtxR, IdeR, and other two-domain homologues, may generate a non-linear, “all-or-none” response to smaller changes in the intracellular Fe(II) levels. It is possible that additional regulatory mechanisms may be operating in the MntR/AntR group of DtxR homologues.

Materials and Methods

Protein expression and Purification

Generation of the PrSH3 and SH3 constructs in pET-15b was described previously⁷. ¹⁵N-Labeled samples of SH3 and PrSH3 were expressed and purified as described previously⁷. NMR samples were prepared by concentrating the protein to ~0.8 mM and dialyzed against NMR buffer (50 mM phosphate buffer containing 0.4% NaN₃ and 10% D₂O at pH 6.5). Previous work established that both proteins are monomeric under these solution conditions.⁷

Peptide Synthesis and Purification

An ¹⁵N-leucine labeled peptide containing the DtxR Pr segment (residues L120-V140: LKDVSRSPFGNPIGLDELGV) was synthesized in an in-house facility at Florida State University. Crude peptide was purified by size exclusion chromatography over a Sephadex G-25 (Sigma) column. Fractions containing peptide were collected, dried, resuspended in NMR buffer and pooled. The peptide mass was determined using matrix-assisted laser desorption ionization mass spectrometry to confirm the amino acid composition.

NMR data collection

All NMR spectra were collected at 30°C on three-channel Varian Unityplus spectrometers equipped with waveform generators and a z-axis pulsed field gradient accessory operating at 600 MHz or 720 MHz proton frequency. The ¹⁵N R₁, R₂ and ¹H-¹⁵N NOE spectra were measured with enhanced sensitivity pulse field gradient pulse sequences⁵⁴. Recycle delays of 1.5 s and 2 s were used in the R₁ and R₂ experiments for the SH3 and Pr-SH3 proteins. Spectra at 600MHz were collected as 200 × 512 complex matrices with spectral widths of 2200 Hz and 10000 Hz in the ¹⁵N and ¹H dimensions, respectively; spectra at 720MHz were collected as 200 × 512 complex matrices with spectral widths of 2500 Hz and 12001 Hz in the ¹⁵N and ¹H dimensions, respectively. On either instrument, spectra were collected using 16 scans per t₁ point for R₁ relaxation delays of 10, 50 (×2), 150 (×2), 250, 400 (×2), 650, 900 (×2), and 1500 milliseconds and R₂ relaxation delays of 10, 30 (×2), 50 (×2), 70, 90, 110, 150, 170 (×2), 210, 250 (×2) milliseconds. The repeated spectra were used to estimate instrumental error. Transverse relaxation optimized spectroscopy (TROSY)-Carr-Purcell-Meiboom-Gill (CPMG) spectra were recorded with 16 scans and a 1.5 s recycle delay. Relaxation delays of 10, 30, 50 (×2), 70 (×2), 90, 150, 170 (×2), 210 ms were used for recording spectra at ν_{cpmg} ~ 1000 Hz and ν_{cpmg} ~ 0 Hz (a 180° pulse replaced the CPMG train)^{55; 56}. The ¹H-¹⁵N NOE spectra^{57; 58} were collected using a 3 s train of 120° proton pulses as 32 scans per t₁ time point, with a 6 s relaxation delay between scans (the ¹H saturation was applied for the last 3s of the relaxation delay). Control spectra were collected using an identical sequence except that the 3s ¹H pulse train was applied at > 60 dB lower power. This sequence begins with a 90° proton pulse followed by a gradient to ensure that magnetization originates on the nitrogen spins. A water-selective 90° flipback pulse was applied immediately prior to the hard proton pulse to minimize radiation damping and to enhance the spectral baseplane.⁵⁸ Careful optimization of the water flipback pulse gave satisfactory performance of the NOE experiment

on the 14.1 T instrument, but the same experiment could not be optimized on the 16.9 T instrument, such that several of the measured NOE values were larger than the theoretical maximum for that field.

$R_{1\rho}$ spectra were recorded at 600MHz for Pr-SH3 for relaxation delays of 10, 30 ($\times 2$), 50 ($\times 2$), 70, 90 ($\times 2$), 150, 170 ($\times 2$), and 210 milliseconds using a 1.5 kHz spin lock and a recycle delay of 2 s. HSQC spectra were collected before and after each relaxation measurement to verify the sample integrity. R_1 , R_2 and ^1H - ^{15}N NOE relaxation data for the ^{15}N -Leu-labeled Pr segment peptide were collected at 30°C on the 600 MHz instrument as 1D spectra using spectral parameters and relaxation delays identical to those given above, except that spectra were collected in 8 scans per spectrum. All data were processed using the nmrPipe and analyzed using NMRView⁵⁹.

Relaxation Data Analysis

The R_1 , R_2 , and $R_{1\rho}$ values for each resonance were determined by fitting the measured peak volumes for each delay time to a single exponential decay curve using tools available in NMRview (version 5).⁵⁹ The uncertainty in the fitted relaxation time was estimated as the geometric mean of the difference between the best fit and upper bound and the difference of the best fit and lower bound confidence levels obtained from Monte Carlo simulation. The ^1H - ^{15}N NOE was obtained from the ratio of the peak volumes with and without the 3 s proton saturation. The NOE error (σ_{NOE}) was calculated as

$$\sigma_{\text{NOE}} = \frac{I_{\text{sat}}}{I_{\text{unsat}}} \left[\left(\frac{\sigma_{\text{sat}}}{I_{\text{sat}}} \right)^2 + \left(\frac{\sigma_{\text{unsat}}}{I_{\text{unsat}}} \right)^2 \right]^{1/2} \quad [1]$$

where σ_{sat} and σ_{unsat} represent the root mean square variation in the noise in empty spectral regions of the spectra with and without proton saturation and I_{sat} and I_{unsat} are the measured intensities of a particular resonance in the presences and absence of proton saturation. The measured $R_{1\rho}^{\text{app}}$ were corrected for resonance offset using the following equation¹⁴

$$R_{1\rho} = \frac{R_{1\rho}^{\text{app}} - R_1 \cos^2 \beta}{\sin^2 \beta} \quad [2]$$

with $\beta = \tan^{-1}(\omega_{\text{SL}}/\Delta\omega)$, where ω_{SL} is the spin lock power and $\Delta\omega$ is the resonance offset. The difference in R_2 relaxation rates (ΔR_2) using $\nu_{\text{cpmg}} \sim 1000$ Hz and $\nu_{\text{cpmg}} \sim 0$ Hz were calculated as the difference in the R_2 values obtained by fitting the TROSY-CPMG data to single exponential decay curves.

Model-Free Analysis

Relaxation rates from two fields and NOE values from one field were analyzed according to the extended Lipari-Szabo formalism^{60; 61} using the Modelfree program (version 4.15).¹² The raw relaxation rates and NOE values were filtered as outlined by Pawley¹⁰. The data was further filtered by removing residues exhibiting high $J(\omega_{\text{N}})$ and R_{ex} values (obtained from ΔR_2 values)¹¹. The diffusion tensor and an initial estimate for the global correlation time were estimated using the *quadric_diffusion* program^{62; 63} (A.G. Palmer, Columbia University) to identify an appropriate diffusion model based on local diffusion properties of individual backbone amides that was then used to parameterize internal motions of the individual amide bond vectors in Modelfree. The relaxation data for each residue were tested against motional models¹² of increasing complexity, based on a spectral density function having the form

$$J(w) = \left(\frac{2}{5}\right) \left[\frac{S_f^2 S_s^2 \tau_c}{1 + (w\tau_c)^2} + \frac{S_f^2 (1 - S_s^2) \tau_{eff}}{1 + (w\tau_{eff})^2} \right] \quad [3]$$

where $S^2 = S_f^2 S_s^2$ is the generalized order parameter, τ_c is the global correlation time, and τ_{eff} is the local effective correlation time given by $\tau_{eff} = (\tau_e \tau_c) / (\tau_e + \tau_c)$ with τ_e being the correlation time for internal motion, S_f^2 is the generalized order parameter for fast internal motions, and S_s^2 is the order parameter for slow internal motions. A particular motional model was assumed to fit the relaxation data for a given residue when the model could reproduce the experimental relaxation data within 90% confidence limits (using critical value $\alpha = 0.1$) and the resolved parameters were larger than their associated errors. Additionally, individual models were compared using Akaike's information criterion to determine the optimal model.¹³ The initial models were combined in a final refinement of all motional parameters, including the global rotational correlation time. All Modelfree calculations used $r_{NH} = 1.02 \text{ \AA}$ and $^{15}\text{N CSA} = -172 \text{ ppm}$.^{64–67}

Reduced Spectral Density Mapping

The 600 MHz relaxation data were also analyzed using reduced spectral densities^{26; 68}. This approach is based on the observation that high-frequency spectral density functions can be approximated by a Taylor expansion around $J(\omega_H)$. The spectral density functions were calculated from the relaxation data as⁶⁹

$$J_{eff}(0) = [6R_2 - 3R_1 - 2.72\sigma_{NH}] / [3d^2 + 4c^2] \quad [4]$$

$$J(\omega_N) = [4R_1 - 5\sigma_{NH}] / [3d^2 + 4c^2] \quad [5]$$

$$J(0.87\omega_H) = 4\sigma_{NH} / (5d^2) \quad [6]$$

where $J_{eff}(0)$, $J(\omega_N)$ and $J(0.87\omega_H)$ are values of spectral density sampling frequencies $\omega = 0$, ω_N (60MHz), and ω_H (516 MHz), respectively; the subscript “eff” in $J_{eff}(0)$ signifies that the spectral density is uncorrected for chemical exchange effects. The other parameters are given

by $\sigma_{NH} = R_1(NOE-1)\gamma_N/\gamma_H$, $d = (\mu_0 h \gamma_N \gamma_H / 8\pi^2) \langle r^{-3} \rangle$, and $c = \omega_N \Delta\sigma / \sqrt{3}$, where the various constants have their normal values.

Molecular Dynamics Simulation

Three independent MD simulations were performed on the PrSH3 and SH3 constructs and the L120-V140 peptide using the AMBER 7 package⁷⁰ with the parm99 force field⁷¹ at constant temperature and pressure. The average temperature and pressure were maintained at 300 K and 1 atm, respectively, using the Berendsen weak-coupling algorithm⁷². Periodic boundary condition was enforced. The nonbonded cutoff was set to 8.0 Å, and the nonbonded list was updated with a 2.0 Å skin width. The particle mesh Ewald (PME) method^{73–76} was used to treat long-range electrostatic interactions. All bond lengths involving hydrogens were constrained, with SHAKE⁷⁷ for the protein and RATTLE⁷⁸ for water molecules. The time step of all simulations was 0.002 ps, and coordinates were saved every 4 ps for analysis.

The starting structure for PrSH3 was the NMR structure (PDB entry 1QW1), in which the Pr segment was bound to the SH3 domain⁷. 9,082 TIP3P⁷⁹ water molecules, plus six sodium ions for charge neutralization, were used to solvate the protein. The simulation started with

3,600 steps of steepest descent and conjugate gradient energy minimization with decreasing harmonic restraints. The system was then heated from 100 K to 300 K for 30 ps, and thereafter the simulation was continued at constant temperature and pressure for up to 51.8 ns. Analysis was only done on the last 49 ns.

Simulations for the other two systems followed similar protocols. The starting structure for the SH3 construct was the NMR structure of PDB entry 1QVP. 4,823 water molecules plus five neutralizing sodium ions were used for solvation. The simulation was continued up to 51.5 ns and analysis was done on the last 45.9 ns. The starting structure for the L120-V140 peptide was taken from the NMR structure of PrSH3. 5,435 water molecules and a neutralizing sodium ion were used for solvation. The simulation was continued to 28.2 ns, with analysis done on the last 26 ns.

iRED analysis of MD trajectories

R_1 , R_2 , and NOE were calculated from the MD trajectories following the isotropic reorientational eigenmode dynamics (iRED) analysis of Brüschweiler.²⁵ The covariance matrix $\mathbf{M}$ was constructed with elements

$$M_{kl} = \langle P_2(\hat{\mu}_k \bullet \hat{\mu}_l) \rangle_{trajectory}$$

where $P_2(x)$ is the second order Legendre polynomial ($P_2(x) = (3x^2 - 1)/2$), $\hat{\mu}_k$ and $\hat{\mu}_l$ are the backbone N-H unit vectors of the residue k and l at the same snapshot. The eigenvectors $|m\rangle$ and eigenvalues λ_m of $\mathbf{M}$ are the reorientational eigenmodes and their amplitudes satisfy the eigenvalue equation, $\mathbf{M}|m\rangle = \lambda_m |m\rangle$. The time-correlation functions,

$$C_j(t) = \sum_m \lambda_m (|m\rangle\langle m|)_{jj} C_m(t)$$

, were generated by modifying the correlation times (from monoexponential fitting, $C_m(t) = \exp(-t/\tau_m)$) of the 10 largest iRED eigenmodes m through minimizing χ^2 defined by

$$\chi^2 = \sum_j (A_j^{calc} - A_j^{exp})^2 / \sigma_j^2$$

where A_j^{calc} are the calculated and A_j^{exp} the experimental T_1 , T_2 , and NOE parameters, respectively, and σ_j are the experimental errors. The spectral density functions were constructed as

$$J_j(\omega) = 2 \int_0^\infty C_j(t) \cos \omega t dt.$$

from which ^{15}N R_1 and R_2 relaxation rates and ^{15}N - ^{13}C NOE values for residue j were calculated as⁸¹

$$\begin{aligned} 1/T_{1,j} &= d^2 [J_j(\omega_H - \omega_N) + 3J_j(\omega_N) + 6J_j(\omega_H + \omega_N)] + c^2 J_j(\omega_N) \\ 1/T_{2,j} &= 0.5d^2 [4J_j(0) + J_j(\omega_H - \omega_N) + 3J_j(\omega_N) + 6J_j(\omega_H) + \\ &6J_j(\omega_H + \omega_N) + 1/6c^2 [3J_j(\omega_N) + 4J_j(0)] \\ \text{NOE}_j &= 1 + (\gamma_H/\gamma_N)d^2 [6J_j(\omega_H + \omega_N) - J_j(\omega_H - \omega_N)]T_{1,j} \end{aligned}$$

where $d^2 = 0.1[(\gamma_H\gamma_N h)/(2\pi r_{NH}^3)]^2$ and $c^2 = (2/15)[\omega_N^2(\sigma_{||} - \sigma_{\perp})^2]$, h is Plank's constant, r_{NH} is the internuclear N-H distance, γ_i is the gyromagnetic ratio of spin i , and $\sigma_{||}$ and $\sigma_{\perp}$ are components of the axially symmetric ^{15}N chemical shift anisotropy (CSA) tensor. Values for r_{NH} and ^{15}N CSA were as used in the Modelfree analysis (*vide supra*). The separability index

$$g_L = \sum_{m=1}^n \lambda_m / \sum_{m=1}^{n-5} \lambda_m$$

indicates the separability of overall and internal motions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ribbon diagram structures of A. PrSH3 and B. SH3. The Pr segment in PrSH3 is indicated in black. Structures are shown in the same orientation and are from PDB files 1QW1 and 1QVP⁷, respectively. The figure was prepared using Molmol⁸².

Figure 2.

Backbone ^{15}N relaxation data for PrSH3 (●) and SH3 (●) constructs. A. Longitudinal relaxation rate; B. Transverse relaxation rate; C. Heteronuclear NOE; D. R_2/R_1 ratio. Error bars in plots A–C indicate the fitting error.

Figure 3. Reduced spectral density analysis of backbone relaxation data for PrSH3 (●) and SH3 (●) constructs. A. $J(0.87\omega_H)$, B. $J(\omega_N)$, and C. $J_{\text{eff}}(0)$.

Figure 4. Microdynamic parameters obtained from Modelfree analysis of backbone relaxation data for the PrSH3 (●) and SH3 (●) constructs. A. Local correlation time, τ_e ; B. Conformational exchange rate, R_{ex} , for PrSH3; C. Conformational exchange rate, R_{ex} , for SH3; D. Order parameters for all residues fit to a Modelfree motional model. Error bars indicating fitting errors.

Figure 5. Analysis of slow motions in PrSH3. A. Difference in transverse relaxation rate (ΔR_2) at $\nu_{\text{cpmg}} \sim 1000$ Hz and $\nu_{\text{cpmg}} \sim 0$ Hz.; B. Ratio of transverse to rotating frame relaxation rate.

Figure 6.

Molecular dynamics trajectories for PrSH3 and SH3. A. Instantaneous C_{α} - C_{α} distance for residues L182- D210 in PrSH3 (black) and SH3 (gray) during a 50 ns molecular dynamics trajectory. B. Ribbon diagram of PrSH3 (yellow) and SH3 (cyan) showing the location of $\alpha 2$ in an “open” structure (structures are taken at the point marked by the asterisk in 6A). The Pr segment (red) is indicated in the PrSH3 structure. Figure 1 shows the location of $\alpha 2$ in the initial, or “closed” structures.

Figure 7. Modeled backbone ^{15}N relaxation data used to estimate the fractional occupancy of the ligand binding site in PrSH3. Experimental (●) and modeled (□) relaxation rates. The error bars for experimental R_2 rates are as in Figure 2B.

Figure 8.

Calculated R_1 and R_2 relaxation rates for an ^{15}N nucleus in 14.1 T external field comparing the relaxation rates of an isotropically tumbling protein experiencing motions in two different time regimes. Solid lines show R_1 and R_2 assuming LS-2 parameterized with $S^2 = 0.90$ and $\tau_e = 50$ ps. This is roughly the behavior seen for the backbone amides in the SH3 portion of the PrSH3 construct. Dashed lines show R_1 and R_2 assuming $S^2 = 0.30$ and $\tau_e = 800$ ps, conditions that approximate those for the backbone amides in the Pr segment of PrSH3. The two arrows indicate the τ_c values for the Pr (2.2 ns) and SH3 (3.8 ns) calculated from the reduced spectral density analysis. The calculated relaxation rates show that R_1 is fairly insensitive to these different motional parameters while R_2 changes significantly.

Figure 9.

Mode collectivity, κ , versus eigenvalue for each eigenmode, λ_i , from the iRED analysis for the PrSH3 trajectory. The absence of a significant gap between the five points with largest λ_i values and the rest of the data indicates that internal and external motions are not separable, as suggested by analysis of the experimental relaxation data.

Figure 10. Reorientational eigenvectors from iRED analysis of the PrSH3 trajectory. Eigenvectors for eigenmode 7 (A) and eigenmode 9 (B) are plotted as the component of the principal order parameter (δS^2) for each residue. Cross-eyed stereo representations of PrSH3 showing the residues contributing to eigenmode 7 (C) or eigenmode 9 (D). In both structures, N atoms of the contributing residues are shown as red spheres.

Table 1
Diffusion parameters for SH3 and PrSH3 from analysis of ¹⁵N relaxation by *quadratic diffusion*.

	Diso (10 ⁻⁷ s ⁻¹)	2D _{zz} /(D _{xx} +D _{yy}) ^c	D _{xx} /D _{yy}	θ(rad) ^d	φ(rad) ^d	ψ(rad) ^d	χ ²	F ^e
SH3 ^a	3.53 ± 0.02	1.36 ± 0.05	—	0.66 ± 0.06	3.1 ± 0.09	—	2.32	11.9
PrSH3 ^b	2.56 ± 0.01	1.21 ± 0.03	—	0.6 ± 0.1	5.89 ± 0.14	—	4.1	3.36

^a Values of D_i for 33 residues were fit using local diffusion approximation;

^b Values of D_i for 26 residues were fit using local diffusion approximation;

^c D_{iso} = D_{xx} = D_{yy} = D_{zz}; ^e D_{||} = D_{zz}, D_⊥ = D_{xx} = D_{yy}, D_{iso} = (D_{||} + 2D_⊥)/3, D_{||}/D_⊥ = 2D_{zz}/(D_{xx} + D_{yy});

^d Orientation angles of Diffusion tensor;

^e F-test to test the significance in addition of extra variable to the fit.

Table 2
Calculated relaxation rates assuming isotropic diffusion tensor.

	R_1 (s ⁻¹)	R_2 (s ⁻¹)	NOE
SH3 ^a	2.31	5.51	0.73
Pr segment ^b	1.92	2.39	-0.07

^aCalculated at 14.1 T for an isotropically diffusing particle with $\tau_c = 3.8$ ns, $S^2 = 0.85$, and $\tau_e = 50$ ps using the two-term spectral density function corresponding to Model 2 of the Modelfree formalism.

^bCalculated at 14.1 T for an isotropically diffusing particle with $\tau_c = 2.2$ ns, $S^2 = 0.30$, and $\tau_e = 800$ ps using the two-term spectral density function corresponding to Model 2 of the Modelfree formalism.