

Defining a length scale for millisecond-timescale protein conformational exchange

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Although atomic resolution 3D structures of protein native states and some folding intermediates are available, the mechanism of interconversion between such states remains poorly understood. Here we study the four-helix bundle FF module, which folds via a transiently formed and sparsely populated compact on-pathway intermediate, I. Relaxation dispersion NMR spectroscopy has previously been used to elucidate the 3D structure of this intermediate and to establish that the conformational exchange between the I and the native, N, states of the FF domain is driven predominantly by water dynamics. In the present study we use NMR methods to define a length scale for the FF I–N transition, namely the effective hydrodynamic radius (EHR) that provides an average measure of the size of the structural units participating in the transition at any given time. Our experiments establish that the EHR is less than 4 Å, on the order of the size of one to two amino acid side chains, much smaller than the FF domain hydrodynamic radius (13 Å). The small magnitude of the EHR provides strong evidence that the I–N interconversion does not proceed via the synchronous motion of large clusters of amino acid residues, but rather by the exposure/burial of one or two side chains from solvent at any given time. Because the hydration of small hydrophobic solutes (< 4 Å) does not involve considerable dewetting or disruption of the water–hydrogen bonding network, the FF domain I–N transition does not require appreciable changes to the structure of the surrounding water.

excited protein states | hydrophobic hydration | CPMG NMR | protein folding

Proteins are inherently dynamic molecules that undergo conformational exchange over a wide range of timescales in solution. Exchange events occurring on the microsecond–second timescale are often relevant for protein folding, a process that has been successfully modeled as a diffusive search occurring on a low-dimensional free-energy surface. Such a reduced dimensional analysis of experimental data is attractive because it provides a framework by which a complex problem can be made tractable, in this case facilitating an understanding of the folding event in terms of the thermodynamics and kinetics that are involved in the process (Fig. 1).

Additional information can also be obtained in the form of the structures that populate the protein's free-energy landscape. Here, relaxation dispersion (RD) NMR spectroscopy (1) has emerged as a valuable tool for determining the structures of transiently formed, sparsely populated protein conformations (referred to as I in what follows) (2–4) that are in equilibrium with the native state (N). These rare states are otherwise invisible to traditional biophysical methods and they do not give rise to cross-peaks in NMR spectra. Nevertheless, their presence can be detected because conformational fluctuations occurring on the millisecond timescale between states N and I lead to a stochastic modulation of NMR chemical shifts of probe nuclei, thereby broadening resonances in NMR spectra of the native conformer (5). The magnitude of exchange broadening can be systematically modulated using a Carr–Purcell–Meiboom–Gill (CPMG) spin relaxation experiment, which consists of a series of chemical shift refocusing pulses applied at varying rates, ν_{CPMG} (5). The resulting RD profile (Fig. 2) for a given nucleus is a graph of its

transverse relaxation rate, $R_{2,\text{eff}}$ (effectively peak linewidth), as a function of ν_{CPMG} . The profile can be fit to a model of chemical exchange to yield the populations of the states, the rates of interconversion, and the differences in chemical shifts at that site in each of the interconverting states (5). Recently, atomic resolution structures of a number of transiently formed, invisible intermediates have been determined (2–4, 6, 7) by using chemical shifts of the I state obtained from RD NMR as input into database-driven structure-prediction algorithms (8, 9).

Conformational exchange in the four-helix bundle FF domain from human HYPA/GBP11 has been extensively characterized by RD NMR spectroscopy (2, 7, 10–12). At 25 °C, FF interconverts between its native state (N) and a compact on-pathway folding intermediate (I), populated to 1%, with an exchange rate constant $k_{\text{ex}} (= k_{\text{IN}} + k_{\text{NI}})$ of 1,940 s^{−1} (Fig. 1) (12). The I-state 3D structure has been elucidated (2) and cross-validated (13), establishing several nonnative structural features. Most notable is that helix 3 (H3) is significantly longer in I than in N, extending to where H4 begins in the native state. This lengthening results in the formation of nonnative hydrophobic contacts at the interface between helices H2 and H3. In contrast to H3, however, H4 is unstructured in the intermediate state. The transition from I to N must, therefore, involve the unraveling of H3 and the formation of H4, as well as the disruption of nonnative hydrophobic interactions. Although atomic resolution 3D structures of the endpoints (I and N in the case of the FF domain) are now available, a description of how structure evolves during the interconversion is still notably lacking.

Some insight can be obtained from a number of theoretical (14, 15) studies in which helix–coil transitions are predicted to be cooperative, but not entirely two-state. Additionally, experimental studies focusing on hydrogen–deuterium exchange using either NMR (16–19) or mass spectrometry (20, 21) provide a further basis for understanding the mechanism of helix folding/unfolding both in the context of peptides and proteins. The denaturant dependence of exchange rates establishes that some protons exchange only upon global unfolding of the entire protein, whereas others exchange as parts of subglobal cooperative secondary structural units called “foldons” (22) that can be tens of amino acids in size. However, other protons, in particular those at the termini of helices, exchange by local dynamic fraying (18, 23) involving breaking one or a few hydrogen bonds at a time. The existence of foldons leads to the creation of partially unfolded forms along a folding pathway, similar to what has been observed for the WT FF domain studied here (22). However, the mechanism by which the WT FF domain transitions from the I to the N state along its folding trajectory remains to be determined. For example, does this interconversion involve the simultaneous rearrangement of foldons with length scales on the order of tens

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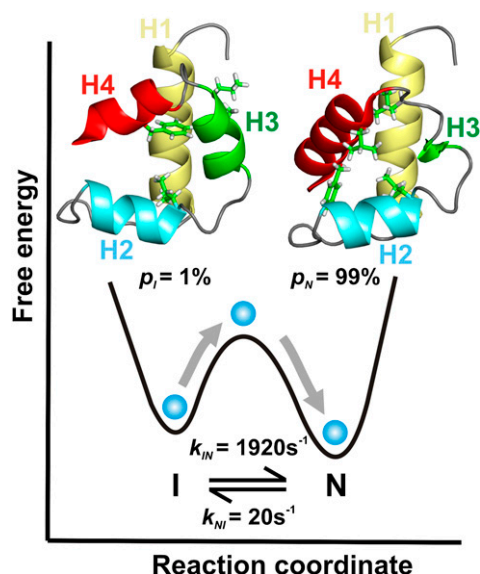


Fig. 1. Schematic representation of the reduced dimensional conformational free-energy landscape for the FF I-N transition. The 3D structures of the I [PDB ID code 2KZG (2)] and N [PDB ID code 1UZC (54)] states are shown above their respective free-energy minima. A few hydrophobic side chains (I43, L52, L55, and F62) that reorganize during the I-N transition are shown as sticks. The I-N interconversion can be described by the diffusion of a “virtual” particle (blue) on the conformational free-energy landscape that governs the folding process, with an EHR whose determination is the subject of the work described here.

of angstroms, or is it a more localized process, like the dynamic fraying of helices characterized previously (18, 23)? Herein we probe the size of the elements that are involved in the elementary steps along the FF domain I-N reaction coordinate using a combined RD and pulsed field gradient (PFG) diffusion NMR study.

In a previous report we determined that the energy driving the interconversion between I and N states of the FF domain originates primarily from water fluctuations (12). This was established by measuring the viscosity dependencies of rates of exchange, k_{IN} and k_{NI} , in viscogens glycerol and BSA that were then modeled using a modified Kramers equation (24–26) for the rate of a reaction in the high friction limit:

$$k_i = \frac{A_i}{(\eta + \sigma)} \exp\left(\frac{-\Delta G_i^\ddagger}{RT}\right), \quad i \in (IN, NI). \quad [1]$$

In Eq. 1, A_i is given by a product of terms that reflect the curvatures of the free-energy surface at the initial state and the barrier, assumed to be independent of viscosity and temperature, ΔG_i^\ddagger is the difference in free energy between the initial state and the barrier, and η and σ are the contributions to the overall viscosity for motion along the free-energy landscape that derive from solvent (η) or that are solvent-independent (σ), respectively. The internal friction for the I-N transition was found to be small ($\sigma \sim 0.2$ cP) in relation to contributions from solvent ($\eta = 0.89$ cP) (12).

Once the values of $A_i \exp(-\Delta G_i^\ddagger/RT)$ and σ have been determined from a viscosity dependence study of rates, Eq. 1 can be used to estimate the “average” solvent viscosity along the landscape for a reaction that occurs in a particular viscogen. The measured viscosity, in turn, can be related to a length scale or the “size” of changes that occur as the reaction proceeds along its trajectory. The inherent relation between microscopic solution viscosity and size can be appreciated in simpler terms by considering a probe molecule as it diffuses in a water–viscogen

mixture. Here, the diffusion rate depends on the relative sizes of the probe and the viscogen. For example, addition of 30% (wt/wt) PEG to water decreases the diffusion coefficient of water (hydrodynamic radius, $R_H = 1.4 \text{ \AA}$) by a factor of 1.5, whereas the diffusion coefficient of the dye carboxytetramethylrhodamine ($R_H \sim 6.5 \text{ \AA}$) decreases by a much larger factor, 4 (27). Once the probe molecule reaches the approximate size of the viscogen, the apparent viscosity is no longer size-dependent (28). Analogously, conformational exchange occurring in the diffusive limit on a reduced dimensional landscape can be formulated in terms of a particle with an effective hydrodynamic radius (EHR) diffusing between each of the states along the reaction coordinate (29). Hence the dependence of reaction rate on viscogen size can be used to estimate the EHR of the particle. However, most viscogens are unsuitable for studies of the kinetics of the FF I–N interconversion because they interact with the protein, altering the relative populations of the interconverting states (12). In the present study we have circumvented this problem by choosing a large, inert viscogen (BSA) and an array of probes with varying R_H to construct a molecular ruler, which is a relationship between η and probe R_H .

Using this molecular ruler relating η and R_H , and I-N exchange rates measured in 200 mg/mL BSA by RD NMR, an upper bound of 4 Å is obtained for the EHR of the I-N transition, which is on the order of the size of one amino acid and much smaller than the FF domain hydrodynamic radius (13 Å). The EHR provides an estimate of the average size of the structural moieties that are actively diffusing in and out of solvent during the conformational rearrangement. Further, the EHR can be interpreted as a measure of the coupling between the solvent and the I-N fluctuations and it is related to the extent of exchange of energy with the solvent that occurs along the reaction (30, 31). The obtained EHR establishes that the transition from I to N proceeds via a series of small steps, involving one or two amino acid side chains on average, rather than a set of more concerted conformational changes involving larger clusters of the protein chain.

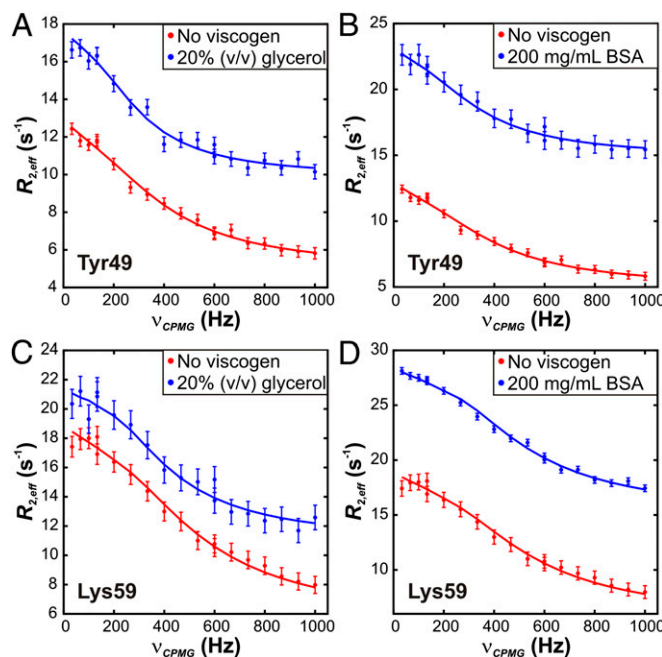


Fig. 2. CPMG RD profiles for the backbone ^{15}N nuclei of Tyr-49 and Lys-59 of the FF domain, 18.8 T, 25 °C without and with (A and C) 20% (vol/vol) glycerol or (B and D) 200 mg/mL BSA. Errors in the data points are shown by the vertical bars and were estimated by acquiring two to four repeat points. Solid lines correspond to fits of data from all residues simultaneously to a two-state exchange model.

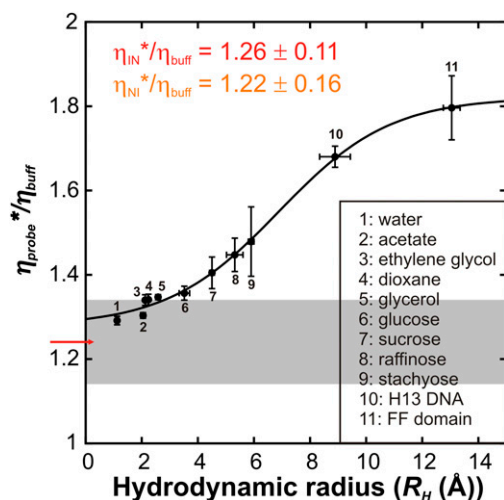


Fig. 4. A molecular ruler for measuring the EHR of the I–N interconversion. Values of $\eta_{\text{probe}}^*/\eta_{\text{buff}}$ are plotted as a function of R_h , as described in the text. The value of $\eta_{\text{ex,FF}}^*/\eta_{\text{buff}} = 1.24 \pm 0.10$, as determined by analysis of RD data and calculated using Eq. 2, is indicated by the red arrow and the associated error (± 1 SD from the mean) is depicted as a gray bar. The solid black line is intended only to guide the eye and does not have any physical significance. All η_{probe}^* and $\eta_{\text{ex,FF}}^*$ values are based on measurements obtained using 200 mg/mL BSA as a viscogen.

a carbonyl vibrational probe attached to lysozyme establish that a 100-fold increase in bulk solution viscosity is accompanied by only a threefold increase in protein dynamics (39). NMR spin relaxation experiments focusing on the amplitudes and time-scales of methyl-containing side-chain dynamics in an SH3 domain show little change upon addition of ~25% (vol/vol) glycerol (40). To supplement these results with further experiments on an additional protein system we have carried out measurements of ps–ns side-chain dynamics in the small protein ubiquitin. We chose ubiquitin rather than the FF domain, which is the focus of the present work, because the pervasive chemical exchange contributions to linewidths of cross-peaks in spectra of the FF domain can complicate the extraction of robust measures of ps–ns motions. In addition, previous detailed NMR relaxation studies have shown that the rotational diffusion of ubiquitin is essentially isotropic (41), simplifying data analysis. A comparison of ps–ns dynamics in aqueous and 25% (vol/vol) glycerol buffers shows that the motion is slowed down by a factor of 1.3 despite the 2.5-fold increase in solvent viscosity (Fig. S1 and SI Text). Taken together, these studies indicate that small-amplitude, rapid side-chain fluctuations occurring in the protein hydration layer are relatively insensitive to increases in bulk solution viscosity. By contrast, we have demonstrated that k_{IN} and k_{NI} for the FF domain I–N interconversion scale essentially inversely with viscosity when the solvent glycerol is used. FF domain folding must, therefore, involve side-chain rearrangements of a length scale that is significantly larger than displacements resulting from ps–ns side-chain dynamics within the protein hydration layer. Thus, the amplitudes spanned by local side-chain motion provide a lower bound for the EHR of the FF domain I to N conformational exchange.

Discussion

We have established an effective length scale or EHR of less than 4 Å for the FF domain conformational exchange process. The method we use for determining the EHR is general and can be applied to any conformational exchange process that can be studied in the presence of a large viscogen. It is, however, predicated upon two assumptions. First, we assume that both glycerol and BSA are “true” viscogens in the sense that they add ruggedness to the FF conformational free-energy surface without

perturbing the landscape. This is supported by the fact that the population of the invisible intermediate state remains unchanged in either viscogen, as established by RD NMR measurements probing the I–N conformational exchange process (12). In addition, backbone ^{15}N chemical shifts of both the ground and the excited states change very little in 200 mg/mL BSA or 25% (vol/vol) glycerol, providing strong evidence that the structures of both states in either viscogen are unchanged (12). Second, our analysis makes the analogy between translational diffusion in 3D space and diffusion along the reduced dimensional landscape in the presence of a potential. In this context we assume that the size dependence of the effective viscosity for diffusing on the conformational landscape is identical to that for translational diffusion (Fig. 4). There are compelling justifications for making this assumption. Computer simulations (36, 42) and experiments (29, 37) have confirmed that protein conformational exchange occurs in the Kramers theory high-friction regime. Thus, both translation and conformational exchange are described by the Smoluchowski equation either without or with an external potential (43). Further, the mechanism of exchange of momentum and kinetic energy between the solvent and the particle for both processes is viscosity-dependent and occurs through collisions with the solvent, given that the FF domain conformational exchange is driven primarily by solvent motion (12). We note that measuring k_{IN} and k_{NI} as a function of viscosity for viscogens of increasing size would be an alternative method of measuring an EHR (31). In the case of the FF domain, however, such measurements are not possible because many viscogens such as ethylene glycol, glucose, sucrose, and trehalose alter the relative stabilities of I and N and thus perturb the free-energy landscape (12).

It is worth emphasizing that we have modeled the relaxation dispersion data probing the WT FF I–N transition as a two-state process to extract viscosity-dependent values of k_{IN} and k_{NI} . A detailed analysis (presented in SI Text and Figs. S2–S7) establishes that the dispersion data can be well explained by a two-site exchange model and that more complex models are not needed. However, we cannot rule out the existence of states at very low fractional populations (less than a few tenths of a percent) that are in fast exchange with I or N. Indeed, the picture that emerges of the I–N interconversion is one involving elementary steps that expose approximately one residue at a time to solvent, suggesting that there are many transiently formed and very sparsely populated microscopic states in solution. We have attempted to evaluate how the presence of these additional conformers might influence the results of the present work by modeling three-site exchange processes, as described in SI Text. For the simulations considered the main result of the present study remains unchanged, namely that the I–N transition can be described using an EHR of <4 Å.

An EHR <4 Å is comparable to the hydrodynamic radius of one amino acid (R_h for Trp is 3.5 Å). Thus, whereas our results are consistent with a cooperative I–N transition for the FF domain interconversion, the small EHR value shows that FF domain folding must proceed at the microscopic level via a conformational search involving the diffusion of one or two protein side-chains in and out of bulk solvent at a given time. This is in contrast to a concerted motion of large structural elements that can be up to tens of amino acids in size. The measured EHR is significantly smaller than the length of an average foldon (22) but is consistent with the length scales observed for end fraying in peptide (18) and protein helices (23).

Because the EHR has been determined from rate constants extracted from fits of RD NMR measurements, this value is an average over multiple barrier crossings. Our results do not preclude variation of the EHR with instantaneous position on the reaction coordinate. Moreover, the generality of the <4 Å length scale for protein conformational exchange reported here remains to be tested. Mukherjee et al. (33) have measured the rates of the helix-to-coil transition for two small helix-forming peptides in solutions containing either of the crowding agents Dextran 70 or Ficoll 70 using fluorescence correlation spectroscopy. They find that the folding lifetimes of these peptides increase by only

20–60% compared with their values in buffer without viscogen, whereas the macroscopic viscosity increases by 10- to 20-fold. The authors note that the polymeric crowding agents used in the study can form porous networks with the effective viscosity highly dependent on the probe size (28), as observed using BSA as a viscogen in our study (Fig. 4). Thus, the small dependence on viscosity reflects the small length scale over which changes take place during the helix–coil transition, far less than the pore sizes in the viscogen polymers. Although the EHR could not be determined from that study, the value of $<4\text{ \AA}$ reported here for a protein domain, involving changes in tertiary interactions, is consistent with the small viscosity dependence noted for the helical peptides in the previous work.

Zwanzig and Harrison (30) have suggested that the EHR of a particle diffusing in solution represents the magnitude of its coupling to solvent. In the reduced dimensional picture of conformational exchange, an effective particle diffuses along a free-energy surface that is modulated by implicit solvent (and protein) degrees of freedom that do not feature in the reaction coordinate. The particle exchanges kinetic energy and momentum with the solvent, providing the driving force for transitioning the activation free-energy barrier. Following the analogy from translational diffusion, the EHR for the FF domain conformational exchange process can be interpreted as the extent of coupling between residues of the FF domain that become accessible during the exchange process and the solvent, defining the scattering cross-section (44) for momentum transfer during a collision between these residues and the solvent molecules. In the case of the FF domain the EHR for the I–N interconversion is at least threefold smaller than the EHR of the domain itself and the scattering cross-section is smaller by an order of magnitude. Thus, momentum is dissipated much less efficiently during the I–N folding reaction than for the diffusing FF module.

The I–N fluctuations for the FF domain, and indeed most folding/unfolding transitions, involve changes in the exposure of hydrophobic residues to water. For example, the conformationally exchanging H3–H4 region (Fig. 1) includes several hydrophobic residues (2 Leu, 4 Ala, 2 Tyr, 1 Val, and 1 Phe) as well as amino acids such as Lys (4) and Arg (1) with long hydrophobic side chains. Interestingly, Φ value analysis of the rate-limiting transition state for folding has established that residues such as Y49, A51, and L52, which form part of helix H3 in I, have low-intermediate Φ values (<0.5) and are thus unstructured in the transition state ensemble (45). It is clear that these residues must, therefore, rearrange as I proceeds to the transition state, with nonnative-like contacts removed (2) and then reorganize further after the transition state to form the native conformation.

The molecular mechanism of solvation of small hydrophobic solutes ($<4\text{ \AA}$), or 1- to 2-aa side chains in the case of the FF domain I–N transition, is very different from the solvation of larger nanometer-scale hydrophobic assemblies (46). Water retains its hydrogen-bonding structure around cavities created by small solutes, whereas large solutes disrupt the hydrogen-bonding network (47). Large hydrophobic solutes are thus drier than small ones and have a significantly lower density of water surrounding them (47). The small EHR for the I–N conformational rearrangement studied here indicates that changes in hydrophobic solvation involving only a very small number of side chains will not appreciably disrupt the water hydrogen-bonding network. Exposure of a large hydrophobic cluster to water during the I–N interconversion seems to be avoided by allowing the reaction to proceed in small ($<4\text{ \AA}$) steps. Thus, depending on the nature of the conformational exchange process, it may be more appropriate to think about hydrophobic contributions from the standpoint of a small hydrophobic solute rather than from the point of view of a nanometer-sized protein.

Finally, our results may have implications for understanding conformational rearrangements of proteins in the cell where the environment is a crowded and heterogeneous mixture of solutes of varying sizes. For processes involving a small EHR, such as a helix folding/unfolding transition, it is likely that large-molecule

viscogens such as proteins, which do not effectively couple to the exchange reaction, will not significantly affect the rates of conformational interconversion.

Concluding Remarks

We have used NMR spectroscopy to determine the EHR for a conformational exchange process in which the native state of the four-helix-bundle FF domain interconverts with a compact on-pathway folding intermediate. The EHR defines a length scale for diffusive barrier crossing between the two states. The EHR for the I–N transition is small ($<4\text{ \AA}$) compared with the protein hydrodynamic radius (13 \AA), establishing that the exchange process occurs via the exposure of one or two amino acid side chains at a time. Because the hydration of small hydrophobic side chains does not involve considerable dewetting, conformational exchange between compact protein states may proceed without significantly affecting the surrounding water structure.

Materials and Methods

Sample Preparation for NMR Spectroscopy. ^{15}N -labeled WT FF domain was overexpressed in *Escherichia coli* and purified as described in detail previously (10). Samples for NMR were prepared in 50 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 1 mM sodium azide (pH 5.7), and 90% $\text{H}_2\text{O}/10\% \text{ } ^2\text{H}_2\text{O}$.

All probe molecules listed in Fig. 4 were purchased from Sigma-Aldrich with the exception of H13 DNA [$d(5'\text{-CGCGTTGTCGCG-3'})$] (48) that was custom-synthesized and HPLC-purified from IDT Inc. and the FF domain that was produced in-house. The concentration of all probe molecules was 10 mM except H13 and the FF domain, which were prepared at concentrations of 400 μM and 1 mM, respectively. For BSA-containing samples, lyophilized BSA powder was dissolved in 50 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 1 mM NaN_3 , and 10% $\text{D}_2\text{O}/90\% \text{H}_2\text{O}$ (vol/vol) (pH 5.7) buffer to a concentration of 200 mg/mL and subsequently dialyzed against the same buffer containing the probe molecule. Raffinose, stachyose, and H13 powders were dissolved directly in 200 mg/mL BSA after dialysis of the BSA sample against buffer. The FF sample was made by dialyzing a solution of 200 mg/mL BSA containing 1 mM ^{15}N -labeled FF domain against buffer.

NMR Spectroscopy and Data Analysis. ^{15}N relaxation dispersion data were acquired on Varian Inova spectrometers operating at magnetic field strengths of 11.7 T and 18.8 T, 25 $^\circ\text{C}$, equipped with standard triple resonance gradient probes, as described previously (12). PFG diffusion experiments were recorded at 11.7 T, 25 $^\circ\text{C}$. Datasets were processed using NMRpipe (49) and visualized using NMRDraw (49) and Sparky (50). Peak intensities were quantified using NMRPipe (1D diffusion spectra) or FUDA (<http://pound.med.utoronto.ca/software.html>; RD and 2D diffusion spectra).

Diffusion coefficients of all probe molecules except the FF domain were measured using a modified water-suppressed longitudinal encode–decode pulse sequence (51). In the case of the FF domain, a ^{15}N -edited 2D diffusion pulse scheme was used (52). Diffusion spectra of each probe sample in the absence of BSA were acquired as a function of gradient strength using a diffusion delay of 100 ms and a decoding/recoding gradient duration of 1 ms. Gradient strengths were calibrated by measuring the diffusion coefficient of the residual HDO in a D_2O sample, using the known diffusion coefficient of $1.9 \times 10^{-5} \text{ cm}^2/\text{s}$ for HDO at 25 $^\circ\text{C}$ (53).

The signal intensity (I) in a PFG diffusion experiment depends on the gradient strength (G) and the diffusion coefficient (D) of the probe molecule as

$$\frac{I}{I_0} = \exp(-cDG^2), \quad [3]$$

where c is a constant that includes the gradient duration and the diffusion delay (34). The variation in I with the square of the gradient strength was fit to a single exponential decay equation to extract a decay constant ($d = cD$) (Fig. 3 and Fig. S8) that was subsequently converted to a hydrodynamic radius (R_H) using the Stokes–Einstein equation and assuming the hydrodynamic radius of dioxane to be 2.12 \AA (Table S1):

$$D_{\text{probe}} = \frac{kT}{6\pi\eta R_{H,\text{probe}}} \quad [4]$$

$$\frac{R_{H,\text{probe}}}{R_{H,\text{dioxane}}} = \frac{D_{\text{dioxane}}}{D_{\text{probe}}} \quad [5]$$

The increase in effective viscosity (η_{probe}^*) resulting from addition of BSA, normalized to the viscosity in the absence of viscogen, as reported for a given probe was extracted from

$$\frac{\eta^*}{\eta_{\text{buff}}} = \frac{D_{\text{probe, buff}}}{D_{\text{probe, BSA}}} = \frac{d_{\text{probe, buff}}}{d_{\text{probe, BSA}}}, \quad [6]$$

where $d_{\text{probe, BSA}}$ and $d_{\text{probe, buff}}$ are the diffusion decay rates measured in the presence and absence of BSA, respectively. Further details are given in [Supporting Information](#).

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