Supporting Information for
Assessing site-specific enhancements imparted by hyperpolarized water in
folded and unfolded proteins by 2D HMQC NMR
Or Szekely†, Gregory Lars Olsen‡, Mihajlo Novakovic‡, Rina Rosenzweig‡, Lucio Frydman*,†
Departments of †Chemical and Biological Physics and ‡Structural Biology, The Weizmann
Institute of Science, 234 Herzl Street, Rehovot 760001, Israel
§Current address: Department of Biochemistry, Duke University School of Medicine, 307 Research Drive,
Durham NC 27710, USA

NMR Spectroscopy. Sample temperature is an important aspect for the claims made in this
study. In principle temperatures should be quite stable and uniform throughout the injections:
both the protein solution waiting inside the NMR and the hyperpolarized water injected into the
NMR are comixed and maintained at the target temperature—the protein pre- and post-injection
solutions as regulated in-situ by the NMR console’s VT system, and the hyperpolarized
water as controlled by a heating-tape-based system. As long as the water is injected above
≈35 °C (the temperature at which it arrives from the Hypersense/Arduino system), this pre-
calibrated approach should give a solution whose temperature matches that of the protein
and hence is constant through space and time.

Figure S1. Examining the reliability of HyperW’s 2D NMR thermal stability experiments at 50 °C. (A, B) Central peak
positions observed along the 1H (F2) dimension of a 50 °C HyperW 2D acquisition, for residue A39 and for the indole
W36 peak of the drkN SH3 (unfolded-form) protein—both of which relatively isolated (Fig. 7) and hence can be followed by
1D 1H projections. The temperature-dependence of these peaks was independently measured by 2D HMQC NMR, leading to
the actual temperatures indicated on the left-hand axes. Shown for completion (dashed lines) are the chemical shifts measured
in the 2D HyperW HMQC spectrum for these residues, leading to ≈49.25±0.25 °C as the representative temperature of this
experiment. (C) 1D traces leading to the results illustrated in panel (B), illustrating both the maxima but also the line shape
changes undergone by the W36 indole (U) peak for two post-injection times. The dashed lines indicate the chemical shifts
measured for the same peak by conventional 2D HMQC NMR at 47 and 50 °C. Notice as well the significant and clearly larger
enhancement shown by the folded (F) signal of this residue, as well as its ensuing line narrowing with time.
Still, driven by the importance that temperatures have in defining folded/unfolded protein ratios, particularly for drkN SH3’s 50 ºC dissolution experiments, ancillary analyses were made. Panels A and B in Figure S1 summarize these, by plotting the central peak positions observed along the $^1$H (F2) dimension of a 50 ºC HyperW 2D acquisition, for two residues of the drkN SH3 protein that are both relatively isolated (and hence can be followed in the 1D projection) and show a relatively strong temperature dependence in their $^1$H chemical shifts. Independent measurements collected by conventional HMQC NMR in the 47-50 ºC range, allowed us to translate these HyperW $^1$H peak positions into sample temperatures as a function of post-dissolution time. These values are also shown in the left-hand axes of these figure’s panels, and paint coinciding pictures regarding the system’s stabilization following the injection. According to these, there is a certain drop in the sample temperature immediately upon injection of the pre-heated water (to ≈48 ºC), but a nearly perfect thermal stabilization at the targeted 50 ºC temperature ca. 15 s past the injection. These examinations can be extended by analyzing the central peak positions displayed by the residues along the $^1$H (F2) dimension, following full 2D processing of the HyperW data. These Fourier-averaged positions should reveal the most representative temperature (and peak intensities) reflected in the 2D HMQC experiments, and they end up being between 49 and 49.5 ºC (dashed lines in Figures S1A, S1B). Further insight into how samples in 2D HyperW HMQC experiments reach their final temperatures can be gathered from the line shape changes exhibited by the traces illustrated in Figure S1C, which focus on the unfolded SH3 W36 indole resonance—one of the thermally-sensitive peaks that were examined. When analyzed in the $(t_1,F2)$-domain for two different post-dissolution times $t_1$ and compared with traces collected for fully-thermally-equilibrated, H$_2$O-dissolved samples, one can notice that peaks are broader at the beginning of the HyperW series (with a maximum at the equivalent of ca. 48 ºC), most likely reflecting a thermal distribution within the NMR tube. Subsequently, peaks sharpen up and coincide with the unfolded chemical shift recorded on a thermally polarized, thermally stabilized sample at 50 ºC. This sharpening leads to dominating peak positions and intensities corresponding to ca. 49.25±0.25 ºC, when Fourier processing the full 2D HyperW HMQC interferogram. In addition, notice how the 1D 17s post-injection trace highlights the orders-of-magnitude enhancements that the folded resonance in this residue gains from the hyperpolarized water over its unfolded counterpart—which of course is one of the paper’s main findings.

**Figure S2.** Pulse sequence for the 2D HyperW $^1$H-$^{15}$N HMQC used in this study. Full bars represent 90° hard pulses, and shapes represent amide-selective 90° and 180° pulses. The recycle delay $d_1$ was typically set to 0.037-0.1 s; water polarization was achieved during $t_{DNP}$ ≈120-180 min, and the subsequent dissolution and injection of hyperpolarized water occurred during $t_{injection}$ ≈2-3 s. Selective excitation of the amide protons was achieved using a PC9 polychromatic pulse, refocusing with a REBURP pulse centered at 8.5 ppm with a 3.0 ppm bandwidth, and typically $N_1$=128 increments were collected. The sequence employed the indicated phase-cycling of the $^{15}$N excitation and storage pulses, to reduce the water background and to deliver by a hypercomplex acquisition purely absorptive lineshapes. Decoupling on the $^{15}$N channel was done using GARP modulation during the acquisition.

The two dimensional spectra were acquired using a 2D HyperW $^1$H-$^{15}$N HMQC sequence, similar to what was used in previous work (Fig. S2). It is a 2D HMQC-based sequence, using a solute-specific
excitation approach. The amide downfield region is excited using a selective 90° pulse in order to maximize the use of the hyperpolarized exchanged sites while minimizing water depolarization.

**PhoA**\(^{(350-471)}\): **Per protio and SSP results.** It is of interest to compare the HyperW HMQC spectrum (Fig. 1, red) not only to a thermal equilibrium spectrum measured on the same post-dissolution sample (Fig. 1, blue), but also against a thermal spectrum measured in an 82.5% H\(_2\)O buffer under otherwise same conditions. A comparison between these data (Fig. S3) and Fig. 1 reveals that indeed with 82.5% water, one could observe peaks that are broadened beyond detection in the thermal post-dissolution sample, albeit with very poor sensitivity. Peaks are observed with a better sensitivity in the HyperW spectrum, thanks to a nearly ~500\(^\times\) enhancement.

![Figure S3](image)

**Figure S3.** Conventional \(^1\)H-\(^{15}\)N HMQC spectrum for 0.125 mM \(^{15}\)N-PhoA4 dissolved in 82.5% H\(_2\)O buffer (50 mM HEPES, pH 7.5, 50 mM KCl). The spectrum was recorded at 50 °C using 64 hypercomplex \(t_1\) increments covering indirect- and direct-domain spectral widths of 6009.6 and 1825.8 Hz. Additional experimental parameters: 14.1 T Prodigy®-equipped Bruker Avance III NMR spectrometer; total experimental time of 42 min 56 sec (16 scans recorded per \(t_1\) increment, acquisition time of 213.0 ms, repetition delay of 1 s). The peaks in the bottom part of the spectrum arise due to slight protein degradation.

Unlike what had been previously reported for α-synuclein, the sensitivity enhancements evidenced by PhoA’s HyperW HMQC, do not appear to correlate with the electrostatic charges in the protein sequence (Fig. 2). To explore potential correlations between the enhancements and the SSP values, Supporting Figure S4 compares both individual enhancements vs SSP scores, as well as the running-average enhancements for every three consecutive residues against the SSP average score of the same three residues. A modest correlation appears to emerge in the latter, but it is hard to ascertain.
Variable temperature ZZ-exchange NMR on drkN SH3. ZZ-exchange is a kinetic experiment based on a 2D NMR $^1$H-X chemical shift correlation,\textsuperscript{9-10} with the addition of a mixing delay $T$ during which the magnetization is stored along the z-axis while dynamics take place. The SH3 domain exists in two exchanging states (U and F), such that a given nucleus resonates at a frequency $\omega_U$ in state U and $\omega_F$ in state F. A 2D ZZ-exchange spectrum for this system (Fig. S5A) will thus contain two diagonal-peaks at $U(\omega_U^{13C}, \omega_U^{1H})$ and $N(\omega_F^{13C}, \omega_F^{1H})$ in the (F1, F2) frequency dimensions, and two cross-peaks at $C1(\omega_F^{13C}, \omega_U^{1H})$ and $C2(\omega_U^{13C}, \omega_F^{1H})$, due to the $U \leftrightarrow F$ exchange occurring during the mixing time. In order to obtain kinetic information, a series of ZZ-exchange spectra are recorded with a range of mixing delays. The dependence of the peak intensities on the mixing delay are then analyzed and fit to a kinetic exchange model (Fig. S5B):\textsuperscript{11}

$$I_U(T) \quad I_U(0) = A_U \cdot \frac{-(\lambda_2 - a_{22}) \cdot e^{-\lambda_1 T} + (\lambda_1 - a_{22}) \cdot e^{-\lambda_2 T}}{\lambda_1 - \lambda_2}$$

\textbf{(S1)}

$$I_N(T) \quad I_N(0) = A_F \cdot \frac{-(\lambda_2 - a_{11}) \cdot e^{-\lambda_1 T} + (\lambda_1 - a_{11}) \cdot e^{-\lambda_2 T}}{\lambda_1 - \lambda_2}$$

\textbf{(S2)}

$$I_{C1}(T) \quad I_U(0) = A_F \cdot \frac{a_{12} \cdot e^{-\lambda_1 T} - a_{22} \cdot e^{-\lambda_2 T}}{\lambda_1 - \lambda_2}$$

\textbf{(S3)}

$$I_{C2}(T) \quad I_N(0) = A_U \cdot \frac{a_{21} \cdot e^{-\lambda_1 T} - a_{22} \cdot e^{-\lambda_2 T}}{\lambda_1 - \lambda_2}$$

\textbf{(S4)}
where \( \lambda_{1,2} = \frac{1}{2} \left\{ (a_{11} + a_{22}) \pm \left[ (a_{11} - a_{22})^2 + 4k_{F \rightarrow U}k_{U \rightarrow F} \right]^{1/2} \right\} \),

\[ a_{11} = R_F^U + k_{U \rightarrow F}, \quad a_{12} = -k_{F \rightarrow U}, \quad a_{22} = R_U^F + k_{F \rightarrow U} \]

and \( a_{21} = -k_{U \rightarrow F} \). \( R_F^U \) and \( R_U^F \) are the longitudinal relaxation rate constants of magnetization in sites U and F, respectively, and \( I_U(0) \) and \( I_N(0) \) are the peak intensities in the unfolded and folded states, respectively, at \( T = 0 \). The factors \( A_F \) and \( A_U \) represent efficiency of coherence transfer after the mixing period \( T \), and were determined as described previously.\(^{12}\) The simultaneous fits to the data yield the first-order rate constants \( k_{F \rightarrow U} = 31.0 \pm 4 \) s\(^{-1}\) and \( k_{U \rightarrow F} = 1.9 \pm 0.4 \) s\(^{-1}\). The populations at 50 °C are therefore: \( p_U = 94.3\% \) and \( p_F = 5.7\% \); these ZZ-exchange results (Fig. S5), which take into account compensation for differences in the relaxation rates (\( R_1 \) and \( R_2 \)) of the peaks. The folded state population of only 5.7% at 50 °C, is to be contrasted to the 55% observed at 27 °C. Populations and exchange rates at 37 °C were also calculated using a \(^1\)H-\(^{15}\)N version of the ZZ-exchange experiment, as \( k_{F \rightarrow U,37C} = 7.9 \pm 2.8 \) s\(^{-1}\), \( k_{U \rightarrow F,37C} = 8.7 \pm 2.9 \) s\(^{-1}\), \( p_{U,37C} = 48\% \) and \( p_{F,37C} = 52\% \). Table S1 summarizes these kinetic and population values, as derived by these measurements on SH3 at the three temperatures that we explored.

![Figure S5](image-url) ZZ-exchange experiment to extract kinetic information on the drkN-SH3 domain U⇌F exchange. (A) Selected regions from one ZZ-exchange spectrum (corresponding to a mixing period of \( T = 0.05 \) s), measured on an 800 MHz spectrometer, equipped with a cryo-cooled HCN probe. Indirect- and direct-domain spectral widths of 12019.2 and 4022 Hz were covered, using 82 hypercomplex \( t \) increments and STATES acquisition.\(^4\) \( N_s = 16 \) scans were collected using a 3 s acquisition time, and a relaxation delay of 1.5 s. Total experimental time was \( \sim 3 \) hrs 20 min for each different mixing time \( T \). The assigned residues are denoted by their residue number, and the peaks are labeled as: U – unfolded diagonal-peak, F – native (folded) diagonal-peak, C1 and C2 – cross-peaks. (B) Normalized peak intensities as a function of mixing times for selected residues, for the diagonal-peak and the C1 and C2 cross peaks. The data points are fitted to Eqs. (S2), (S3) and (S4) to extract kinetic information.

**Table S1.** Kinetic parameters for the U⇌F process of drkN SH3 domain, as derived from ZZ-exchange measurements for 50 °C, 100% D\(_2\)O; and 37 °C, 90% H\(_2\)O; and from HMQC peak intensity ratios at 27 °C, 90% H\(_2\)O.

| Temperature (°C) | Relative population of the folded state (\( p_U \), %) | Relative population of the unfolded state (\( p_F \), %) | \( U \leftrightarrow F \) exchange rate \( k = k_{F \rightarrow U} + k_{U \rightarrow F} \) (s\(^{-1}\)) | \( k_{F \rightarrow U} \) (s\(^{-1}\)) | \( k_{U \rightarrow F} \) (s\(^{-1}\)) |
|-----------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-----------------|-----------------|
| 50              | 6 ± 10                                          | 94 ± 10                                         | 33 ± 4                                          | 31 ± 4          | 1.9 ± 0.4       |
| 37              | 52 ± 10                                         | 48 ± 10                                         | 17 ± 3                                          | 8 ± 3           | 9 ± 3           |
| 27              | 60 ± 20                                         | 40 ± 20                                         | 33 ± 4                                          | 31 ± 4          | 1.9 ± 0.4       |
Methyl-TROSY NMR experiments on drkN SH3. The relative populations in Table S1 were derived from ZZ-exchange experiments performed on mostly deuterated (90% D$_2$O) solutions. D$_2$O as a solvent, however, has been reported to stabilize certain protein structures when compared to H$_2$O, and to affect protein folding-unfolding kinetics.$^{21,22}$ The dDNP enhancements reported for drkN SH3, however, are done by comparing HyperW results arising from deuterated solutions, against thermal results arising from mostly protonated ones. It follows that in order to properly quantify the signal enhancement upon hyperpolarization, one must also take into account the potential differences in the populations observed for the folded states in D$_2$O (the solvent used in the HyperW measurements) vs. in H$_2$O (the solvent used for the thermal equilibrium measurements). As solvent exchanges prevent us from measuring the populations of drkN SH3’s folded and unfolded forms by relying on the amide group resonances using D$_2$O as solvent, a methyl labeled ($^{13}$CH$_3$-ILVM, $^2$H) drkN SH3 protein was expressed, and the populations of these two forms were quantified by integrating the peak intensities of ten residues in the folded and unfolded states using methyl-TROSY $^1$H-$^{13}$C.$^{23}$ These experiments were carried out at 50 °C in both 90% H$_2$O and in 100% D$_2$O. The ensuing results are summarized in Table S2. As can be appreciated from these results, the populations of the folded states at 50 °C in D$_2$O were indeed higher than those in H$_2$O: 5.7 ± 0.6%, vs 4.0 ± 0.7%. At the same time, the deuterated solvent methyl-TROSY results were in excellent agreement with the ZZ-exchange measurements. These methyl-TROSY-derived populations were used in the simulations described throughout the Supporting and the Main texts; these populations were also used to rescale the kinetic rates in Table S1, as appropriate.

**Table S2.** Folded-state populations extracted for various methyl residues of drkN SH3, as derived from methyl-TROSY measurements performed at 50 °C and 90/10% mixtures of mostly per-deutero and per-protoio aqueous solutions. Data were recorded at 800 MHz using a TCI Cryoprobe®.

| drkN SH3 Residue | % - Folded state population |
|------------------|-----------------------------|
|                  | Solvent – D$_2$O | Solvent – H$_2$O |
| Ile4 δ$_1$       | 5.9             | 3.8             |
| Leu17 δ          | 5.8             | 4.2             |
| Leu25 δ          | 5.8             | 3.3             |
| Ile27 δ          | 4.2             | 2.9             |
| Leu41 δ$_1$      | 5.3             | 4.0             |
| Leu41 δ$_2$      | 5.7             | 3.4             |
| Ile48 δ$_1$      | 6.3             | 5.6             |
| Ile53 δ$_1$      | 6.8             | 3.2             |
| Leu30 δ          | 5.6             | 3.8             |
| Leu50 δ          | 5.9             | 4.9             |
Supporting Figure S6 compares CLEANEX-PM measurements at 37 °C, with the HyperW enhancements observed for the folded and unfolded residues of the drkN-SH3 domain.

**Figure S6.** Comparison of HyperW enhancements and CLEANEX-derived exchange rates for the folded (left) and unfolded (right) states of drkN SH3 domain at 37 °C. (A) Comparison of amide proton exchange rates $k_{FW}$ arising for different drkN SH3 residues in the folded state as extracted from CLEANEX-PM experiments at 14.1 T and 37 °C (black squares), with the corresponding HyperW HMQC sensitivity enhancements at 37 °C (blue circles, taken from Fig. 8B). Orange and green shaded areas are drawn in the bottom panel for regions which correspond to the secondary structure elements depicted in the top panel (as in Figs. 8A and 8B). The linear correlation coefficient (bottom), between the HyperW enhancements and CLEANEX-PM exchange rates for the folded state at 37 °C is 0.85. (B) Comparison of amide proton exchange rates $k_{UW}$ arising for different drkN SH3 residues in the unfolded state as extracted from CLEANEX-PM experiments at 14.1 T and 37 °C (black squares), with the corresponding HyperW HMQC sensitivity enhancements at 37 °C (red circles, taken from Fig. 8C). The correlation coefficient (again calculated for the data in a linear plot) between the HyperW enhancements and CLEANEX exchange rates for the unfolded state at 37 °C (bottom) was 0.49.
Figure S7. Same as Figure 8E in the main text, but assuming that the HyperW injection temperature had been misscalibrated and actually took place at 47 °C.

Supporting Figure S7 re-examines drkN SH3 HyperW’s enhancements measured at 50 °C, assuming that post-injection temperatures were not as believed but instead lower by 3 °C – a difference that is still compatible with the peak positions recorded in the HMQC NMR spectra. Indeed, although Fig. S1 attests to the good thermal reliability of our setup, the linewidths of the HyperW NMR data yield a certain uncertainty in the temperature, which is bound by a lower limit of 47 °C. This plot is a recalculation of the enhancement data presented in Figure 8E, but with enhancements renormalized according to thermally polarized reference spectra measured at 47 °C. As evidenced by this plot, this still leads to a picture where folded-residue peaks are more enhanced by the hyperpolarized solvent than their unfolded-state counterparts.

Table S3 summarizes the enhancements observed for the various folded and unfolded drkN SH3 residues at 50 °C, taking into account multiple dissolutions and the population considerations in Table S2. Comments indicate why the corresponding residues were not utilized in the paper’s discourse/conclusions.

Table S3. Average enhancements ± deviation obtained from three separate hyperpolarized water injections for folded and unfolded residues of the drkN SH3 domain at 50 °C.

| Folded/Unfolded | Residue | Enhancement-deviation | Comments |
|-----------------|---------|-----------------------|----------|
| F               | E2      | 170 ± 50              |          |
| F               | A3      | 200 ± 60              |          |
| F               | I4      | 70 ± 10               |          |
| F               | A5      | 100 ± 20              |          |
| F               | K6      | 120 ± 20              |          |
| F               | H7      | 30 ± 40               |          |
| F               | D8      | 90 ± 40               |          |
| F               | F9      | 120 ± 20              |          |
| F               | S10     | 230 ± 20              |          |
| F               | A11     | 50 ± 30               |          |
| F               | T12     | 160 ± 40              |          |
| F               | A13     | 210 ± 90              |          |
| F               | D14     | 90 ± 40               |          |
| F               | D15     | 100 ± 20              |          |
| F               | E16     | 49 ± 8                |          |
|     |     |     |
|-----|-----|-----|
| F   | L17 | 58 ± 5 |
| F   | S18 | 340 ± 20 |
| F   | F19 | 240 ± 70 |
| F   | R20 | 75 ± 8 |
| F   | K21 | 75 ± 1 |
| F   | T22 | 200 ± 40 |
| F   | Q22 |     |
| F   | I23 |     |
| F   | L25 | 50 ± 20 |
| F   | K26 |     |
| F   | I27 | 58 ± 5 |
| F   | L28 | 42 ± 4 |
| F   | N29 | 96 ± 9 |
| F   | M30 | 130 ± 50 |
| F   | E31 | Not identified |
| F   | D32 | 23 ± 3 |
| F   | D33 | Not identified |
| F   | S34 | 180 ± 200 Heavy overlap |
| F   | N35 | 70 ± 10 |
| F   | W36 | Not identified |
| F   | W36_INDOLE | 70 ± 30 |
| F   | Y37 | 24 ± 2 |
| F   | R38 | 57 ± 6 |
| F   | A39 | 90 ± 50 |
| F   | E40 | 100 ± 10 |
| F   | L41 | 80 ± 20 |
| F   | D42 | 300 ± 40 |
| F   | G43 | 130 ± 20 |
| F   | K44 | 39.1 ± 0.8 |
| F   | E45 | 23 ± 3 |
| F   | G46 | 120 ± 10 |
| F   | L47 | 75.4 ± 0.9 |
| F   | I48 | 29 ± 6 |
| F   | P49 | Not identified |
| F   | S50 | 31 ± 2 |
| F   | N51 | 140 ± 20 |
| F   | Y52 | 110 ± 10 |
| F   | I53 | Not identified |
| F   | E54 | 31.0 ± 0.8 |
| F   | M55 | 190 ± 20 |
| F   | K56 | 65 ± 10 |
| F   | N57 | 30 ± 2 |
| F   | H58 | Not identified |
| Position | Name | Value | Comment |
|----------|------|-------|---------|
| F        | D59  | 19 ± 4|         |
| U        | E2   | Not identified |      |
| U        | A3   | Not identified |      |
| U        | I4   | Not identified |      |
| U        | A5   | 55 ± 20 |         |
| U        | K6   | Not identified |      |
| U        | H7   | Not identified |      |
| U        | D8   | Not identified |      |
| U        | F9   | Not identified |      |
| U        | S10  | 60 ± 50 | Hard to identify |
| U        | A11  | 100 ± 90 | Overlap |
| U        | T12  | 60 ± 20 |         |
| U        | A13  | 75 ± 70 | Overlap |
| U        | D14  | Not identified |      |
| U        | D15  | Not identified |      |
| U        | E16  | Not identified |      |
| U        | L17  | Not identified |      |
| U        | S18  | 200 ± 200 | Heavy overlap |
| U        | F19  | Not identified |      |
| U        | R20  | Not identified |      |
| U        | K21  | Not identified |      |
| U        | T22  | 85 ± 70 | Ambiguous assignment |
| U        | Q22  | Not identified |      |
| U        | I23  | Not identified |      |
| U        | L25  | 18.7 ± 0.5 |         |
| U        | K26  | Not identified |      |
| U        | I27  | Not identified |      |
| U        | L28  | 18 ± 2 |         |
| U        | N29  | 53 ± 2 |         |
| U        | M30  | Not identified |      |
| U        | E31  | 33 ± 2 |         |
| U        | D32  | Not identified |      |
| U        | D33  | Not identified |      |
| U        | S34  | 40 ± 10 |         |
| U        | N35  | Not identified |      |
| U        | W36  | 36 ± 1 |         |
| U        | W36_INDOLE | 13.75 ± 0.04 |         |
| U        | Y37  | 19 ± 2 |         |
| U        | R38  | 16 ± 2 |         |
| U        | A39  | 18.7 ± 0.5 |         |
| U        | E40  | 53 ± 2 |         |
| U        | L41  | Not identified |      |
| U        | D42  | Not identified |      |
| U   |     |     |
|-----|-----|-----|
|     | G43 | 39 ± 6 |
| U   | K44 | Not identified |
| U   | E45 | 44 ± 2 |
| U   | G46 | 39 ± 6 |
| U   | L47 | Not identified |
| U   | I48 | 9 ± 2 |
| U   | P49 | Not identified |
| U   | S50 | Not identified |
| U   | N51 | Not identified |
| U   | Y52 | Not identified |
| U   | I53 | 16 ± 2 |
| U   | E54 | 16 ± 6 |
| U   | M55 | 31 ± 8 |
| U   | K56 | 31 ± 8 |
| U   | N57 | 36 ± 1 |
| U   | H58 | Not identified |
| U   | D59 | 27 ± 3 |
| U   | Overlap of multiple sites | 35 ± 1 |
| U   | Overlap of multiple sites | 46 ± 2 |
| U   | Overlap of multiple sites | 58 ± 1 |

\(^1\text{H}-^{15}\text{N} \text{HyperW HMQC for a three-site exchanging system incorporating cross-relaxation: Theoretical Considerations.}\) Site-specific amide-water exchange rates lead to heterogeneities in the HyperW enhancement. However, the exchange rates in the folded state were expected to be slower relative to the unfolded state, due to protection factors and hydrogen bonds. Scheme 1 suggests that an additional magnetization transfer from an enhanced unfolded state residue to the same residue in the folded state can explain its observed sensitivity enhancements. Biases in the hyperpolarization of folded and unfolded residues could also arise from the different cross-relaxation behavior of these systems. To estimate how the HyperW signal enhancements will be affected by these exchanges, we computed the water and amide magnetizations for each conformation \(<\text{H}_2\text{O}>_z, <\text{H}_\text{N}^U>_z, <\text{H}_\text{N}^F>_z\) expected to arise in a process characterized by a forward reaction rate (proton transfers from \(\text{H}_2\text{O}\) to \(\text{H}_\text{N}\)) \(k_{\text{WU}}, k_{\text{WF}}\), and a backward reaction rate \(k_{\text{UW}}, k_{\text{FW}}\). These exchange rates are in fact related to each other by the water and protein molar fraction ratios \(X\):

\[
\begin{align*}
 k_{\text{UW}} &= \frac{X_{\text{H}_2\text{O}}}{X_{\text{H}_\text{N}^U}} \cdot k_{\text{WU}}, \\
 k_{\text{FW}} &= \frac{X_{\text{H}_2\text{O}}}{X_{\text{H}_\text{N}^F}} \cdot k_{\text{WF}} \\
\end{align*}
\]

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 k_{\text{FW}} &= \frac{X_{\text{H}_2\text{O}}}{X_{\text{H}_\text{N}^F}} \cdot k_{\text{WF}} \\
\end{align*}
\]

A model based on the McConnell-Solomon equations\(^{14-16}\) was implemented within a home-written Matlab\textsuperscript{®} (The Mathworks Inc.) code that involved numerical solution of the system of differential equations for different proton reservoirs, including chemical exchange and cross-relaxation.
between amide and aliphatic proton pools as well as with protons in the hyperpolarized H\textsubscript{2}O pool. This leads to the system of 7 differential equations:

\[
\frac{d}{dt} \begin{pmatrix}
\langle H_N^F \rangle_z(t) - \langle H_N^F \rangle_z(eq) \\
\langle H_N^U \rangle_z(t) - \langle H_N^U \rangle_z(eq) \\
\langle H_C^F \rangle_z(t) - \langle H_C^F \rangle_z(eq) \\
\langle H_C^U \rangle_z(t) - \langle H_C^U \rangle_z(eq) \\
\langle H_X^F \rangle_z(t) - \langle H_X^F \rangle_z(eq) \\
\langle H_X^U \rangle_z(t) - \langle H_X^U \rangle_z(eq) \\
\langle H_2O \rangle_z(t) - \langle H_2O \rangle_z(eq)
\end{pmatrix} = \begin{pmatrix}
-r_F & k_{UF} & \sigma_F & 0 & \sigma_{XF} & 0 & k_{WF} + \sigma_{WF} \\
k_{FU} & -r_U & 0 & -R_{1H}^{HF} & \sigma_{CU} & 0 & k_{WF} + \sigma_{WU} \\
\sigma_F & 0 & -R_{1H}^{HF} & k_{UF} & 0 & \sigma_{WU} & 0 \\
0 & \sigma_U & k_{FU} & -R_{1H}^{HF} & 0 & 0 & \sigma_{WU} \\
\sigma_{XF} & 0 & \sigma_{XF} & 0 & -R_{1H}^{HF} & k_{UF} & k_{WX} + \sigma_{WFX} \\
0 & \sigma_{XU} & 0 & \sigma_{XU} & k_{FU} & -R_{1H}^{HF} & k_{WX} + \sigma_{WX} \\
k_{FW} + \sigma_{FW} & k_{WF} + \sigma_{WF} & 0 & 0 & k_{WX} + \sigma_{WFX} & k_{WX} + \sigma_{WX} & -r_W
\end{pmatrix}
\]

(S6)

The relaxation matrix used in this model was generated using the Bloch-Redfield-Wangsness theory on a reduced spin system\textsuperscript{17} in combination with SpinDynamica\textsuperscript{18} Five spins were included to account for the polypeptide backbone and sidechain (H\textsubscript{N}, N\textsubscript{H}, two aliphatic sidechain protons H\textsubscript{C} and H\textsubscript{X}, one labile sidechain protons H\textsubscript{X}) and a reduced relaxation matrix with only longitudinal terms for each spin present was utilized. A model-free approach with order parameters for each interaction was adopted\textsuperscript{19} with spectral densities given by the general form:

\[
J_i(\omega) = \frac{2}{5}S_i^2 \frac{\tau_c}{1 + (\omega \tau_c)^2}
\]

(S7)

The final relaxation matrix includes two spin order longitudinal terms of folded and unfolded conformations for the amide protons, two corresponding terms for the aliphatic protons, one for the sidechain labile proton, and one for the external water proton. Cross-relaxation between the
amide and aliphatic spin pools were assumed to differ for the folded and unfolded states, given in each case by:

$$\sigma = \frac{1}{10} \delta_{HH}^2 [f(2\omega_H) - f(0)]$$  \hspace{1cm} (S8)

where $\delta_{HH} = (\mu_0 \gamma_H^2 h)/(8\pi d_{HH}^3)$. Diagonal elements in the relaxation matrix are given by $r_F = k_{FW} + k_{FU} + R_{1F}^{HF} + \frac{1}{\tau_1^R}$, $r_U = k_{UW} + k_{UF} + R_{1U}^{HnU} + \frac{1}{\tau_1^U}$, $R_{1F}^{HF}$, $R_{1U}^{HnU}$ and $r_W = \frac{1}{\tau_1^W} + k_{WU} + k_{WF}$.

The rates $k_{FW}$ and $k_{UF}$ represent the exchange rates between the folded and unfolded states (see Scheme 1). $R_{1F}^{HF}$, $R_{1U}^{HnU}$, $R_{1F}^{HF}$ and $R_{1U}^{HnU}$ are the corresponding auto-relaxation rates of the amide and aliphatic protons in the folded and unfolded states, including dipolar interactions between the $^{15}$N and H$_N$, H$_N$ and H$_C$, as well as between pairs H$_C$–H$_C$ of aliphatic protons. Based on this, the rates are then given by

$$R_{1F}^{Hn} = \frac{1}{10} \left( \delta_{HnH}^2 [J_2(0) + 3J_2(\omega_H) + 6J_2(2\omega_H)] \right)$$

$$R_{1F}^{HF} = \frac{1}{10} \left( \delta_{HnH}^2 [J_2(0) + 3J_2(\omega_H) + 6J_2(2\omega_H)] \right)$$

Additional intrinsic relaxation rates $1/T_1^{UF}$ were also added to the relaxation terms of each amide proton, in a search for an additional ad hoc parameter that might potentially explain drkN SH3’s anomalous HyperW behavior. Order parameters and internuclear distances were chosen from the literature for Ubiquitin at room temperature, which is a fair assumption based on the very similar molecular weights of Ubiquitin and drkN-SH3 domain. The $R$ and $\sigma$ rates will mostly depend on the internuclear amide/aliphatic distance $d_{HH}$ (kept constant at 2.3 Å for the folded and unfolded states for simplicity) and on the internuclear correlation time $\tau_c$, which was taken to be 3.4 ns for the folded state and 0.8 ns for the unfolded state of the protein. As purely intramolecular cross-relaxation models failed to predict larger folded than unfolded enhancements unless exchange rates $k_{FW}, k_{UF}$ were invoked, Eq. (S6) was modified to enable the presence of intermolecular water-amide proton-proton cross-relaxation. This interaction was incorporated into the simulations in a manner similar to that in Eq. (S8); for simplicity, the same correlation times were assumed to control the intra- and inter-molecular cross-relaxation processes (3.4 ns for the folded, 0.8 ns for the unfolded H—H vectors). $\langle H_2O \rangle_z(eq), \langle H_N \rangle_z(eq)$ and $\langle H_C \rangle_z(eq)$ in Eq. (S6) are the water and protein amide and aliphatic magnetizations at thermal equilibrium. Complementing Eq. (S6)’s time-dependence, the evolution of $\langle H_N \rangle_z(t)$ was artificially set to zero at $t = n TR$ (where $TR$ is the experimental repetition time) to account for the depletion of protein magnetization arising due to the selective excitation pulses applied. Equation (S6) plus this reset condition were used for analyzing both the HyperW (Hyp) and the thermal equilibrium (TE) experiments that were carried out, which were recorded on the same samples under identical conditions –apart from their initial water.
polarization. The initial water magnetization was \( \langle H_2O \rangle_z(0) = e \langle H_2O \rangle_z(eq) \), where \( e \) is the enhancement factor over the thermal equilibrium polarization (\( e = 200 \) for the Hyp experiment; and \( e = 1 \) for the TE experiment). The initial polarization for the amide protons in the protein was assumed to be \( \langle H_N \rangle_z(0) = 0 \); \( \langle H_C \rangle_z(0) \) and \( \langle H_C \rangle_z(eq) \) were set equal to unity. For both cases (Hyp and TE) the equilibrium polarization was scaled according to the concentrations:

\[
\langle H_2O \rangle_z(eq) = \frac{X_{H_2O}}{X_{H_2O}} \equiv X_{WF} ; \langle H_N^U \rangle_z(eq) = \frac{X_{H_N^U}}{X_{H_N^U}} \equiv X_{UF} ; \langle H_N^F \rangle_z(eq) = 1 \] and same holds for aliphatic spin pools.

In order to translate the magnetizations that will be predicted by these equations into observable signals, we further considered that in the full 2D HyperW \(^1\text{H}^-15\text{N}\) HMQC experiment these will have to be converted into a \(^1\text{H}\) coherence that transfers to and from the amide nitrogens:

\[
\langle H_N \rangle_z^{\text{pulse}} \rightarrow (H_N)_{x/z}^{15N} \rightarrow (15N)_{x/z}^{15N} \rightarrow (15N)_{x/y}(t) \rightarrow (H_N)_{x/y}(\text{detect}) \] (S10).

Besides \( T_2 \)-derived losses that for simplicity were ignored, the efficiency of these coherence transfers/encodings will also depend on the inverse \( H_N^-H_2O \) rate constant \( (k_{UF}, k_{FW}) \): indeed, rapid exchanges of the amide proton with the solvent will preclude an efficient coherence transfer to the \(^{15}\text{N} \) evolving during \( t_I \). This will lead to an overall exponential signal decay, where the duration of the decay period for the \( n \)th \( t_I \) increment can be expressed as (see Fig. S2):

\[
t_{acq}(n) = P90H + \frac{1}{t_{HN}} + 2 \cdot P90N + t_I(n) + P180H \] (S11).

Accordingly, we express the average signal per scan after a total of \( N_I \) increments \( t_I \) as:

\[
S_U(TR,k_{UF},k_{FW}) = \frac{1}{N_I} \sum_{n=1}^{N_I} \langle H_N^U \rangle_z(nTR,k_{UF},k_{FW}) \cdot e^{-k_{UF} \cdot t_{acq}(n)} \] (S12a)

\[
S_F(TR,k_{UF},k_{FW}) = \frac{1}{N_I} \sum_{n=1}^{N_I} \langle H_N^F \rangle_z(nTR,k_{UF},k_{FW}) \cdot e^{-k_{UF} \cdot t_{acq}(n)} \] (S12b)

where we stress the potential dependence of the amide magnetization on the time \( t \) that each \( t_I(n) \) increment will have associated since the injection of the hyperpolarized solvent. On the basis of all these considerations, the various 3D plots shown in Fig. 10 of the main text were computed.

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