ON-LINE METHODS

NMR spectroscopy. Uniformly ^{15}N-labeled or ^{2}H,^{15}N-labeled ubiquitin was prepared as described previously [40]. ^{2}H,^{15}N-labeled ubiquitin in 50 mM sodium acetate, pH 5, with 50 mM NaCl was encapsulated in 75 mM AOT reverse micelles in 99% perdeuterated pentane at W_0 = 9.0. Water loading was determined by ^{1}H NMR. For both aqueous and reverse micelle samples, the pH of the sample was confirmed using the pH-dependent ^{1}H chemical shifts of the buffers as internal NMR standards, acetate (pH 5–7) or imidazole (pH 7–9)[41]. Three-dimensional sensitivity-enhanced[42] ^{15}N-resolved NOESY-HSQC[29,43-45] and ROESY-HSQC[30,46,47] spectra were collected at 500 MHz (^{1}H) on a Bruker AVANCE III spectrometer equipped with a cryoprobe. Three-dimensional NOESY and ROESY spectra were collected with 64 (RM) or 48 (aqueous) and 80 complex increments in the indirect ^{15}N and ^{1}H dimensions, respectively. The ROESY experiment employed a 8.33 kHz CW spin-lock field with the 90x–SLy–90x scheme in order avoid spin-lock offset effects[48]. Mixing times for all three-dimensional experiments were 40 ms with a recycle delay of 1.4 s. Water suppression was achieved using a water-selective sin x/x flip back pulse after the mixing time. Quadrature detection was achieved by gradient selection and States-TPPI in the ^{15}N and ^{1}H indirect dimensions, respectively.

NOE/ROE ratios were calculated by fitting the NOESY and ROESY peaks to Gaussian functions with Sparky[49] after processing with FELIX. A few weak peaks gave fits with large errors, therefore maximum peak intensities were used for these sites, as indicated in Supplementary Table 2. In the limit of short τ_m, the cross peak intensities are directly representative of the cross relaxation rates[50]. ^{15}N T_1 (ref. [51]) and H_zN_z (ref. [52,53]) relaxation times were measured and found to be long (> 500 ms and > 100 ms, respectively) compared to the mixing time of the three-dimensional NOESY experiment, thus auto-relaxation effects are minimal in the measured NOE

peaks. As a result, the fit volumes of the NOE signals in the water ^{1}H plane (4.32 p.p.m.) were directly proportional to the NOE cross-relaxation rate and were used without additional manipulation. Longitudinal relaxation in the transverse plane ($T_{1\rho}$) was measured using two-dimensional HSQC experiments for spectral resolution with spin locking as described above for the three-dimensional experiment. Nine mixing times were measured ranging from 2.9 ms to 80 ms with three times measured in duplicate. The measured $T_{1\rho}$ values (**Supplementary Table 1**) were comparable to the mixing time, thus the contributions of auto relaxation to the ROE cross peaks had to be taken into account. NOE/ROE ratios were calculated as shown in equation 1, which simply adjusts the intensity of the ROE cross peaks to account for the attenuation caused by auto relaxation[50].

$$\frac{NOE}{ROE} = \frac{NOE_{pv} e^{-\frac{\tau_{mix}}{T_{1\rho}}}}{ROE_{pv}} \qquad (1)$$

where the subscript '*pv*' denotes the fitted peak volumes of water cross peaks from the three-dimensional experiments. Cross peaks to water were identified by comparison of the ^{15}N-resolved NOESY spectrum of encapsulated ubiquitin to that of the protein in free aqueous solution. Calculated NOE/ROE ratios are given in Supplementary Table 2.

Structural analysis. Structural analysis of the hydration data obtained in the NOESY and ROESY experiments was performed using the reverse micelle ubiquitin NMR structure (PDB 1G6J). Identification of buried amide hydrogens was done using the Travel Distance Suite[54,55] using a 1.4 Å probe to represent water. This water radius was chosen to produce a liberal evaluation of water accessibility to the backbone amides. The minimum distance from each atom to the molecular surface was averaged over the 32 structures in the NMR-based ensemble [14] and

any amide hydrogen that was 4.0 Å or more from the molecular surface were considered to be buried, i.e. outside potential NOE-detection distance from water hydrogens. This distance was chosen to account for the 4.3–4.5 Å maximum range of the intramolecular NOEs detected with a 40 ms mixing time. Because the distances calculated are to the Connolly molecular surface[56] rather than the Lee-Richards solvent accessible surface[57], an additional ~0.5 Å must be added to account for the effective van der Waals radius of the water hydrogen. Of the eight amide hydrogens which are considered to be buried by these criteria, three show cross peaks to water. The water cross peaks for I3 and L56 were weak in both the NOESY and ROESY spectra, suggesting that these sites have more long-range interactions with water. I23 showed a strong ROE but a very weak NOE at the water resonance. This indicates that it has a more short-range interaction with water than other buried residues, but the mobility of the water interaction at this site must be quite high.

For calculation of the backbone amide hydrogen – labile hydrogen distances, the interatomic distance was calculated from every backbone amide hydrogen to every labile side chain hydrogen for all 32 structures in the 1G6J ensemble. The average of the distance minima over the 32 structures was then calculated. Of the 54 detected protein-water cross peaks, 32 come from backbone amide hydrogens which have an average minimum distance greater than 4.3 Å from any labile side chain hydrogen.

For comparison of the hydration data to the locations of crystallographic water molecules, the two available structures of wild type human ubiquitin (PDB 1UBQ and 1UBI) were compared. Both of these structures were solved using room temperature x-ray scattering and share the same space group and the same mother liquor composition[27,33]. The protein structures are virtually identical. Following superposition of protein coordinates, waters common to the two structures

were identified as those having oxygen coordinate differences of 1 Å or less. Of the 58 waters in 1UBQ and 81 waters in 1UBI, 36 were defined by this criterion as being common to both crystal structures (**Supplementary Fig. 4**).

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