Supporting Information for the manuscript


**Identification of Hydroxyl Protons, Determination of their Exchange Dynamics and Characterization of Hydrogen Bonding in a Microcrystallin Protein**

by

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**NMR experiments**
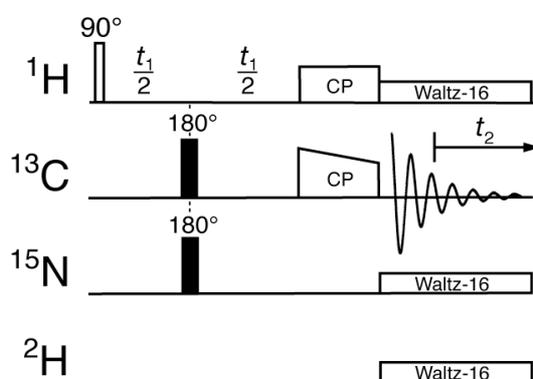


**Supporting Figure 1:** Pulse sequence for the two-dimensional HETCOR experiment. [13]C and ^{15}N scalar couplings were refocused in the indirect dimension by employing a 180° pulse on the ^{13}C and ^{15}N channel in the center of the evolution period. The rf carrier frequencies on the ^{1}H and ^{13}C channel were set to water and to 100 ppm, respectively. For ^{1}H,[13]C CP ($\tau_{CP}$ = 2.2 ms), the RF field on the carbon channel was ramped from 65.5 kHz to 45.5 kHz, and the proton field was adjusted in each experiment individually to match the (n=+1) rotary resonance condition. [1]H, ^{15}N and ^{2}H scalar decoupling during acquisition was achieved using WALTZ-16.[1] The decoupling rf field strength on proton and nitrogen was set to 2-2.5 kHz, respectively, while a rf field on the order of 1.5 kHz was employed on the deuterium channel. Figure 1 (main manuscript) was acquired within 40 h, with $t_1^{max}$ = 21.3 ms, acquiring 160 scans per increment on a Bruker 700 MHz quadruple resonance probe in which a deuterium coil was mounted onto the standard triple resonance setup for locking and deuterium decoupling. Except for T37OH, all other hydroxyl resonances decay within 10 ms of the indirect proton evolution period. In all experiments, the MAS frequency was set to 24 kHz. Spectra were measured at three different nominal temperatures, namely 245 K, 251 K and 271 K, which correspond to an actual sample temperature of 269 K, 278 K and 298 K, respectively. The effect of MAS on the sample temperature was evaluated independently using methanol as an external standard. The uncertainty in the estimation of the temperature is on the order of ± 2 K. Employing the empirical formula for the temperature dependence of the water frequency,[2,3] we calibrated the water chemical shift assuming the same temperature in both the methanol and the SH3 sample. In all cases, the flow rate of the cooling air was kept constant.
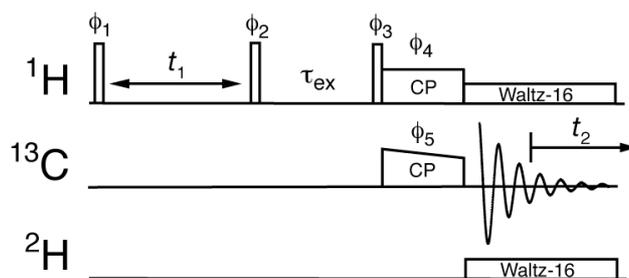
**Supporting Figure 2:** Pulse sequence for the quantification of hydroxyl proton exchange rates. The rf carrier on the $^1$H, $^2$H and $^{13}$C channel was set 5.1, 4.0 and 98 ppm, respectively. 90° rf pulses on the $^1$H channel were applied with an rf field of 70 kHz. During CP, a constant rf field of 70 kHz was applied on protons while the rf field on the carbon channel was ramped with a span of 10 kHz, achieving a frequency match at the (-1) rotary resonance condition. During $^{13}$C detection, WALTZ-16 decoupling was applied on the $^1$H and $^2$H channel applying a rf field strength below 5 kHz. Nine exchange experiments were recorded corresponding to mixing times of 10 μs, 1.0 ms, 2.5 ms, 5.0 ms, 7.5 ms, 10 ms, 25 ms, 50 ms and 100 ms. After every 2D exchange experiment, a 1D $^1$H,$^{13}$C correlation spectrum was recorded to check the integrity of the sample and the stability of the experimental conditions. Additionally, the 25 ms exchange spectrum was re-recorded and the integrated intensity was within experimental error. A recycle delay of 4 s was used between transients. The spectra were acquired as matrices with 160*5992 complex points with spectral widths of 7142.8 Hz and 50000 Hz in the $^1$H and $^{13}$C dimensions, respectively. The phase cycle was as follows: $\phi_1$=+y,-y, $\phi_2$=y, $\phi_3$=4(-y),4(+y), $\phi_4$=4(-x),4(+x), $\phi_5$= 2(+x),2(-x), $\phi_{rec}$= -x,+x,+x,-x, +x,-x,-x,+x. Phase sensitive detection in $t_1$ was achieved by means of TPPI. The experimental time per exchange point was on the order of 17 h. The exchange experiments were recorded on a Bruker 600 MHz Avance spectrometer using a conventional triple resonance 3.2 mm probe tuned to $^1$H, $^{13}$C and $^2$H. The MAS frequency was adjusted to 20 kHz. The effective temperature of the sample was maintained at 275 K.
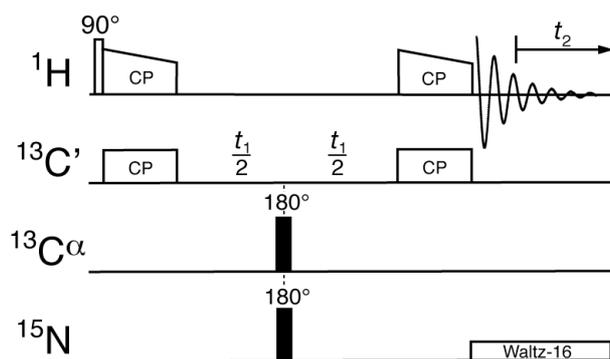
**Supporting Figure 3.** Pulse scheme to identify backbone hydrogen bonds in perdeuterated proteins. The rf carrier on the $^1$H, $^{13}$C and $^{15}$N channels was positioned at 4.8 ppm, 175 ppm and 110 ppm, respectively. Narrow (wide) bars indicate 90° (180°) pulses, using rf field strengths of 57, 48, and 51 kHz on the $^1$H, $^{13}$C and $^{15}$N channel, respectively. During the $t_1$ evolution period, the $^{13}$C carrier frequency was switched to the aliphatic region, and a low power 180° pulse was applied to decouple $C^\alpha$ from carbonyls. The two CP elements were identical ($\tau_{CP}$ = 10 ms), applying a constant rf field of 6 kHz on the $^{13}$C channel, while the rf field on the $^1$H channel was swept from 36 to 25 kHz, achieving a frequency match at the (-1) rotary resonance condition.[4,5] During detection, WALTZ-16 decoupling[1] on the $^{15}$N channel was applied using a rf field strengths of 2.0 kHz. The recycle delay between two consecutive scans was set to 2.8 s. The spectrum was acquired as a complex matrix with 128x24448 points, employing a spectral width of 3125 and 81521 Hz in the $^1$H and $^{13}$C dimensions, respectively. Phase-sensitive detection in $t_1$ was achieved using TPPI. The MAS frequency was adjusted to 24 kHz. The set temperature of the experiment was 274 K.
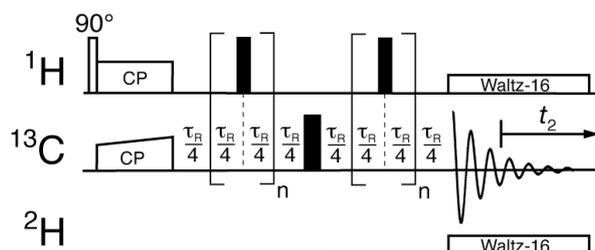


**Supporting Figure 4.** REDOR pulse scheme to quantitate interresidual longrange $^1$H,$^{13}$C distances. The experiments were acquired at a static magnetic field of 9.4 T, corresponding to a proton Larmor frequency of 400.13 MHz. The experiments were performed using a 3.2 mm HXY triple-resonance Bruker magic-angle-spinning probe, adjusting the MAS frequency to 22222 ± 1 Hz. All experiments were performed at a set temperature of 281 K, using a sample crystallized from a buffer containing 10% $H_2O$. $^1$H,$^{13}$C CP was used to transfer magnetization from protons to carbons. For REDOR, the carbon magnetization was dephased by applying proton π-pulses every half rotor period using XY-8 phase cycling. Narrow (wide) pulses were applied with a flip angle of 90° (180°) using a rf field strength of 71.5 and 76.9 kHz on the $^1$H and $^{13}$C channel, respectively. The 180° pulse on carbon was applied as a composite $(90)_x(180)_y(90)_x$ pulse in order to achieve broadband refocussing of the carbon resonances. Again, Waltz-16 decoupling was utilized to decouple $^1$H and $^2$H, respectively. A recycle delay of 3 seconds was used between two transients. The REDOR mixing time was incremented in steps of 2 rotor periods, recording 22 REDOR mixing times in total. 6144 scans were acquired for each mixing time, resulting in an experimental time of approximately 5.1 hours per point. The carbon resonances of Y15Cζ, E22Cδ, T24Cβ and E17Cδ are resolved in a $^{13}$C-1D spectrum. A second carbon

evolution period is easily implemented, in case the resolution in one carbon dimension is not sufficient.
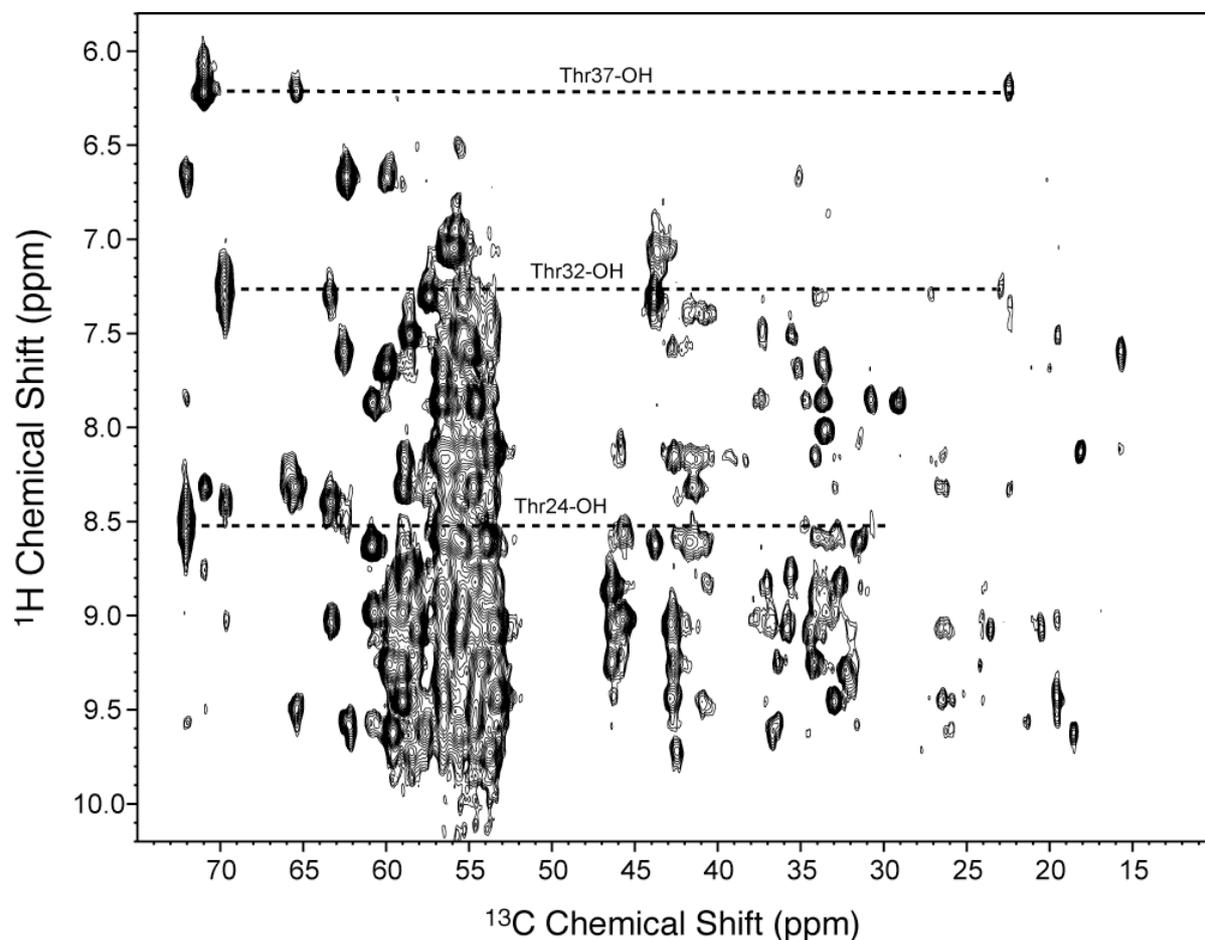


**Supporting Figure 5.** [13]C detected HETCOR highlighting correlations between threonine hydroxyl protons and methyl carbons. Correlations involving magnetization transfer between the hydroxyl proton and the methyl carbon have relatively low intensity as the associated ^{1}H,[13]C distance is on the order of 3.3 Å and depends on the X-C-O-H torsional angle.

## Integration of NMR data

The series of 2D heteronuclear exchange spectra were integrated using Sparky employing the built-in integration routine. For REDOR, integration of peaks was carried out using the 1D integration routine in TOPSPIN. The reference spectrum $S_0$ is obtained by implementing an additional [1]H 180° pulse in the center of the mixing period. The REDOR difference spectrum $(S_0-\Delta S)$ is obtained by subtracting the REDOR dephased spectrum $(\Delta S)$ from the reference spectrum $S_0$. The integral region for each peak was defined in the reference spectrum. The same integral width was used for integration of all reference $(S_0)$ and REDOR $(S_0-\Delta S)$ spectra. In total, 22 REDOR mixing times were recorded. The average of the intensities of the last twelve points in the REDOR experiments $\Delta S$ was employed to normalize $(S_0-\Delta S)/S_0$ and to compensate for the low signal-to-noise of the carbon detected experiments.

## References

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