Supporting Information

Dependence of Distance Distributions Derived from Double Electron–Electron Resonance Pulsed EPR Spectroscopy on Pulse-Sequence Time**

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Protein Preparation

Protein expression and purification.

Two constructs AviTag-^{C}ABD^C and AviTag-^{C}ProteinA^C, previously designed for single molecule FRET studies, were used. DNA inserts encoding 6H-GB1-Fax-AviTag-Spacer-^{C}ABD^C (Avi-^{C}ABD^C) and 6H-AviTag-spacer-^{C}ProteinA^C (Avi-^{C}ProteinA^C) were synthesized and cloned between Nco1 and BamH1 sites in pET15b vector (Novagen, San Diego, CA). 6H, GB1 and AviTag denote the 6His-tag, the B1 domain of immunoglobulin binding protein G and the biotin acceptor peptide (Avidity LLC, Aurora, CO), respectively. Cysteine residues were substituted towards the N- and C-termini of ABD and Protein A domains as indicated in the sequence (Fig. S1). Two more constructs, Avi-^{C}ProteinA^A and Avi-^{A}ProteinA^C, in which one of the cysteine residues was substituted to Ala as a control for intermolecular DEER effects, were created by site-directed mutagenesis using the Quik-Change mutagenesis kit (Stratagene). *E. coli* (BL21-DE3) bearing the appropriate plasmid were grown either in Luria-Bertani medium in H_2O to generated protonated protein, or in minimal medium substituting H_2O for 99% D_2O (Cambridge Isotope Laboratories) to generate ~80% deuterated protein (as judged by mass spectrometery),[S1] or in minimal medium with 99.9% D_2O, 2g/l deuterated (97-98%) d_7-glucose (Cambridge Isotope laboratories)) and 1g/l isogro-D powder growth medium (Aldrich) to generate 99% perdeuterated protein.

The expression construct and a plasmid with an isopropylthiogalactoside (IPTG) inducible birA gene to overexpress the biotin ligase (Avidity LLC) were co-transformed into *E.coli* BL-21 (DE3; Stratagene, La Jolla, CA). Cells were grown and expression induced at an A_{600} of 0.7 with a final concentration of 1 mM IPTG for a period of 3-4 h. A final concentration of 50 µM d-biotin (Sigma, St. Louis, MO) was added to the medium ~ 30 min before induction. Typically, cells harvested from a 500-mL culture were lysed by uniform suspension in 90 mL of bacterial protein extraction reagent (B-PER, Pierce, Rockford, IL) containing 5 mM benzamidine and sonication. The lysate was centrifuged at 12,800 rpm (SS-34 rotor, ThermoFisher Scientific, Asheville, NC) for 30 min at 4ºC. The supernatant was subjected to affinity chromatography using streptavidin Mutein matrix (Roche Diagnostics GmbH, Mannheim, Germany). The column was equilibrated and washed extensively, after passing the lysate, with 1x PBS (1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) and the biotinylated protein was eluted in 1x PBS containing 2 mM d-biotin. Peak fractions were pooled, dialyzed against 25 mM Tris-HCl at pH 8, 100 mM NaCl, 2 mM $CaCl_2$ and 20 mM imidazole, and subjected to Fxa protease cleavage at a concentration of 1-2 mg/ml fusion protein to 0.1-0.2% Fxa overnight at room temperature. The extent of cleavage was monitored by SDS-PAGE and the cleaved Avi-ABD and Avi-ProteinA were obtained in the flow-through by passing the digest on a Ni-NTA agarose affinity column. The flow-through fraction was incubated with 5 mM dithiothreitol for 1 hr at room temperature and further purified on reverse-phase HPLC (POROS 20 R2 resin, Perspective Biosystems, Framington, MA) by eluting using a linear gradient from 99.95% water (v/v) and 0.05% trifluroacetic acid (TFA) to 60% acetonitrile (v/v), 0.05% TFA (v/v) and 39.95% water (v/v) over a period of 16 min at a flow rate of 4 mL/min. Aliquots of the peak fraction were lyophilized and stored at -70ºC.

MTSL labeling

200-300 µg of protein was reacted with 0.5 mg (1-Oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate (MTSL, Toronto Research Chemicals) in 6M guanidine hydrochloride, 50 mM Tris-HCl, pH 8, for 2.5 hours followed by subjecting the reaction mixture to size-exclusion chromatography on Superdex-peptide (1 x 30 com, GE Healthcare) equilibrated in 0.5x PBS. Peaks fractions were combined, concentrated and adjusted to give a final protein concentration of 50 µM in 0.5x PBS/99.9% D_2O containing 30% deuterated glycerol (99%, Cambridge Isotope Laboratories). In the case
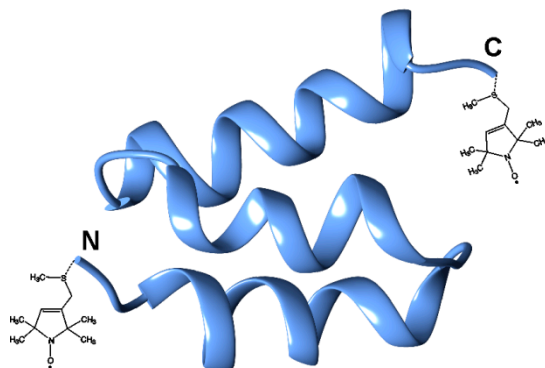
of the experiments on perdeuterated protein A, deuterated MTSL (Toronto Research Chemicals) was employed.

AviTag-^{C}ABD^C

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         10        20        30        40        50        60
GHMGMSGLND IFEAQKIEWH ESSGLVAGGC LKNAKEDAIA ELKKAGITSD FYFNAINKAK
         70
TVEEVNALKN EILKAHAC
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AviTag-^{C}ProteinA^C

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         10        20        30        40        50        60
GHMGMSGLND IFEAQKIEWH ESSGLVAGGT ADNKFNKECQ NAFYEILHAP NLNEEQRNGF
         70        80
IQSLKDDPSQ SANLLAEAKK LNDAQAPCA
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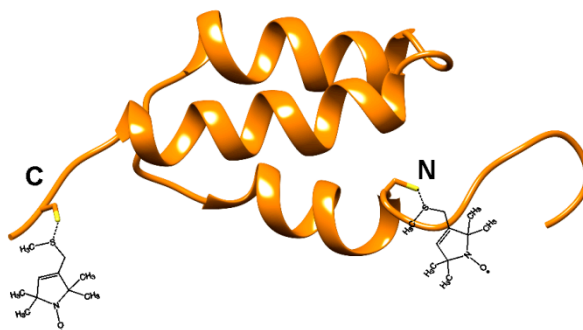

Figure S1. Sequence and ribbon representation of AviTag-^{C}ABD^C and AviTag-^{C}ProteinA^C. In the sequences, the AviTag is shown in highlighted yellow followed by the ABD or protein A domains, and the engineered surface cysteine residues are indicated in red. The ABD and Protein A ribbon diagrams were generated from the PDB coordinates 1PRB[S2] and 1BDD,[S3] respectively, and the sites of attachment of the MTSL side chain are shown. (Note that DEER experiments on control samples in which one cysteine is replaced by alanine so that only a single site is spin-labeled show no evidence of dipolar modulation and only the exponential background decay is observed).
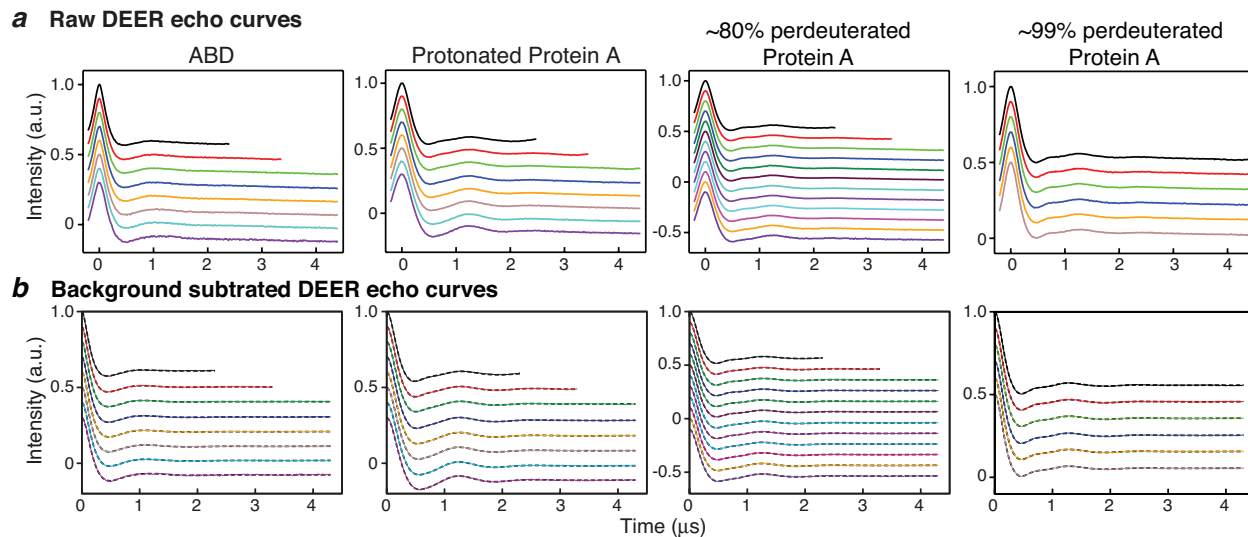
a **Raw DEER echo curves**

b **Background subtrated DEER echo curves**

Figure S2. Stacked plots of the (*a*) raw and (*b*) background subtracted DEER echo curves of the data presented in Fig. 2 of the main text. The color-coding corresponds to that used in Fig. 2 of the main text and is presented again below for convenience. DeerAnalysis2013[S4] Tikhonov Regularization fits are shown by the black dashed lines in panel *b*. Protonated ABD protein: T (total duration of second echo period = $2\tau_2$; cf. Figure 1, main text) = 6 (black), 8 (red), 10 (green), 12 (blue), 14 (orange), 16 (brown), 18 (turquoise) and 20 μs (violet). Protonated Protein A: T = 6 (black), 8 (red), 10 (green), 12 (blue), 14 (orange), 16 (brown), 18 (turquoise) and 20 μs (violet). ~80% perdeuterated Protein A. T = 6 (black), 8 (red), 10 (green), 12 (blue), 15 (dark green), 20 (maroon), 24 (turquoise), 30 (violet), 35 (cyan), 40 (magenta), 50 (orange) and 60 μs (indigo). 99% perdeuterated Protein A, T = 10 (black), 20 (red), 30 (green), 40 (blue), 50 (orange) and 60 (brown) μs.
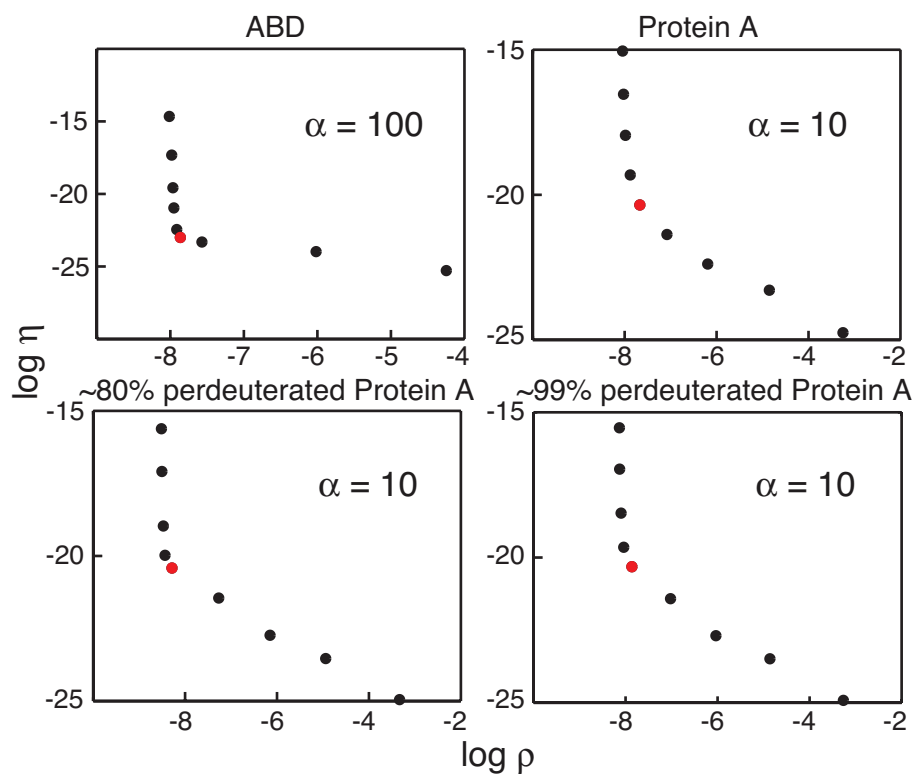
Figure S3. Representative DeerAnalysis2013[S4] L-curves used to select the optimal Tikhonov Regularization parameter α. Background subtracted DEER echo curves with $T = 10$ μs (shown in Fig. S2) were used to generate these plots. The α values selected are represented by the red circles.
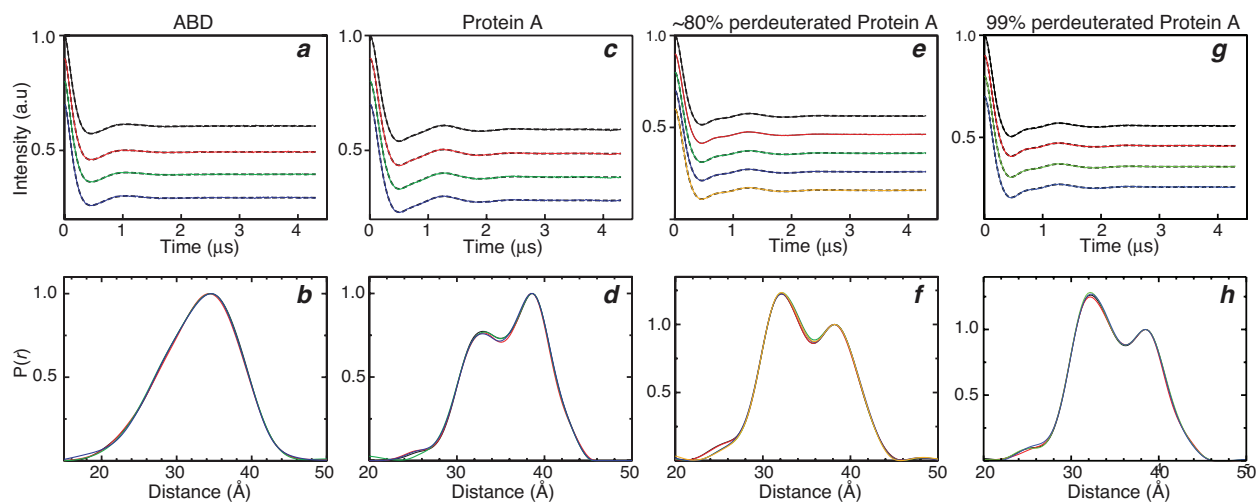
Figure S4. Reproducibility of DEER data. For each system studied, the DEER experiment was performed and analyzed with DeerAnalyis2013[S4] multiple times using one set of parameters to gauge data reproducibility. Background subtracted DEER echo curves are shown in (*a*), (*c*), (*e*) and (*g*). For protonated ABD, protonated Protein A and 99% perdeuterated Protein A, $T = 10$ μs. For ~80% perdeuterated Protein A, $T = 12$ μs. All other experimental and fitting parameters are identical to those listed in the main text. Results from the data for ABD and 99% perdeuterated protein A are plotted as open circles in panels (*c*) and (*k*), respectively, of Figure 2 in the main text. (For protonated Protein A and ~80% perdeuterated Protein A, the variation is too small to be seen in panels (*f*) and (*i*) of the main text). The black dashed lines superimposed on the DEER echo curves are the results of DeerAnalysis2013[S4] Tikhonov Regularization fits.
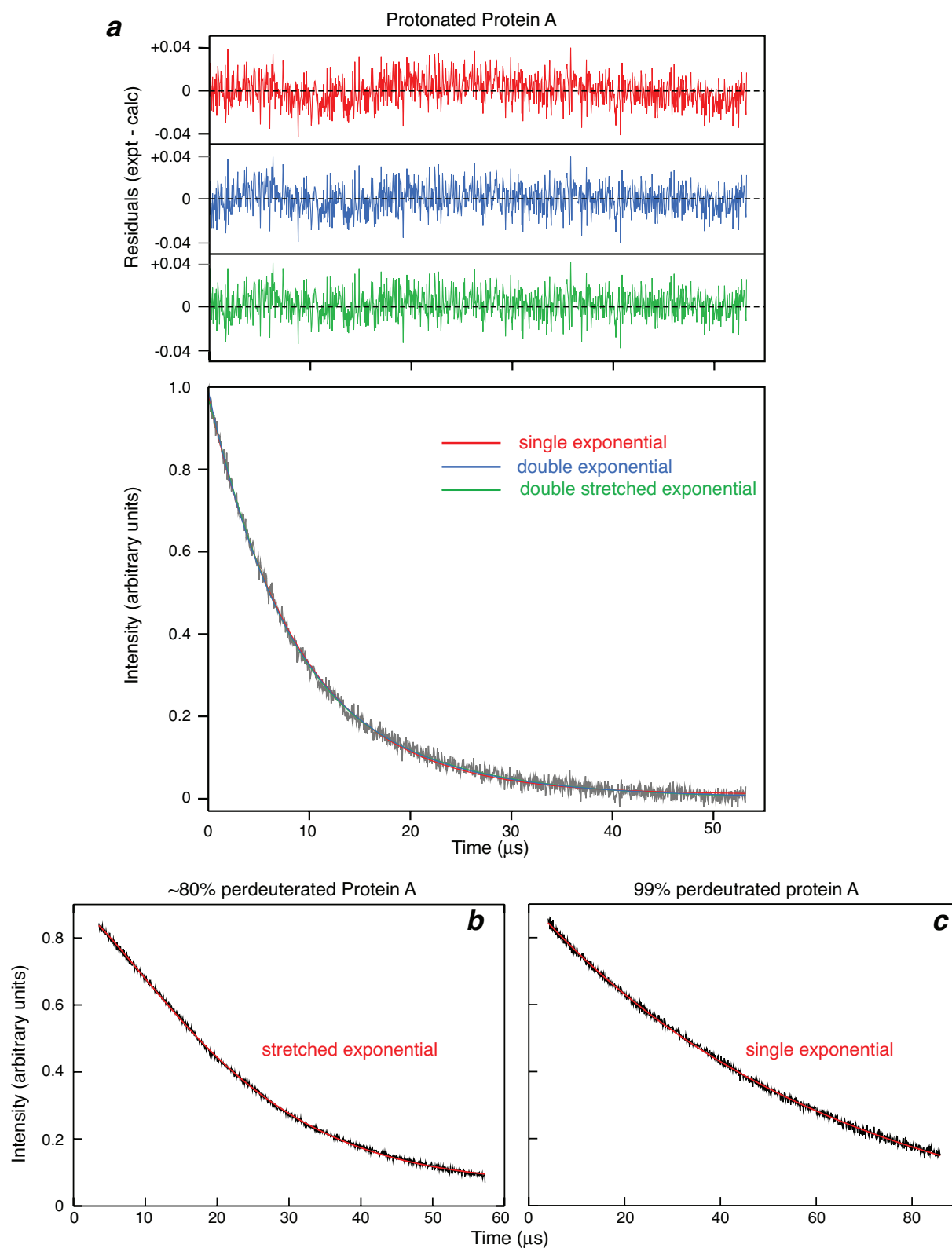
Figure S5 Measurement of apparent phase memory relaxation times T_m on doubly spin-labeled protein A using a two pulse Hahn spin-echo experiment. (a) Protonated Protein A; (b) ~80% perdeuterated Protein A; and (c) 99% perdeuterated Protein A. Spin echo-decay curves were measured using the Assisted T_2

Figure S5 legend (cont.)

relaxation experiment included in the Bruker Xepr software (Version 2.6b.69). This is a standard two-pulse Hahn echo experiment with the echo signal recorded as a function of the echo delay time.[S5] The field was set to the same spectrum position that was used for the observe signal in the DEER experiment, 34G upfield of the spectrum maximum. (*a*) For protonated protein A, a $\pi/2$ pulse of 80 ns was used to avoid ^2H electron spin echo envelope modulation (ESEEM). The short repetition time (SRT) was 20 ms. Fits are shown to a single exponential (red curve: T_{m1} = 8.8 μs), a double exponential (blue curve: T_{m1} = 7.7 μs and T_{m2} = 15.9 μs with normalized amplitudes of 0.79 and 0.21, respectively), and a double stretched exponential (green curve: T_{m1} = 6.4 μs and T_{m2} = 15.0 μs with normalized amplitudes of 0.59 and 0.41, respectively, and a stretch factor of 1.1). The top panel shows a plot of the residuals: although all three fits are almost indistinguishable in the lower panel, it is clear that from the distribution of residuals in the top panel that the quality of the fit for the double stretched exponential is better than for the double exponential which in turn is better than for the single exponential. (*b*) For the ~80% perdeuterated Protein A sample, a $\pi/2$ pulse of 12 ns was used and the first 3.5 μs of the decay was discarded for fitting because of ^2H ESEEM; SRT = 20 ms. The red curve shows the fit to a single stretched exponential with T_m = 24 μs and a stretch factor of 1.4. (*c*) For 99% perdeuterated Protein A, a $\pi/2$ pulse of 12 ns was used and the first 4 μs of the decay was discarded for fitting because of ^2H ESEEM; SRT = 15 ms. The red curve show the fit to a single exponential with a T_m of 64 μs.
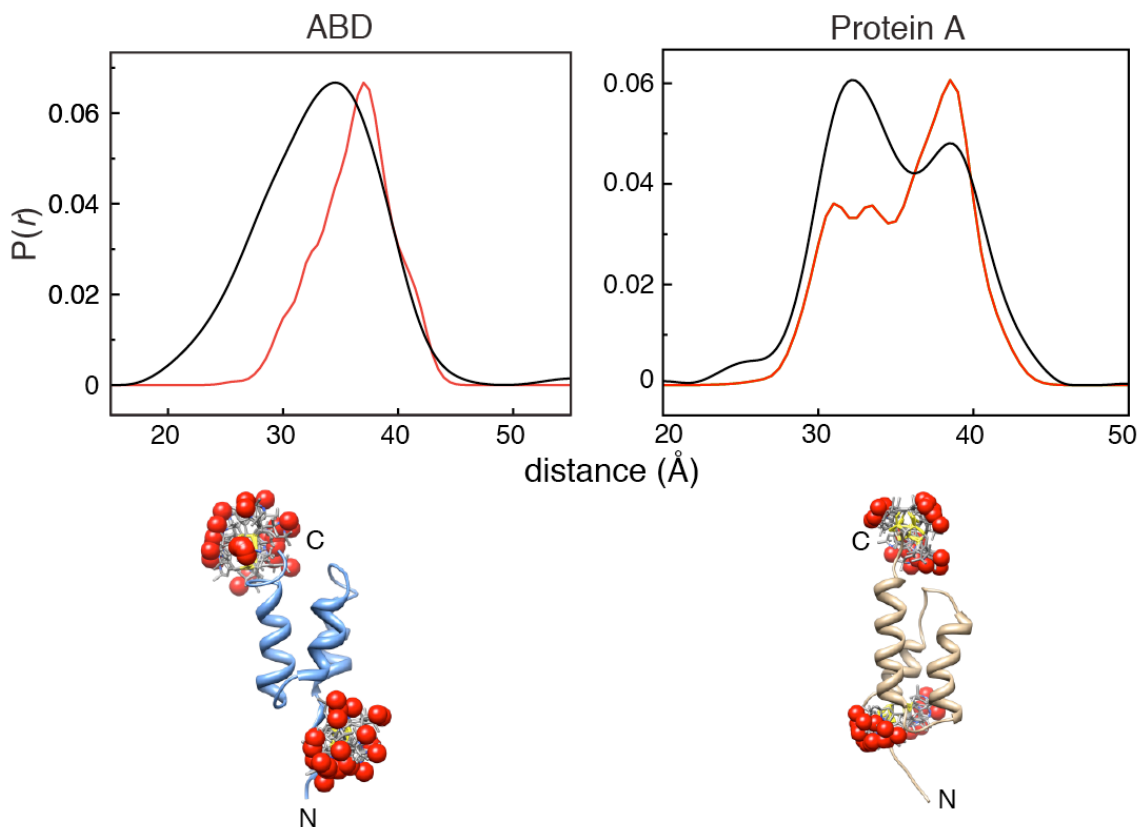
Figure S6. Comparison of observed distributions derived from experimental DEER measurements with those predicted on the basis of rotamer probabilities using the program MMM version2013.2.[S6] The starting PDB coordinates were 1PRB[S2] and 1BDD[S3] for ABD and Protein A, respectively. The software program SCWRL4.0[S7] was used to optimize the side chains of both coordinate files before they were loaded into the MMM software. MTSL was added to residues 6 and 54 of ABD and residues 10 and 59 of protein A using the program MMMv2013.2.[S6] The resulting MMM models of nitroxide distance distributions are shown in red. Experimentally derived distance distributions from DEER are shown in black. For ABD, the experimental distribution presented is identical to that shown in Figure 2*b* of the main text for a total second echo period time $T = 10$ µs. For protein A, the experimental distribution shown is identical to that shown in Figure 2*k* of the main text (99% perdeuterated Protein A) for $T = 10$ µs. The distribution of the nitroxide labels (with the oxygen atom bearing the unpaired electron represented by a red sphere) on the coordinates is shown in the lower panels. In each instance the shorter pairwise nitroxide-nitroxide distances involve rotamers in which the nitroxide is in closer proximity to the protein surface, consistent with the correspondingly shorter T_m values.

Supplementary references:

[S1]   a) A. Shekhtman, R. Ghose, M. Goger, D. Cowburn, *FEBS Lett*. **2002**, *524*, 177-182. b) A. L. Hansen, P. Lundström, A. Velyvis, L. E. Kay, *J. Am. Chem. Soc*. **2012**, *134*, 3178-3189.

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