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Supporting Information

Long Distance Measurements up to 160 Å in the GroEL Tetradecamer Using Q-Band DEER EPR Spectroscopy

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SUPPORTING INFORMATION

Experimental

Mutations. The wild type GroEL DNA sequence was synthesized by GenScript and cloned into the pET-21(a+) vector. The E315C and R268C mutations were introduced using the QuickChange site-directed mutagenesis kit (Strategene) with polyacrylamide gel electrophoresis-purified primers from Quiagen.

Expression and Purification of GroEL mutants. The plasmids were transformed into *E.coli* BL21(DE3*) cells, and expressed and purified as previously published.[S1] Briefly, the cells were grown at 37 °C in 3 ml Luria-Bertini medium for 6-8 hours and centrifuged for 3 min at 3,000xg. The pellet was resuspended in 1 L standard M9 minimal medium in 99.9% D_2O and grown at 37°C to an OD_{600} of 0.7. Expression was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for a period of 24 hours. The cells were harvested and lysed in buffer A comprising 50 mM Tris, pH 8, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT) and 1 mM tris(2-carboxyethyl)phosphine (TCEP). After a streptomycin sulfate precipitation the supernatant was loaded on a self-packed 60 ml ion exchange column (Q Sepharose Fast Flow resin from GE Healthcare) equilibrated with buffer A and eluted with a gradient to 0.56 M NaCl. Fractions containing GroEL, verified by SDS-PAGE electrophoresis, were pooled and salted out using ammonium sulfate. The precipitate was resuspended in buffer B (10 mM Tris, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, and 1 mM TCEP) and loaded onto a Superdex S300 gel filtration column. The GroEL peak was collected and the purity of the mutants were verified by SDS-PAGE and liquid chromatography (Waters 1500)-positive ion electron spray mass spectrometry (LC-MS).

EPR Sample Preparation. Fully deuterated GroEL(E315C) and GroEL(R268C) were dialysed into 20 mM Tris, pH 8, 20mM MgCl resulting in final concentrations of 1.43 μM (equivalent to 20 μM in subunits) in a volume of 1 mL. Various concentrations and dilutions of protonated paramagnetic/diamagnetic spin labels were added and allowed to incubate overnight at 4 °C. (Note that deuterated MTSL is not required owing to the large hyperfine couplings of the protons in the nitroxide spin-label,[S3] and the presence of protonated MTS does not present an issue as the distance between labeling sites is >10 Å[S3]). The spin-label incorporation obtained with a 20-fold excess of MTSL per subunit (i.e. full spin-labeling) was confirmed by LC-MS. The spin-labeled species was buffer exchanged into 99.996% D_2O and concentrated to 100 μM, after which it was diluted with a 60%/40% (v/v) d8-glycerol-D8/D_2O mixture to a final EPR sample concentration of 50 μM GroEL in 10mM Tris pH 8, 20 mM $MgCl_2$ and 30%/70% (v/v) d8-glycerol/D_2O. The resulting solution was pipetted into a 1 mm internal diameter quartz EPR tube (VitroCom inc.) and flash frozen in liquid nitrogen.

Extent of spin-labeling and deuteration. The level of spin-labeling and deuteration for GroEL was assessed by LC-MS. The electrospray mass spectrometry data were deconvoluted using the Waters MaxEnt I program from Mass Lynx Version 4.1. For fully spin-labeled samples only a single species was observed with masses of 60451 and 60554 Da for GroEL(E315C) and GroEL(R268C), respectively, corresponding to 100% MTSL-labeling and 100% deuteration.

Protein A. Fully deuterated protein A (the immunoglobulin B domain of protein A), with two surface exposed, cysteine residues engineered close to the N- and C-termini (separation 30-40 Å) was expressed, purified and MTSL spin-labeled, as described previously.[S2] The sample for EPR comprised 50 μM protein A, 0.85 mM KH_2PO_4, 2.5 mM Na_2HPO_4, pH 7.4, 75 mM NaCl, and 30%/70% (v/v) d8-glycerol/D_2O.

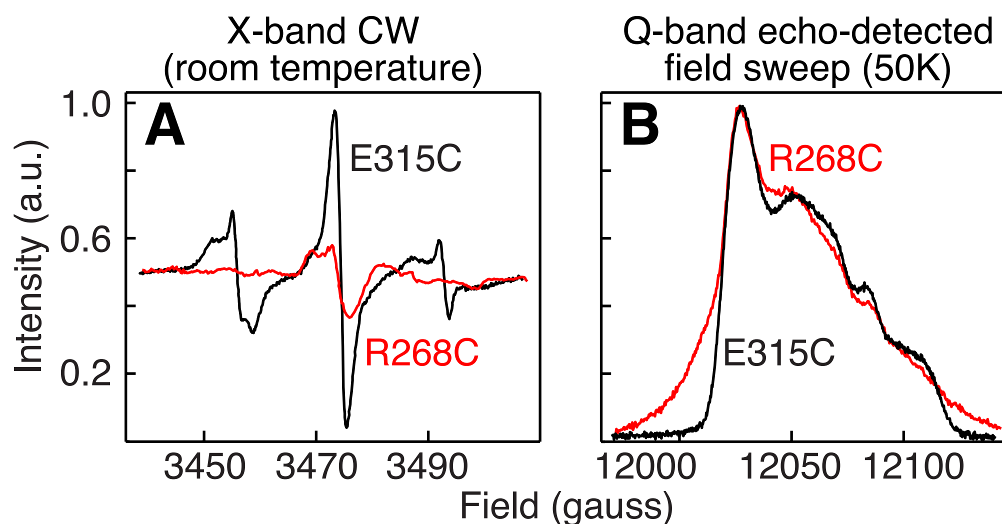

Figure S1 X-band CW and Q-band echo-detected field sweep spectra of nitroxide spin-labeled GroEL. (A) Room temperature X-band (9.3 GHz) CW spectra of GroEL nitroxide spin-labeled at E315C (black) and R268C (red). Data were recorded on a Varian E-104 spectrometer (kindly made available to us by Dr. M.K. Cherukuri, National Cancer Institute, NIH) with a scan width of 80 G. (C) Q-band echo-detected field sweep EPR experiment at 50 K for spin-labeled GroEL(R268C) (red) and GroEL(E315C) (black). The actual scale of each spectrum was adjusted slightly (by a few Gauss) to facilitate comparison. A Hahn echo with $\pi/2$ and π pulses of 12 and 24 ns, respectively, a half echo period of 400 ns, and an integration window of 32 ns was used to collect the Q band data. Q-band (33.8 GHz) data were acquired on a Bruker E-580 spectrometer equipped with a 150 W traveling-wave tube amplifier, a model ER5107D2 resonator, and a cryofree cooling unit operating at 50K. Sample conditions were 50 μM spin-labeled, fully deuterated GroEL 14mer, 10 mM Tris pH 8, 20 mM $MgCl_2$, 30%/70% (v/v) d8-glycerol/D_2O.

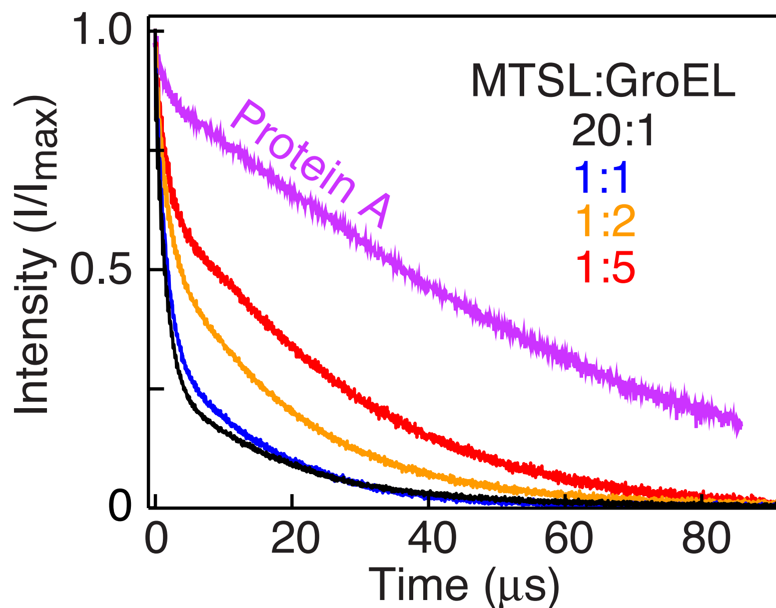

Figure S2. Impact of fractional spin-labeling on the phase memory time T_m. Q-band spin echo decay curves for fully deuterated GroEL(E315C) showing the increase in apparent T_m accompanying a reduction in the number of spin-labeled subunits obtained by decreasing the ratio of MTSL to GroEL (by reducing the concentration of MTSL for a given GroEL concentration). Also shown in purple is the spin-echo decay curve for doubly nitroxide labeled fully deuterated protein A.[S3] Pulse sequence and experimental conditions are given in Fig. 2 of the main text. In practical terms restricting access of labeling sites by reducing the concentration of MTSL below stoichiometric levels may have a number of drawbacks: specifically, the possibility of intermolecular disulphide bond formation between exposed unlabelled cysteines, uncertainties in labelling percentages owing to pipetting errors and errors in accurately determining the protein concentration, and potentially preferential labeling of some cysteines over others. Thus spin dilution of MTSL with its diamagnetic analog MTS (cf. main text Fig. 2) is preferable in most circumstances, especially since the reactivity of the two reagents is essentially the same, and therefore the average number of spin labels per oligomer can be easily controlled.

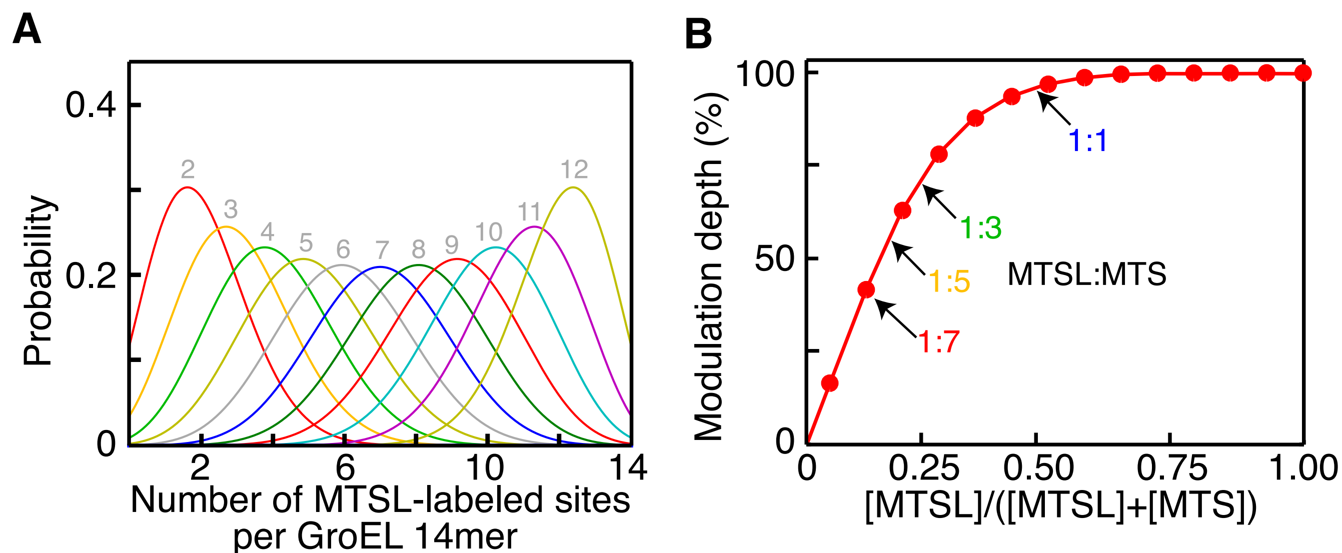

Figure S3. Impact on modulation depth of fractional spin-labeling of the GroEL 14mer. (A) Probability distributions for the number of MTSL-labeled sites per GroEL 14-mer for ratios of MTSL to total label (MTSL+MTS) ranging from 2:14 to 12:14 (only the numerator values are indicated in the plot). (B) Calculated modulation depth as a function of the ratio of MTSL to total label (MTSL + MTS) based on the probability distributions shown in panel A. The modulation depth (Δ) for n sites spin-labeled per GroEL 14-mer is given by $\Delta_n = 1 - \lambda_B{}^{n-1}$, where λ_B is the fraction of B spins (dipolar coupled to the observer spin) inverted by the pump pulse (assumed to be equal to the maximum value of 0.5).[S4]

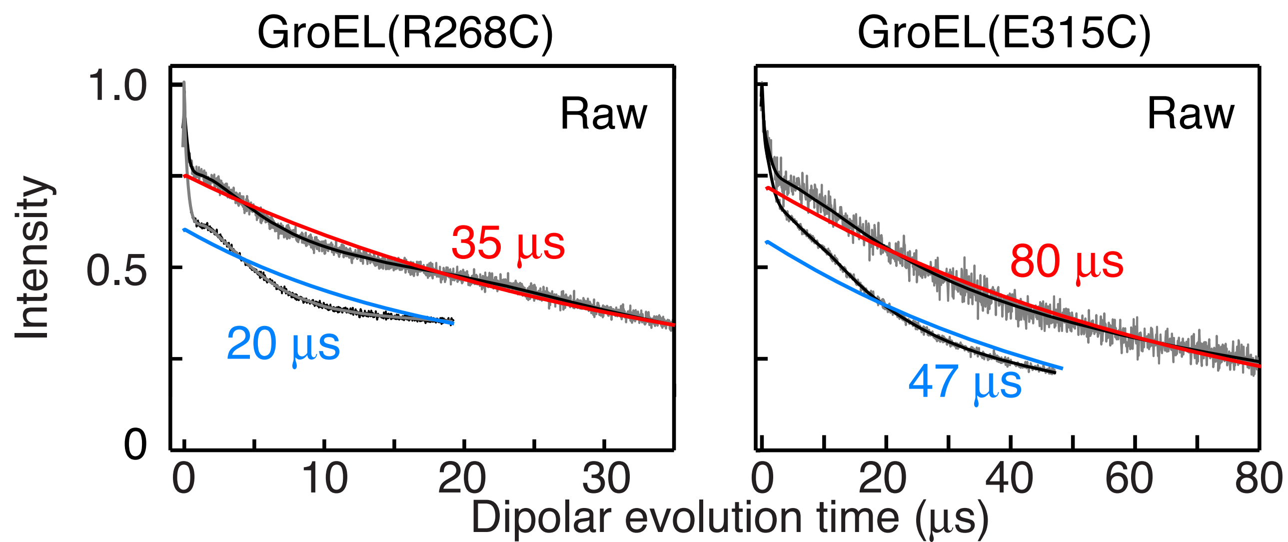

Figure S4. Overlay of the background functions on the raw DEER traces. The background function obtained using the DD program[S5] is an exponential with the best-fit decay rate (shown by the red and blue curves). The raw DEER traces are shown in black.

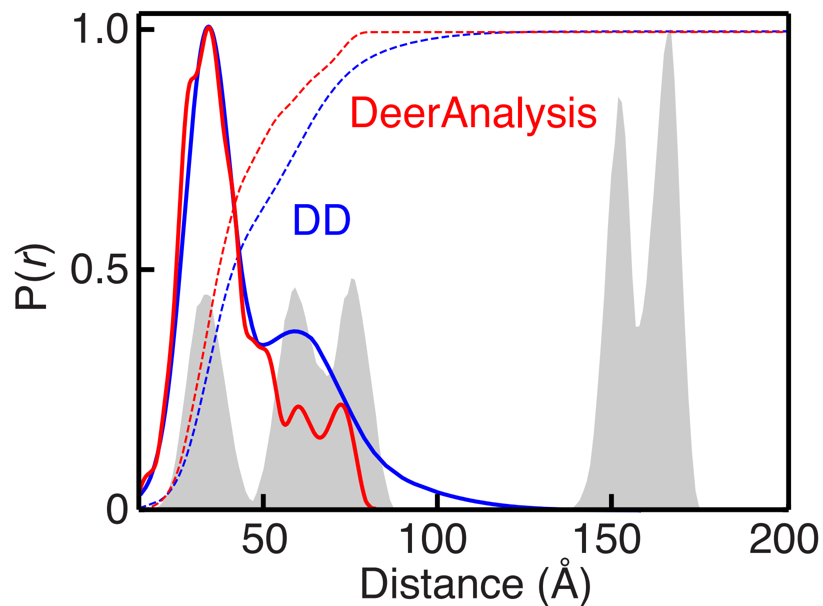

Figure S5. P(r) probability distance distributions obtained by DD (blue) and DEERAnalysis (red) of the DEER curve recorded on fully spin-labeled deuterated GroEL(E315C) with a t_{max} value of 18 µs (black curves in right-hand panels of Fig. 2A of the main text). The integrals of the P(r) distributions are shown as dashed line. The theoretical P(r) distribution calculated from the crystal structure is shown by the grey envelopes. The long inter-ring distance of 160 Å cannot be extracted from the t_{max} = 18 µs DEER data.

Table S1. Effect of sparse spin-labeling on the decay rates (T_m) and amplitudes (A) of the fast and slow components of phase memory relaxation at 50 K for fully deuterated spin-labeled GroEL(E315C) obtained by restricting access of the spin label to GroEL.

[MTSL]:[GroEL subunit]	T_m^{fast} (ms) / A_{fast} (%)	T_m^{slow} (ms) / A_{slow} (%)
20:1	1.5±0.1 / 70.4±0.1	18.4±0.1 / 29.6±0.1
1:1	1.5±0.1 / 61.3±0.1	15.5±0.1 / 38.7±0.1
1:2	1.5±0.1 / 40.4±0.1	19.4±0.1 / 59.6±0.1
1:5	1.2±0.1 / 25.6±0.1	26.8±0.1 / 73.2±0.1

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[S3] J. L. Baber, J. M. Louis, G. M. Clore, *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 5336-5339.
[S4] B.E. Bode, D. MArgraf, J. Plackmeyer, G. Dürner, T.F. Prisner, O. Schiemann, *J. Am. Chem. Soc.* **2007**, **129**, 6736-6745.
[S5] S. Brandon, A. H. Beth, E. J. Hustedt, *J. Magn. Reson.* **2012**, *218*, 93-104.