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Supporting Information

Submillisecond Freezing Permits Cryoprotectant-Free EPR Double Electron–Electron Resonance Spectroscopy

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Supporting Information
Experimental Procedures

Expression, purification and nitroxide spin-labeling of AviTag-protein A. Fully deuterated (99%) immunoglobulin-binding B domain of protein A with a 29 residue AviTag at the N-terminus and two surface exposed, cysteine residues engineered close to the N- (Q39C) and C- (K88C) termini of the protein A sequence (separation 30-40 Å) was expressed and purified as described previously.[1] Nitroxide (R1) spin-labeling with (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl)methanethiosulfonate (MTSL; Toronto Research Chemicals) was carried out as described previously[1] and free MTSL was removed by dialysis. The sample for EPR comprised 50 µM protein A in 0.85 mM KH2PO4, 2.5 mM Na2HPO4, pH 7.4, 75 mM NaCl, dissolved in a mixture of d8-glycerol (98 atom % D) and D2O (99.9% atom D) at various (v/v) percentages.

Expression, purification and nitroxide spin-labeling of drkN SH3. The construct encoding His6-spacer-drkN SH3 contained two surface-exposed cysteines for spin-labeling at M2C and C61. Expression, uniform deuterium labeling (99%), purification and nitroxide (R1) spin-labeling with MTSL was carried out as described previously.[1] In brief, cells were grown to an OD600 of 0.7 and the induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for a period of 4 h. Cells were harvested by centrifugation, resuspended in denaturing buffer (50 mM Tris, 150 mM NaCl, 20 mM imidazole, 8 M urea, pH 8), lysed by sonication, and initially fractionated by passing the cleared lysate over a 5 mL HisTrap column (GE Healthcare) equilibrated with 50 mM Tris, 150 mM NaCl, 60 mM imidazole, 8 M urea, pH 8. The bound fraction was washed with 50 mM Tris, 150 mM NaCl, 60 mM imidazole, 8 M urea, pH 8, and eluted with 50 mM Tris, 150 mM NaCl, 300 mM imidazole, 8 M urea, pH 8. Fractions containing the drkN SH3 were pooled and nitroxide spin-labeled with MTSL, as described previously.[1] Free MTSL was removed and drkN SH3 refolded by step dialysis from 8 to 0 M urea in 50 mM Tris, 50 mM NaCl, pH 8. The His6-spacer sequence was cleaved using TEV protease. The cleaved mixture was passed through a 5 mL HisTrap column (GE Healthcare) and drkN SH3 was eluted with 50 mM Tris, 50 mM NaCl, and d6-glycerol/D2O at various (v/v) percentages.

Expression, purification and nitroxide spin-labeling of HIV-1 reverse transcriptase. The base construct comprised the His-tagged p66 subunit of HIV-1 reverse transcriptase (RT) with a HRV 3C protease cleavage site between the tag and the N-terminus of p66, and the naturally occurring cysteines of p66 mutated to Ala.[5] The construct employed contained a K308C mutation for nitroxide spin-labeling. Expression, uniform deuterium labeling (99%), purification and nitroxide spin-labeling with MTSL was carried out as described previously.[2] The protein concentration for EPR samples was 50 µM. The buffer for all EPR samples comprised 25 mM Tris-HCl, pH 8, 100 mM NaCl, 20 mM MgCl2, and d6-glycerol/D2O at various (v/v) percentages.

Purification of HIV-1 gp41 transmembrane helix. The single R707C cysteine mutant of the HIV-1 p41 transmembrane helix (gp41 TM) was chemically synthesized, purified using reverse phase HPLC, and nitroxide spin-labeled as described previously.[3] gp41 TM (R707C) in 50 mM Tris (pH 7.5), 60% isopropanol and 20% acetonitrile was incubated overnight with a 10-fold excess of MTSL at room temperature. Lyophilized gp41 TM (R707C-R1) was dissolved in 25 mM MES buffer, pH 6.5, containing 25 mM DMPC/DHPC (qeff ~ 0.5), and d8-glycerol/D2O at various v/v percentages. The lipid to peptide ratio was 500:1.

Regular flash freezing in liquid N2. Regular (‘slow’) flash freezing was carried out by placing the EPR tube (1 mm ID/1.6 mm OD) containing 15 µl of sample into liquid N2. Freezing in this manner takes about 1.5 s.

Rapid freeze quenching. Rapid freeze-quenching (RFQ) was carried out with some minor modifications of the apparatus (Fig. S1) and procedure previously described for solid-state NMR studies.[4] Two high pressure HPLC systems (LU05S, Scientific Systems, Inc.) are used to pump water as a driving solution at flow rate of 2 ml/min. The water from the pumps is directed to a blank nozzle that is initially matched with the pressure at the RFQ nozzle determined by the viscosity the sample until the dynamic pressure reaches a stable equilibrium; the flow is then directed to a Y-junction (Valco Instrument Company, part number C-NYXP) by turning a 6 channel valve (Rheodyne, LLC). 75 µl sample was loaded followed by 25 µl hexane in each of two 100 µl sample lines as shown in Fig. S1B. Spraying of the sample onto the pre-cooled rotating Cu-disk at 80 K and 200 rpm was controlled using a stepper motor that swings the nozzle-mounted rod, operated by programmable controller (Anaheim automation, Inc.). The RFQ sample was scrapped and collected into a brass funnel, and packed into the 1 mm ID (1.6 mm OD) quartz EPR tube (VitriCom Inc.) placed in the center hole of the brass funnel using the customized tools shown in Fig S1C.


**Pulsed Q-band EPR spectroscopy.** Pulsed EPR data were collected at Q-band (33.8 GHz) and 50 K on a Bruker E-580 spectrometer equipped with a 150 W traveling-wave tube amplifier, a model ER5107D2 resonator, and a cryofree cooling unit, as described previously.\[1, 2\] DEER experiments were acquired using a conventional four-pulse sequence.\[3\] The observer and ELDOR pump pulses were separated by ca. 90 MHz with the observe π/2 and π pulses set to 12 and 24 ns, respectively, and the ELDOR π pulse to 10 ns. The pump frequency was centered at the Q-band nitroxide spectrum located at +40 MHz from the center of the resonator frequency. The τ₁ value of 400 ns for the first echo-period time was incremented eight times in 16 ns steps to average 2H modulation; the position of the ELDOR pump pulse was incremented in steps of Δt = 14 ns. The bandwidth of the overcoupled resonator was 120 MHz. The second echo period time τ₂ was set to t_max+ 700 ns, where t_max is the maximum dipolar acquisition time; data collection was not extended to the full τ₂ range because of a persistent “2+1” echo perturbation of the DEER echo curves at a time of about τ₁ from the final observe π pulse.

**P(𝑟)** distributions from DEER data. \(P(𝑟)\) distributions from the DEER echo curves were obtained using three different methods: (a) the program DD\[6\] which uses a sum of Gaussians to directly fit the experimental DEER data (including automated background correction with a best-fit exponential decay); (b) validated Tikhonov regularization using DeerAnalysis 2016;\[7\] and (c) the program WavPDS which uses a wavelet approach to filter out noise and obtains \(P(𝑟)\) via singular value decomposition (SVD) analysis.\[8\] The optimal number of Gaussians used by the program DD to represent the DEER data was assessed using the Akaike information criterion corrected for finite sample size (AICc) and the Bayesian information criterion (BIC).\[6\] Validated Tikhonov regularization was carried out using the DeerAnalysis\[7\] validation tool varying the modulation depth (11 steps), background density (11 steps), background start (11 steps) and noise sensitivity (5 steps) for a total of 6655 permutations (see Fig. S11). The Tikhonov regularization parameter \(\alpha\) was automatically determined by DeerAnalysis for each iteration. The WavPDS method uses the Daubechis 6 wavelet family to filter out noise; post-denoising, the traces were analyzed by SVD reconstruction, and the SVD cutoff was assessed from a Picard plot.\[8\] In the case of both DeerAnalysis and WavPDS/SVD, background subtraction was carried either with a single exponential, or, in the case of gp41 TM in lipid bicelles, with a stretched exponential.\[5\]

**Molecular graphics.** The PyMol molecular graphics system (version 2.0 Schrödinger, LLC) was used to visualize and render the various proteins used in the current work.
Figure S1. Apparatus used for RFQ EPR. (A) Photograph of the apparatus used for rapid mixing and freeze-trapping. A more detailed description is given in ref. [4]. The mixer is contained within a temperature-controlled polytetrafluoroethylene (PTFE) chamber (1). The copper plate (2) is cooled in a bucket of nitrogen bucket (3) and the electric motor (4) drives plate rotation. The stepper motor (5) that sweeps the mixer across the copper plate is controlled by the programmable driver (6). A brass fixture (7) with a funnel-shaped opening at the top contains the EPR tube, into which frozen solutions are packed into after freeze-trapping. When the high-pressure pumps (8) are switched on, water is initially pumped through a 100 µm ID tube at the output of a T-junction (9) until the desired flow rates and pressures are reached. The valve (10) is then switched on manually to apply pressure to the input lines going to the mixer. Items 1-5 and 7 are contained within a nitrogen-filled glove box while samples are prepared. (B) Schematic description of the flow path. (C) The EPR tube (3) (1 mm ID, 1.6 mm OD) is held by a brass funnel/holder (1) that is attached to the copper disc in (A). The frozen material is scrapped from the copper disc and carefully inserted into the EPR tube using the hollow needle (2).
Figure S2. Impact of the percentage (v/v) d8-glycerol in the EPR sample on the phase memory time $T_m$. Q-band spin-echo decay curves for fully deuterated AviTag-protein A (Q39C-R1/K88C-R1) showing the increase in $T_m$ accompanying an increase in the percentage glycerol in the sample. Samples were flash frozen in liquid N2 (referred to as SF for “slow freezing” in the main text). The pulse sequence employed to acquire the spin-echo decay curves was a standard two-pulse Hahn echo experiment\cite{10} with the echo signal recorded as a function of the echo delay time with time steps of 20 ns up to an evolution time of 90 μs, limited by the traveling-wave tube amplifier. The length of the $\pi/2$ pulse was 10 ns, the shot repetition time was set to 10 ms, and the pulse gate time used for integration was 120 ns. Sample conditions were 50 μM nitroxide spin-labeled, fully deuterated protein A in 20 mM NaPO4, pH 6.5, 50 mM NaCl and varying d8-glycerol/D2O (v/v) percentages as indicated in the figure.
Figure S3. Q-band DEER echo curves for nitroxide spin-labeled AviTag-protein A (Q39C-R1/K88C-R1) in the presence of varying d8-glycerol percentages (v/v), flash frozen (SF) in liquid N2. Raw (grey) and background-corrected DEER echo curves for protein A in the presence of varying amounts of glycerol; the fitted background is shown in black. The color coding for the background-corrected DEER echo curves is the same as in Fig. 1 of the main paper with the color gradation from red (5% glycerol) to blue (80% glycerol). The background, as obtained from DeerAnalysis,[7] is shown in black. Protein A was nitroxide spin-labeled at sites close to the N- and C-termini of the protein A sequence.[1]
Figure S4. Q-band background-corrected DEER echo curves for rapid freeze quenched (RFQ) fully deuterated AviTag-protein A (Q39C-R1/K88C-R1) in the absence (red) and presence (30% v/v) of d₈-glycerol (blue). Background correction was carried out with the program DeerAnalysis. The corresponding $P(r)$ distributions are shown in Figs. 1E and S5.
Figure S5. DEER-derived $P(r)$ distributions for AviTag-protein A (Q39C-R1/K88C-R1) obtained using (A) SVD and (B) DD. The left panels show the $P(r)$ distributions of samples at various percentages (v/v) $d_8$ glycerol (ranging from 5 to 80%) flash frozen (SF) in liquid N<sub>2</sub>. The left panels show a comparison of the $P(r)$ distributions obtained by rapid freeze quenching (RFQ) of samples in either 0 % (red) or 30% (blue) $d_8$-glycerol (v/v), together with a comparison of the $P(r)$ distribution obtained for a SF sample in 30% (v/v) $d_8$-glycerol. The $P(r)$ distributions are normalized to the peak height at 38 Å. For the $P(r)$ distributions shown in (A), the raw DEER traces were initially ‘denoised’ using the program WavPDS with the Daubechis 6 wavelet family; post ‘denoising’ the traces were analyzed using the program SVDReconstruction; the SVD cutoff was judged from a Picard plot. The $P(r)$ distributions in (B) were obtained using the program DD as the sum of two Gaussians directly fit to the experimental DEER data. The optimal number of Gaussians was assessed using the Akaike and Bayesian information criteria.
Figure S6. Comparison of observed and MMM modeled $P(r)$ distributions for aviTag protein A (Q39C-R1/K88C-R1). (A) RFQ with 30% (left) and 0% (right) glycerol (v/v). Black, DEER-derived $P(r)$ distributions obtained by Tikhonov regularization using DeerAnalysis 2016;[7] blue, raw $P(r)$ distribution obtained using MMM;[11] red, MMM rotamer optimized $P(r)$ distribution to best approximate the experimental $P(r)$ distribution. In the case of the RFQ 0% glycerol $P(r)$ distribution, a 5 Å reduction in the Cα-Cα separation between residues 39 and 88 is required to reproduce the experimental $P(r)$ distribution. This is not unreasonable, given that K88C-R1 is located in a short flexible C-terminal tail (see Fig. 1). The starting coordinates were PDB 1BDD;[12] the program SCWRL4.0[13] was first used to optimize side rotamers prior to loading the coordinates into MMM.
Figure S7. Q-band DEER echo curves for nitroxide spin-labeled drkN SH3 (M2C-R1/61C-R1) in the presence of varying $d_8$-glycerol percentages (v/v), flash frozen (SF) in liquid $N_2$. Both raw (grey) and background-corrected DEER echo curves are displayed; the fitted background is shown in black.
Figure S8. Q- and DEER echo curves for nitroxide spin-labeled drkN SH3 (M2C-R1/61C-R1) obtained by rapid freeze quenching (RFQ). Raw (grey) and background-corrected DEER echo curves for samples in (A) 30% (blue) and (B) 0% (red) d8-glycerol (v/v). The fitted background is shown in black.
Figure S9. DEER-derived $P(r)$ distributions for nitroxide spin-labeled drkN SH3 (M2C-R1/61C-R1) obtained using (A) SVD and (B) DD. The left panels show the $P(r)$ distributions of samples at various percentages (v/v) $d_8$ glycerol (ranging from 5 to 30%) flash frozen (SF) in liquid $N_2$. The left panels show a comparison of the $P(r)$ distributions obtained by rapid freeze quenching (RFQ) of samples in either 0% (red) or 10% (blue) $d_8$-glycerol (v/v), together with a comparison of the $P(r)$ distribution obtained for a SF sample in 10% (v/v) $d_8$-glycerol. The $P(r)$ distributions are normalized to the peak maximum. For the $P(r)$ distributions shown in (A), the Raw DEER traces were initially ‘denoised’ using the program WavPDS with the Daubechis 6 wavelet family; post ‘denoising’ the traces were analyzed using the program SVDReconstruction; the SVD cutoff was judged from a Picard plot.[8] The $P(r)$ in (B) were obtained using the program DD[6] as the sum of two Gaussians directly fit to the experimental DEER data. The optimal number of Gaussians was assessed using the Akaike and Bayesian information criteria.
Figure S10. Cylindrical model used to simulate the expected $P(r)$ distributions for two gp41 TM (R707C-R1) molecules randomly embedded in a DMPC/DHPC bicelle ($q_{eff} = 0.5$). (A) The upper and lower surfaces of the cylinder depict the DMPC leaflets (width = 25 Å). Parallel and anti-parallel orientations of two gp41 TMs in the bicelle give rise to intra- and inter-leaflet pairwise distances between nitroxide spin-labels. The model discards orientations associated with the DHPC rim. (B) The diameter of the bicelle ($q_{eff} = 0.5$) is 45 Å. The predicted $P(r)$ distance distribution maximum is 22 Å for the parallel orientation and 31 Å for the antiparallel orientation (taking into account a 6 Å distribution for MTSL rotamers).
Figure S11. Results of DeerAnalysis\cite{7} validation of $P(r)$ distributions obtained using Tikhonov regularization. (A) AviTag-protein A (Q39C-R1/K88C-R1) (Fig. 1, main text), (B) drkN SH3 (M2C-R1/61C-R1) (Fig. 2, main text), (C) urea-denatured drkN SH3 (M2C-R1/61C-R1) (Fig. 3, main text); (D) HIV-1 p66/p66' reverse transcriptase precursor (Fig. 4, main text), and (E) gp41-TM in DMPC/DHPC bicelles (Fig. 5, main text). In each instance the $P(r)$ distribution for rapid freeze quenching (RFQ) in the absence of glycerol is shown on the left, and that for slow freezing (SF) in the presence of glycerol (with percentage indicated) on the right. Validated Tikhonov regularization was carried out varying the modulation depth (11 steps), background density (11 steps), background start (11 steps) and noise sensitivity (5 steps) for a total of 6655 permutations. The $P(r)$ distribution with the best r.m.s.d. is displayed in black; the grey regions indicate the full $P(r)$ variation over all trials; and the red and blue dotted lines are the upper (mean value probability plus two times its S.D.) and lower (mean value minus two times its S.D.) error estimates, respectively.
Author Contributions

T.S., J.J., and GMC designed research; T.S., J.J. and G.M.C. performed research; S.C.C. and Y.O. contributed reagents; T.S. and G.M.C. analyzed data; T.S. and G.M.C. wrote paper.

Supplementary references