# Supporting information

## In-Cell Trityl-Trityl Distance Measurements on Proteins

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## Experimental details

Protein expression, purification, and labeling

The double mutations K25C/E153C of the peptidyl-prolyl cis-trans isomerase B (PpiB) from E. coli and T11C/V21C of streptococcal β1 immunoglobulin binding domain of protein G (GB1) were prepared in a pET28a vector, respectively. Briefly, the E. coli BL21 (DE3) Rosetta cells transformed with the plasmid of interest were grown in 2 L LB medium at 37 °C to an OD600 of 0.6 and then induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for protein expression at 37 °C for 6 hours. The bacterial cells were collected by centrifugation and then lysed by sonication. The target proteins were both purified by DEAE column (DEAE Sepharose FF, GE Healthcare Biosciences), followed by a size exclusion column (HiLoad 16/600 Superdex 75, GE Healthcare Biosciences). $^{15}$N-labled PpiB was made following the same protocol as previously reported.[1]

Labeling with CT02MA was performed as described earlier.[2] 0.2 mL of a 0.1 mM solution of the target mutant in 20 mM tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.5) was first treated with 0.2 mM tris(2-carboxyethyl)phosphine (TCEP) and then removing it using a Micro Bio-Spin 6 Size Exclusion Spin Columns (Bio-Rad Laboratories Ltd.). Then 10 equivalents of CT02MA (in 50 mM stock) (per one cysteine) were added to the mixture gradually. The labeling mixture was incubated at 4 °C for about 8 h. It is noted that for PpiB four cysteines were modified with CT02MA when the reaction was carried out at 30°C (Figure S1). BrPSPy-DO3A-Gd(III) was prepared as previously reported.[3] 0.1 mL of a 0.2 mM solution of target mutant was mixed with 2 mM BrPSPy-DO3A-Gd(III) and 0.4 mM TCEP in 20 mM Tris buffer. The pH was adjusted to ~8.5 and the mixture was incubated at 4 °C for about 12 h. For labeling with M-proxyl, 0.1 mL 0.1 mM PpiB K25C/E153C was mixed with 0.6 mM 0.1 mL M-proxyl (Sigma Aldrich) and 0.2 mM TCEP in 20 mM MES buffer at pH 6.5. The mixture was incubated at 4 °C for 4 h. For all the labelling experiments mentioned in this work, the excess of free ligands were removed by passing through a Bio-spin columns twice.

For EPR measurements in solution, the spin-labeled proteins were in Tris-HCl (pD 7.0) in $D_2O$/glycerol-d8 (7/3 v/v) and loaded into EPR quartz capillary (0.6 ID × 0.84 OD mm). For stability test, 30 μM PpiB-CT02MA was mixed with Glutathione (GSH). Then the samples were incubated under the same conditions as for the living cell for different times.

NMR measurements

All NMR experiments were performed on a Bruker Avance 600 MHz NMR spectrometer equipped with a QCI CryoProbe. Unless noted otherwise, the NMR spectra of PpiB K25C were recorded in 20 mM **Tris** (hydroxymethyl) aminomethane (Tris) buffer for the $^{15}$N-labeled protein sample at a concentration of 0.08 mM, a pH of 7.2, and a temperature of 298 K.

Electroporation delivery

HeLa cell culturing and electroporation was carried out as reported earlier.[4] Briefly, HeLa cells were suspended in 100 μL of PBS electroporation buffer (100 mM sodium phosphate, 5 mM KCl, 15 mM MgCl2, 15 mM HEPES, 0.1 mM ATP, 0.1 mM reduced glutathione, pH 7.4) with 0.2 mM spin labeled protein, then transferred into 2 mm cuvettes and electroporated by a Nucleofector™ 2b (Lonza) with pulse program B28. The cells were then transferred to collagen treated dishes and incubate at 37 °C for 4-5 h. Then the HeLa cells were detached and washed twice in PBS buffer (100 mM, pH 7.2) to remove the non-internalized protein and dead cells, and incubated at 37 °C for 5 min in PBS-$D_2O$ buffer and glycerol-$d_8$ (8/2 v/v), and then transferred to the EPR capillary (0.6 ID × 0.84 OD mm) and centrifuged at 1500 g for 30 min. Finally, the capillary was slowly frozen in an isopropanol rack at −80 °C. The control experiment was performed as described above except no electroporation pulse was used.

EPR measurements and data processing

EPR measurements were carried out on a home-built W-band spectrometer (94.9 GHz).[5-6] Echo-detected EPR (ED-EPR) spectra were recorded using π/2 and π pulse durations of 20 and 40 ns for CT02MA and M-Proxyl, and 15 and 30 ns for BrPy-DO3A-Gd(III) labeled samples, respectively, with an echo delay of 550 ns and a repetition time of 1 ms. Echo decays were measured at the maximum of the trityl or Gd(III) signal in the ED-PR spectrum using the Hahn echo sequence (π/2-τ-π-τ-echo). The π/2 and π pulse durations were 20 and 40 ns for CT02MA and 15 and 30 ns for BrPy-DO3A-Gd(III) labeled samples, respectively, with an echo delay of 150 ns and a repetition time of 1 ms.

Chirp-pulse DEER measurements were recorded using the 'reversed' four-pulse DEER sequence ($\pi/2(\nu_{obs}) - \tau_1 - \pi(\nu_{obs}) - (\tau_1\text{-}t) - \pi(\nu_{pump}) - (\tau_2+t) - \pi(\nu_{obs}) - \tau_2 -$ echo) with two chirp pump pulses.[2,

[7-9] Standard four-pulse DEER ($\pi/2(\nu_{obs}) - \tau_1 - \pi(\nu_{obs}) - (\tau_1+t) - \pi(\nu_{pump}) - (\tau_2 - t) - \pi(\nu_{obs}) - \tau_2 - echo$) using a rectangular pump pulse was also used for comparison.[10] An eight-step phase cycle was applied to remove the instrumental artefacts and to compensate for DC offset. The parameters of the DEER measurements were shown is in Table S1 and Figure S5.

Table S1. The experimental parameters used for the DEER measurements.

| **set-up** | **Chirp-pulse 1** | **Rectangular-pulse 1** | **Chirp-pulse 2** | **Chirp-pulse 1** |
|---|---|---|---|---|
| Spin label | CT02MA | CT02MA | BrPy-DO3A-Gd(III) | M-Proxyl |
| Temperature | 50K | 50K | 10 K | 25 K |
| $\pi/2$-$\pi$ pulse length / ns ($\nu_{obs}$ / GHz) | 25-50 (94.91) | 25-50 (94.91) | 15-30 (94.9) | 20-40 (94.85) |
| pump pulse length / ns ($\nu_{pump}$ / GHz) | 96 (94.84-94.89) | 40 94.88 or 94.89 | 96 (94.5-94.8, 95-95.3) | 96 (94.9-95) |
| $\tau_1$ / µs | 5 / 4* | 0.35 | 5.5 / 5* | 5.5 |
| $\tau_2$ / µs | 4.5 / 3.8* | 5 | 5 / 4.5* | 5.0 |
| $\Delta t$ / ns | 30 | 30 | 30 | 25 |
| repetition time / ms | 3 | 3 | 0.8 | 3 |
| Recording time / min | 60 or 120 / 1200* | 60 | 120 | 120 |

* The parameters used for in-cell DEER measurements.

The DEER data were analyzed using the program DeerAnalysis2018 software[11]. Distance distributions were obtained using Tikhonov regularization with a regularization parameter of 10 for solution and 50 for in-cell experiments respectively. The background validation was performed with a starting time of 15% to 80% of the DEER time trace in 16 trials.

Signal-to-noise values (SNR) per unit time were calculated as follows:

$$\mathrm{SNR} = \frac{\Delta}{\sigma \cdot \sqrt{t}}$$

where Δ is the fraction of the modulation depth, *t* is the accumulation time in minutes and σ is the standard deviation of the noise, obtained as the standard deviation of the difference between the background corrected DEER traces and their corresponding fits as obtained with the DeerAnalysis in the range of 1.5 – 3 μs. For the estimation of error, Δ was varied by fitting the background with a starting time of 1.6 to 2.2 μs in the DEER time trace.

Estimation of in-cell spin concentrations

The ED-EPR spectra of HeLa cells without EP delivery ($S_{HeLa}(x)$) and of 100 μM free spin labels in solution ($S_{vitro}$ (x)) were recorded. The ED-EPR spectra of in-cell samples ($S_{exp}$ (x)) were simulated as $S_{exp}(x) = a * S_{HeLa}(x) + b * S_{vitro}(x)$ with $a$ and $b$ as fitting parameters. The intracellular concentrations, $C_{cell}$(EPR) was estimated by using the Mn(II) signal as an internal standard as described earlier[3]:

$C_{cell}(EPR) = b$*100 μM*$V_{HeLa}/(a$*$V_{cell}$*$N_{HeLa})$ where $V_{cell}$ is the volume of a single HeLa cell taken as ~2,000 $\mu m^3$, $V_{HeLa}$ is the pellet volume of the sample without electroporation sample and $N_{HeLa}$ is the corresponding cell number in EPR the capillary. The error was estimated from the experimental uncertainties in the ED-EPR echo intensity, cell number counting and an assumed 5% uncertainty in the spectra-superimpose simulation.

## MALDI-TOF spectra

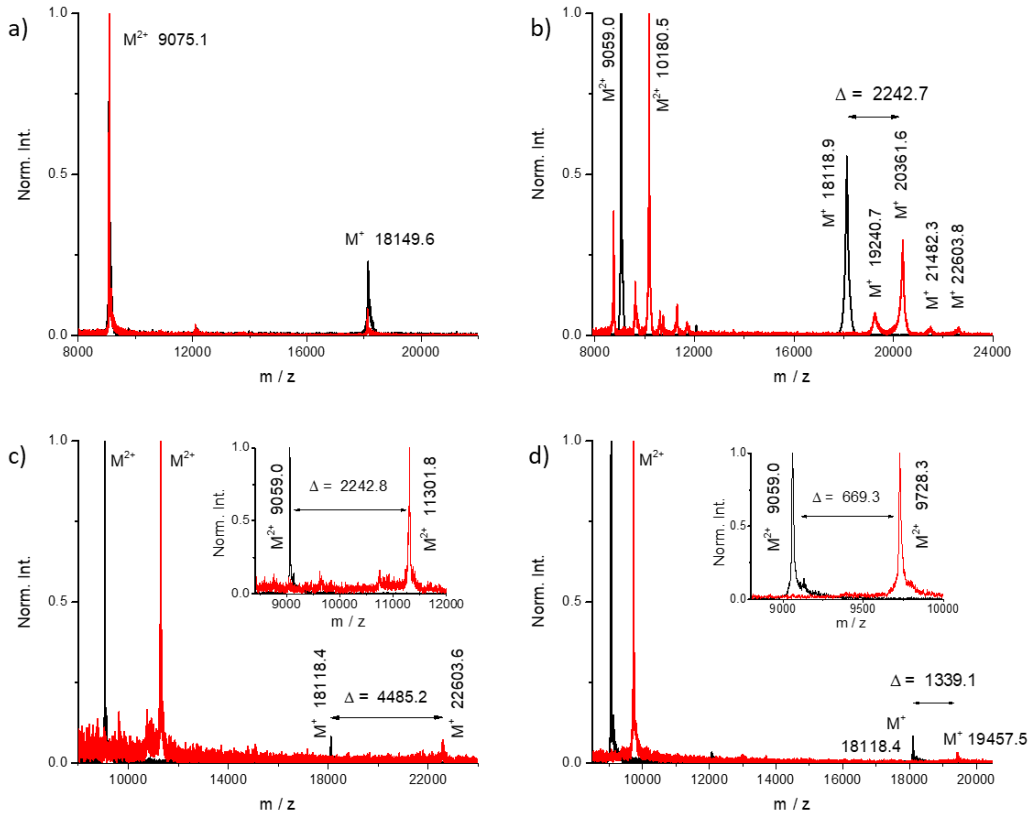


**Figure S1.** MALDI-TOF mass spectra of PpiB constructs before (black) and after (red) ligation. (a) Wild type PpiB labeled with CT02MA at 30ºC for 24 h. (b) PpiB K25C/E153C labeled with CT02MA at 4ºC for 6 h or at 30ºC for 15 h (c), and with BrPSPy-DO3A-Gd(III) at 30ºC for 15 h (d). The inserts focus on the double-charged molecular ions. The molecular masses of mono- and multi-labeling are indicated. The calculated molecular mass difference is 1121 mass units between PpiB and its mono-CT02MA conjugate, and 669 for mono-BrPy-DO3A-Gd(III) conjugate.

## NMR measurements

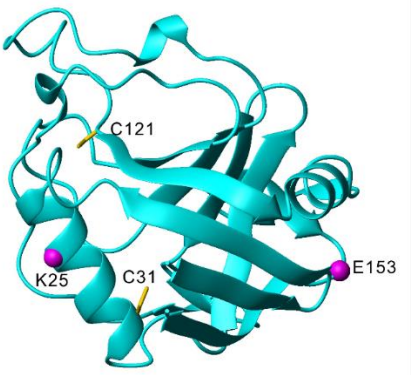


**Figure S2.** Structural representation of PpiB (PDB code: 2NUL), of which the $C_\alpha$ atoms of K25 and E153 used for mutation are shown as magenta spheres and the side chains of native cysteine C31 and C121 are shown as yellow sticks.

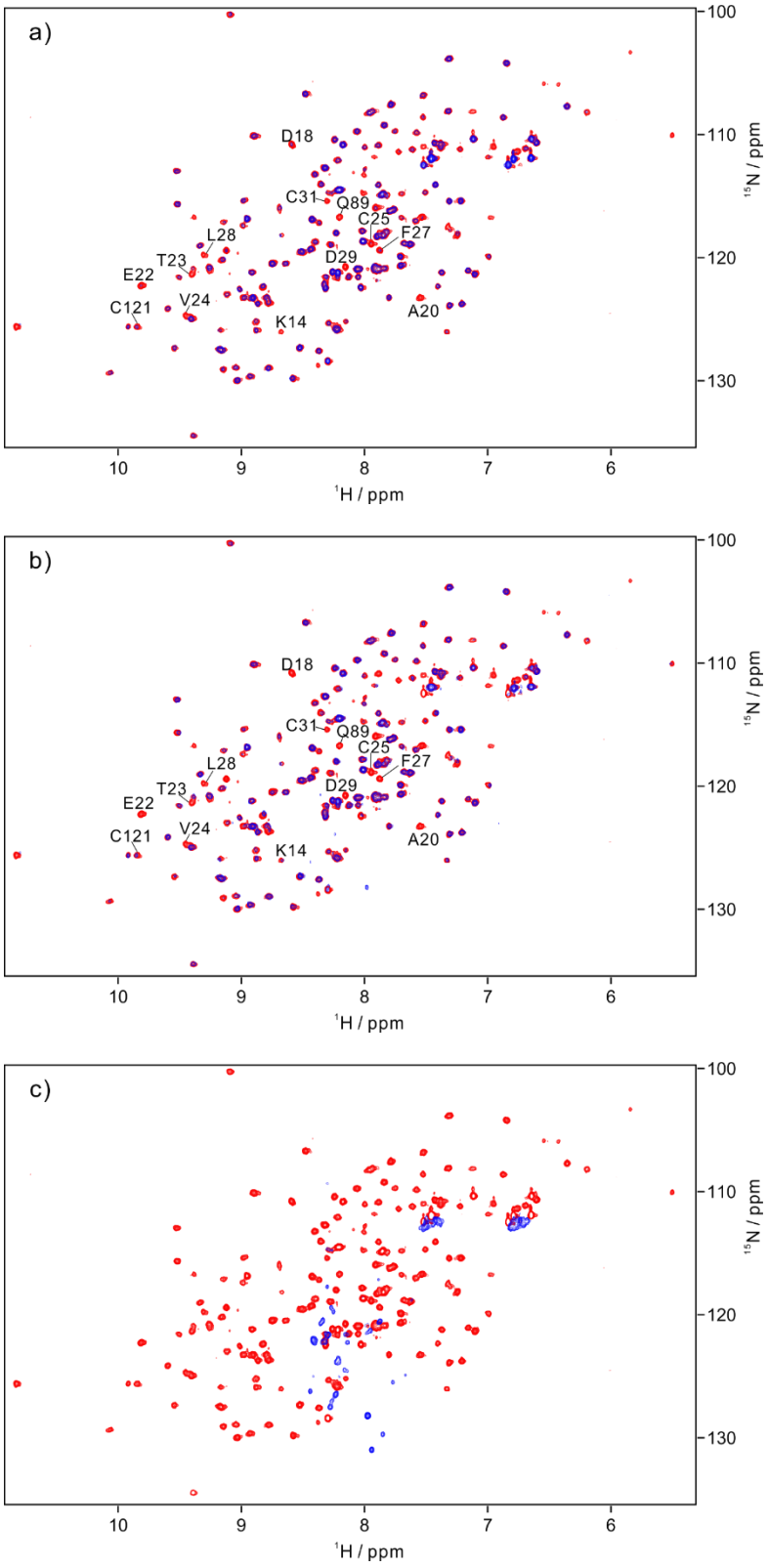


**Figure S3**. Superimposition of $^{15}$N-HSQC spectra of 0.08 mM PpiB K25C (red) and after incubation with varied concentration of CT02MA in 20 mM Tris buffer, pH 7.2 and at different temperature (blue): a) incubation with 0.08 mM CT02MA at 293 K for 5 hours, of which the residues experiencing significant PRE effects were labeled; b) incubation with 0.28 mM CT02MA at 293 K for 10 hour; c) incubation with 0.28 mM CT02MA at 308 K for 10 hour. The spectra were recorded at 298K.

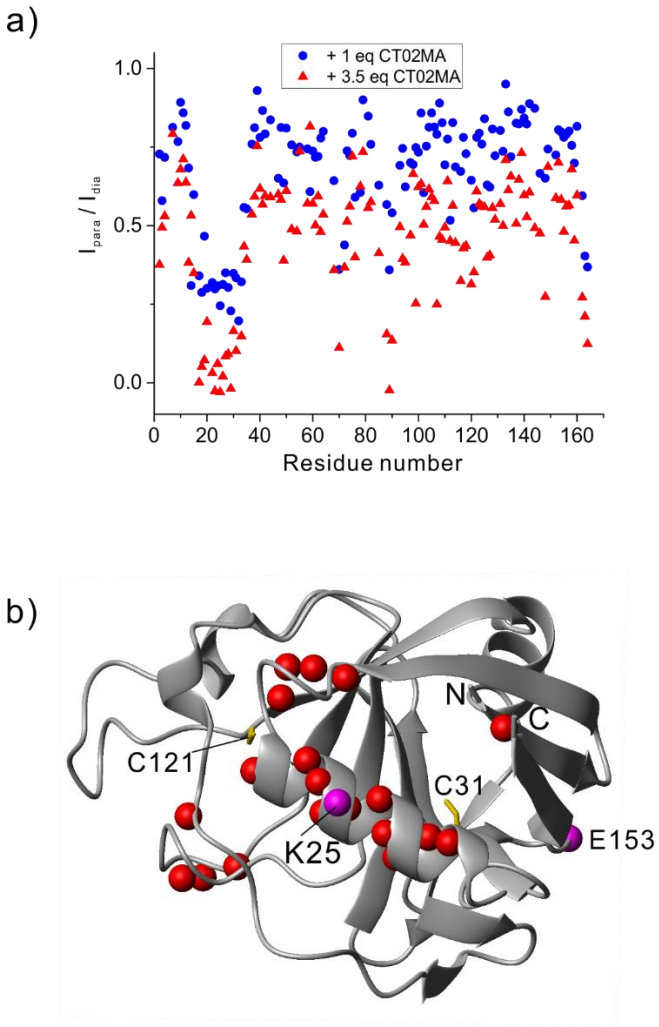


**Figure S4**. a) Plot of cross-peak intensity ratio as a the function of amino acid sequence of PpiB, of which $I_{dia}$ depicts the cross-peak intensities as shown in Figure S3 in the absence of CT02MA and $I_{para}$ are the cross-peak intensities in the blue spectra as shown in Figure 3a and S3b, respectively. b) The residues of backbone amide nitrogen atoms with $I_{para}/I_{dia}$ < 20% in Figure S3b are shown as red spheres, the sidechains of C31 and C121 are shown as yellow sticks, and the $C_\alpha$ atoms of the ligation sites are magenta spheres. It is evident that incubation of K25C with 3.5 equivalents of trityl tag below 293 K only results in selectively labeling at K25C at pH 7.2, of which the decreased cross-peak intensities for the residues containing 70, 88, 90 and 163 are likely caused by non-specific PRE effects.

## ED-EPR spectra, echo decays, comparison of DEER data

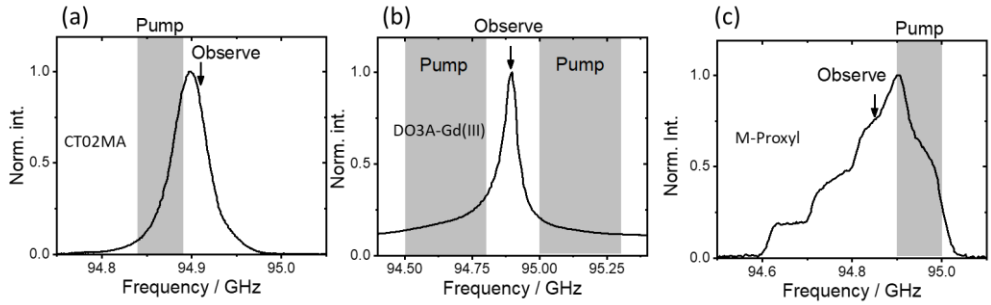


**Figure S5.** W-band ED-EPR spectra of (a) PpiB-CT02MA (50 K) (b) PpiB-DO3A-Gd(III) (10 K) and (c) PpiB-M-proxyl (25 K).The positions of the observe and chirp pump pulses are shown as indicated.

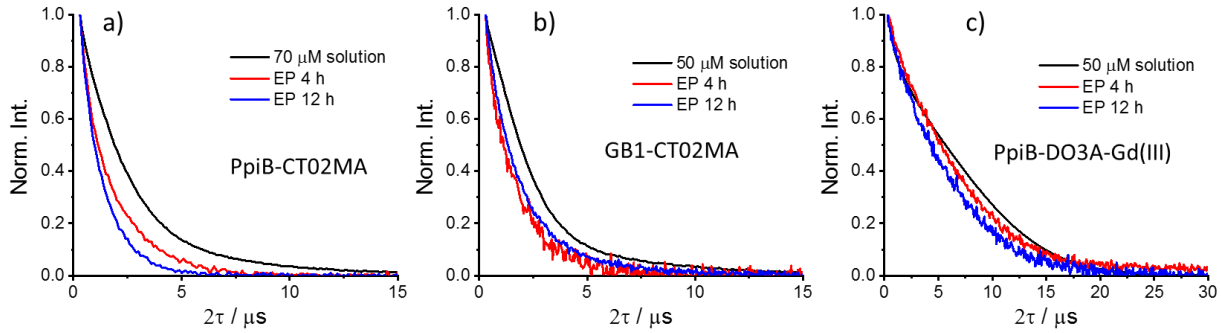


**Figure S6.** W-band echo decay traces measured at the maximum of the ED-EPR spectrum for (a) PpiB-CT02MA, (b) GB1-CT02MA, and (c) PpiB-BrPy-DO3A-Gd(III) in solution and in cells. EP stands for electroporation and the time indicates the incubation time before freezing the cells.

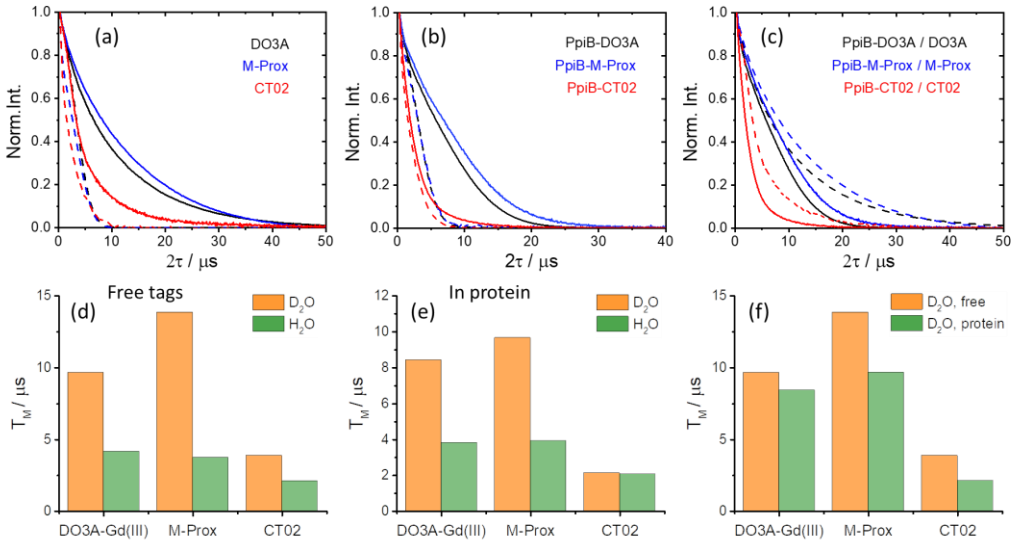


**Figure S7.** W-band echo decay traces measured at the maximum of the ED-EPR spectra for (a) the free tags (100 μM) as noted on the figure (abbreviated names), in $H_2O$/glycerol (dashed line) and $D_2O$/ glycerol-$d_8$ (solid line), (b) the same for PpiB labeled with the spin labels noted on the figure. The measurements were taken at the maximum of the ED-EPR intensity and temperatures of 10, 25 and 50 K for BrPy-DO3A-Gd(III), M-Proxyl and CT02MA, respectively. (c) Comparison of the echo decay for the three different tags when free in solution (dashed line) and in the labeled protein (solid line). Comparison of the $T_M$ values are given in (d), (e) and (f).

**Table S2.** Summary of the echo decay analysis of the data shown in Fig. S6. The echo-decays were fitted to a stretched exponent $V(2\tau) = Aexp(-\frac{(2\tau)^{\beta}}{T_M})$.

| | $D_2O$/glycerol-$d_8$ | | In $H_2O$/ glycerol | |
|---|---|---|---|---|
| | $T_M$ / µs | β | $T_M$ / µs | β |
| CT02MA | 3.86 | 0.84 | 2.10 | 0.86 |
| PpiB-CT02MA | 2.12 | 0.86 | 2.06 | 1.08 |
| BrPSPy-DO3A-Gd(III) | 9.62 | 0.88 | 4.14 | 1.96 |
| PpiB – BrPy-DO3A-Gd(III) | 8.42 | 1.35 | 3.80 | 1.82 |
| M-Proxyl | 13.84 | 1.03 | 3.72 | 1.52 |
| PpiB -M-Proxyl | 9.66 | 1.32 | 3.91 | 1.74 |

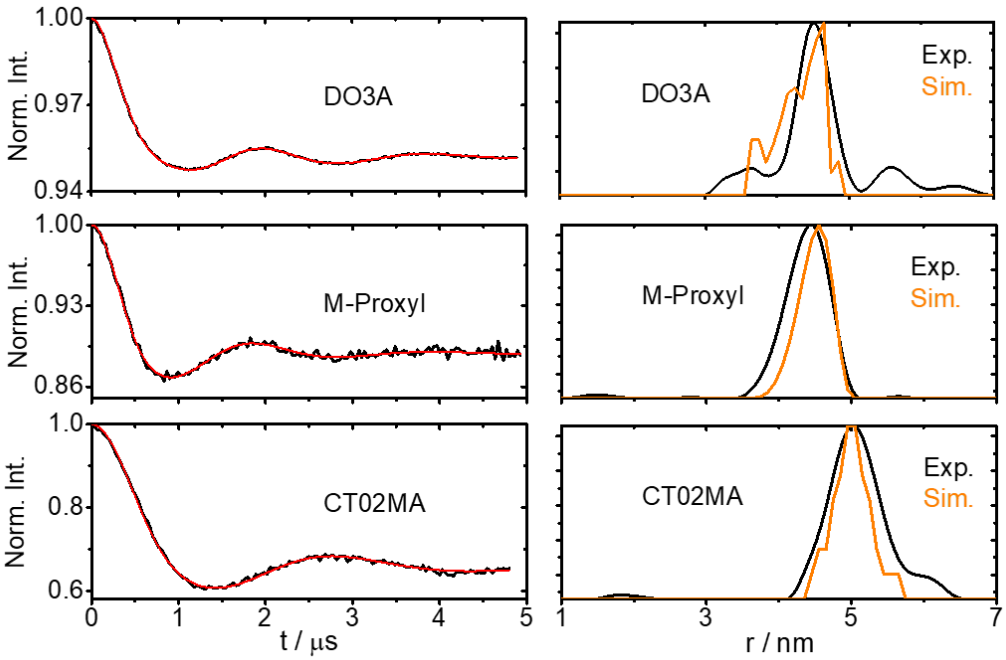


**Figure S8.** Comparison of the W-band DEER data of PpiB K25C/E153C labeled with CT02MA (50 K), BrPy-DO3A-Gd(III) (10 K) and M-Proxyl (25 K). The left panels show the DEER trace after background removal and the red lines are the fitted data obtained with Tikhonov regularization using DeerAnalysis and the distance distributions shown on the left panels. The distance distributions are compared with those obtained using mtsslWizard[12] shown in orange. The primary DEER trace of the M-Proxyl labeled protein is given in Fig. S9.

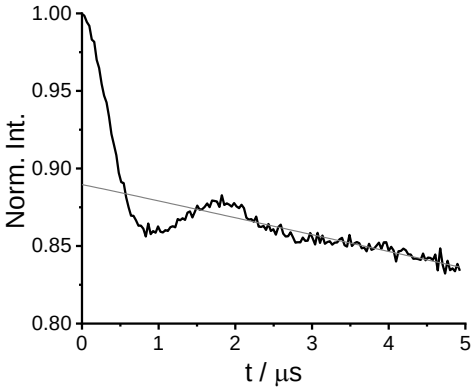


**Figure S9.** Primary DEER data of PpiB K25C/E153C-M-Proxyl (25 K) with the background decay curve in gray. Experimental conditions are given in Table S1.

## Comparison of DEERs with chirp-pulse and rectangular-pulse

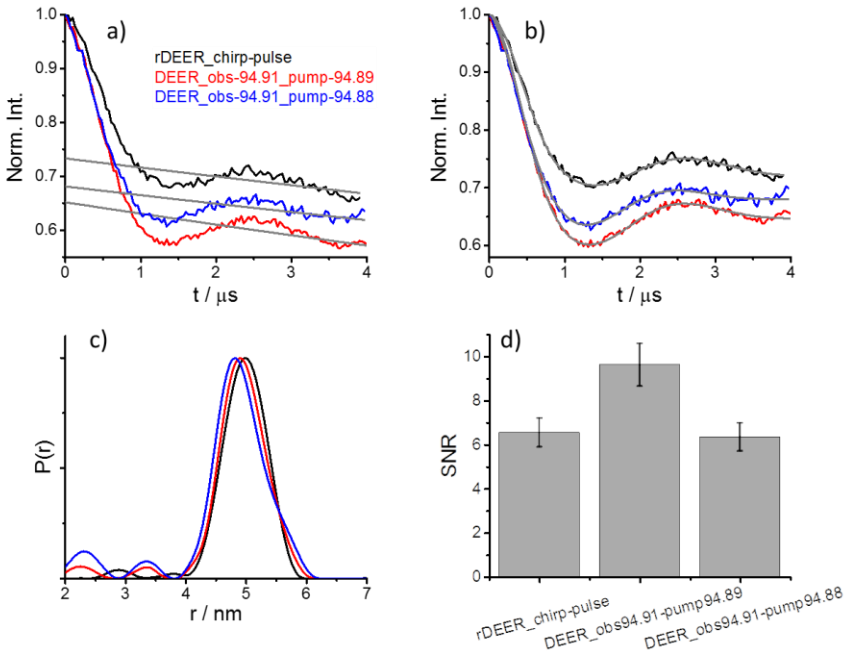


**Figure S10.** Comparison of W-band DEER results on PpiB-CT02MA (50 µM) in solution measured using the rDEER sequence with chirp pulses (black) and standard DEER sequence with rectangular pulses, observing at 94.91 GHz and pumping at 94.89 GHz (red) and at 94.88 GHz (blue) respectively. (a) The primary DEER traces and background fitting (gray). (b) The background corrected traces and their fits obtained with Tikhonov regularization using DeerAnalysis (gray). (c) The obtained distance distributions. (d) The SNR per unit time (min).

## Stability of CT02MA labeled PpiB in reducing conditions

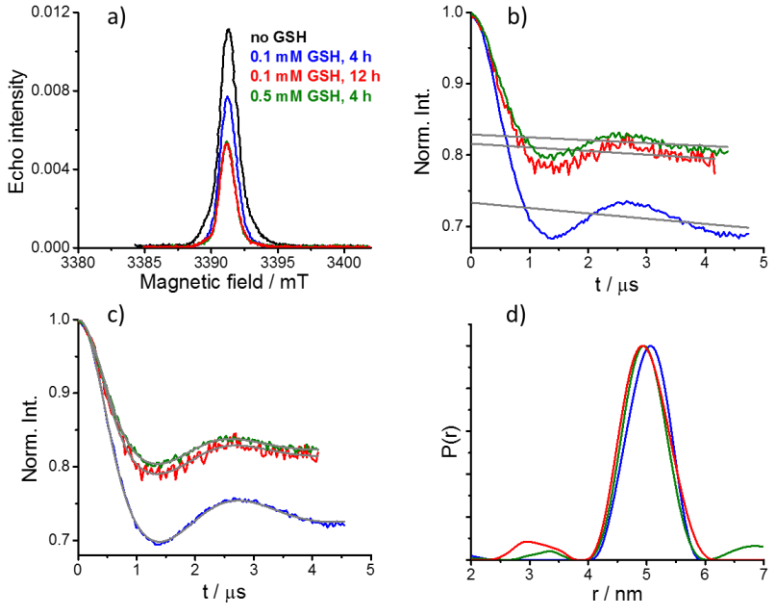


**Figure S11.** W-band DEER results on PpiB-CT02MA (30 µM) incubated with different concentrations of glutathione (GSH) for different times. (a) ED-EPR spectra. (b) The primary DEER traces and the background fitting (gray). (c) The background corrected traces and their fits obtained with Tikhonov regularization using DeerAnalysis (gray). (d) The obtained distance distributions.

## References


1. Cao, C.; Chen, J. L.; Yang, Y.; Huang, F.; Otting, G.; Su, X. C., Selective $^{15}$N-Labeling of the Side-Chain Amide Groups of Asparagine and Glutamine for Applications in Paramagnetic Nmr Spectroscopy. *J. Biomol. NMR* **2014,** *59* (4), 251-61.
2. Giannoulis, A.; Yang, Y.; Gong, Y.-J.; Tan, X.; Feintuch, A.; Carmieli, R.; Bahrenberg, T.; Liu, Y.; Su, X.-C.; Goldfarb, D., Deer Distance Measurements on Trityl/Trityl and Gd(III)/Trityl Labelled Proteins. *Phys. Chem. Chem. Phys.* **2019,** *21*, 10217 - 10227.
3. Yang, Y.; Yang, F.; Gong, Y.-J.; Bahrenberg, T.; Feintuch, A.; Su, X.-C.; Goldfarb, D., High Sensitivity in-Cell Epr Distance Measurements on Proteins Using an Optimized Gd(III) Spin Label. *J. Phys. Chem. Lett.* **2018,** *9* (20), 6119-6123.
4. Theillet, F. X.; Binolfi, A.; Bekei, B.; Martorana, A.; Rose, H. M.; Stuiver, M.; Verzini, S.; Lorenz, D.; van Rossum, M.; Goldfarb, D.; Selenko, P., Structural Disorder of Monomeric Alpha-Synuclein Persists in Mammalian Cells. *Nature* **2016,** *530* (7588), 45-50.
5. Goldfarb, D.; Lipkin, Y.; Potapov, A.; Gorodetsky, Y.; Epel, B.; Raitsimring, A. M.; Radoul, M.; Kaminker, I., HYSCORE and DEER with an Upgraded 95 GHz Pulse EPR Spectrometer. *J. Magn. Reson.* **2008,** *194* (1), 8-15.
6. Mentink-Vigier, F.; Collauto, A.; Feintuch, A.; Kaminker, I.; Tarle, V.; Goldfarb, D., Increasing Sensitivity of Pulse EPR Experiments Using Echo Train Detection Schemes. *J. Magn. Reson.* **2013,** *236*, 117-25.
7. Bahrenberg, T.; Rosenski, Y.; Carmieli, R.; Zibzener, K.; Qi, M.; Frydman, V.; Godt, A.; Goldfarb, D.; Feintuch, A., Improved Sensitivity for W-Band Gd(III)-Gd(III) and Nitroxide-Nitroxide DEER Measurements with Shaped Pulses. *J. Magn. Reson.* **2017,** *283*, 1-13.
8. Bahrenberg T, Y. Y., Goldfarb D, Feintuch A. , rDEER: A Modified DEER Sequence for Distance

Measurements Using Shaped Pulses. *Magnetochemistry* **2019,** *5* (1), 20.
9. Doll, A.; Qi, M.; Wili, N.; Pribitzer, S.; Godt, A.; Jeschke, G., Gd(III)-Gd(III) Distance Measurements with Chirp Pump Pulses. *J. Magn. Reson.* **2015,** *259*, 153-62.
10. Pannier, M.; Veit, S.; Godt, A.; Jeschke, G.; Spiess, H. W., Dead-Time Free Measurement of Dipole-Dipole Interactions between Electron Spins. 2000. *J. Magn. Reson.* **2011,** *213* (2), 316-25.
11. Jeschke, G.; Chechik, V.; Ionita, P.; Godt, A.; Zimmermann, H.; Banham, J.; Timmel, C. R.; Hilger, D.; Jung, H., Deeranalysis2006 - A Comprehensive Software Package for Analyzing Pulsed ELDOR Data. *Appl. Magn. Reson.* **2006,** *30* (3-4), 473-498.
12. G. Hagelueken, R. W., J. H. Naismith and O. Schiemann, MtsslWizard: In Silico Spin-Labeling and Generation of Distance Distributions in Pymol. *Appl. Magn. Reson.* **2012,** *42* 377-391.