

Supporting information

Probing the solution structure of the E. coli multidrug transporter MdfA using DEER distance measurements with nitroxide and Gd(III) spin labels

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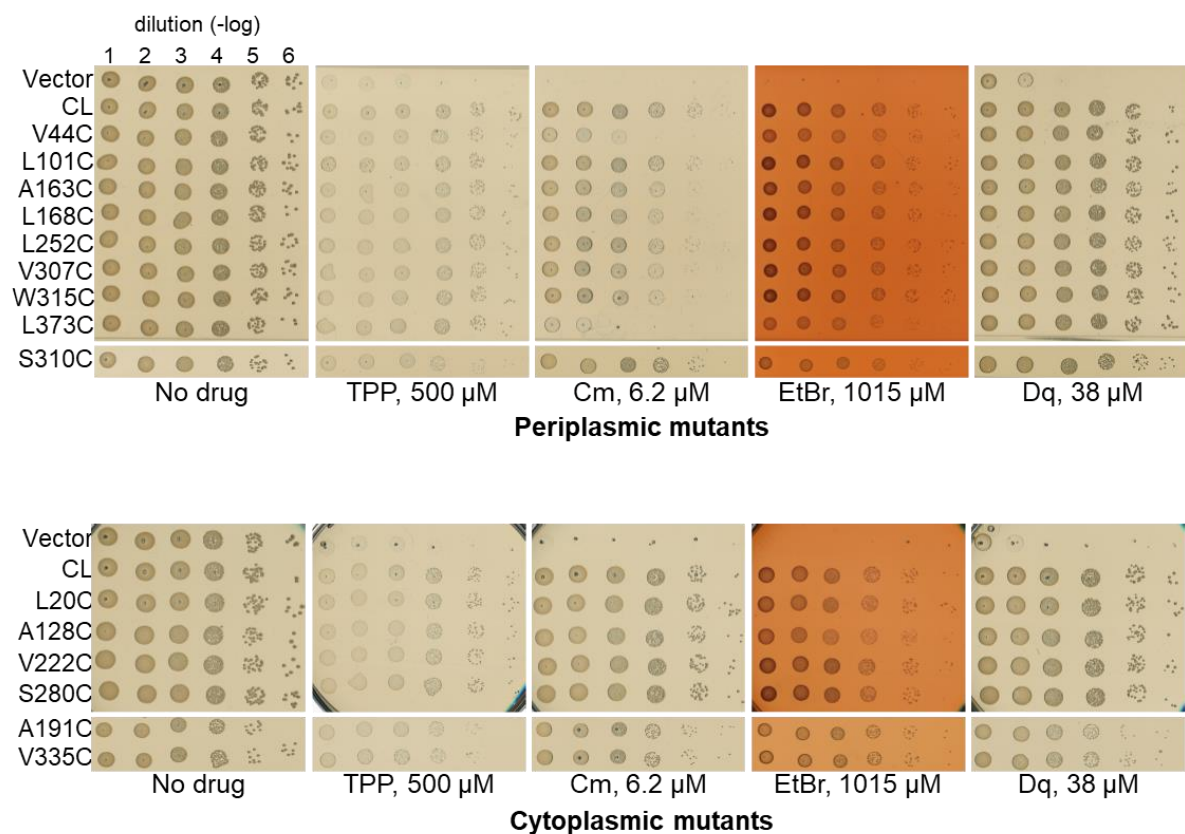


Figure S1: Mdr activity of single cysteine mutants. Serial dilutions of cells, expressing the indicated constructs (CL = cysteine-less) were spotted on LB-agar plates containing the indicated drugs. Upper panel, mutant S310C was analyzed in a separate plate, as shown by the space line. Lower panel, mutants A191C and V335C were analyzed in a separate plate, as shown by the space line.

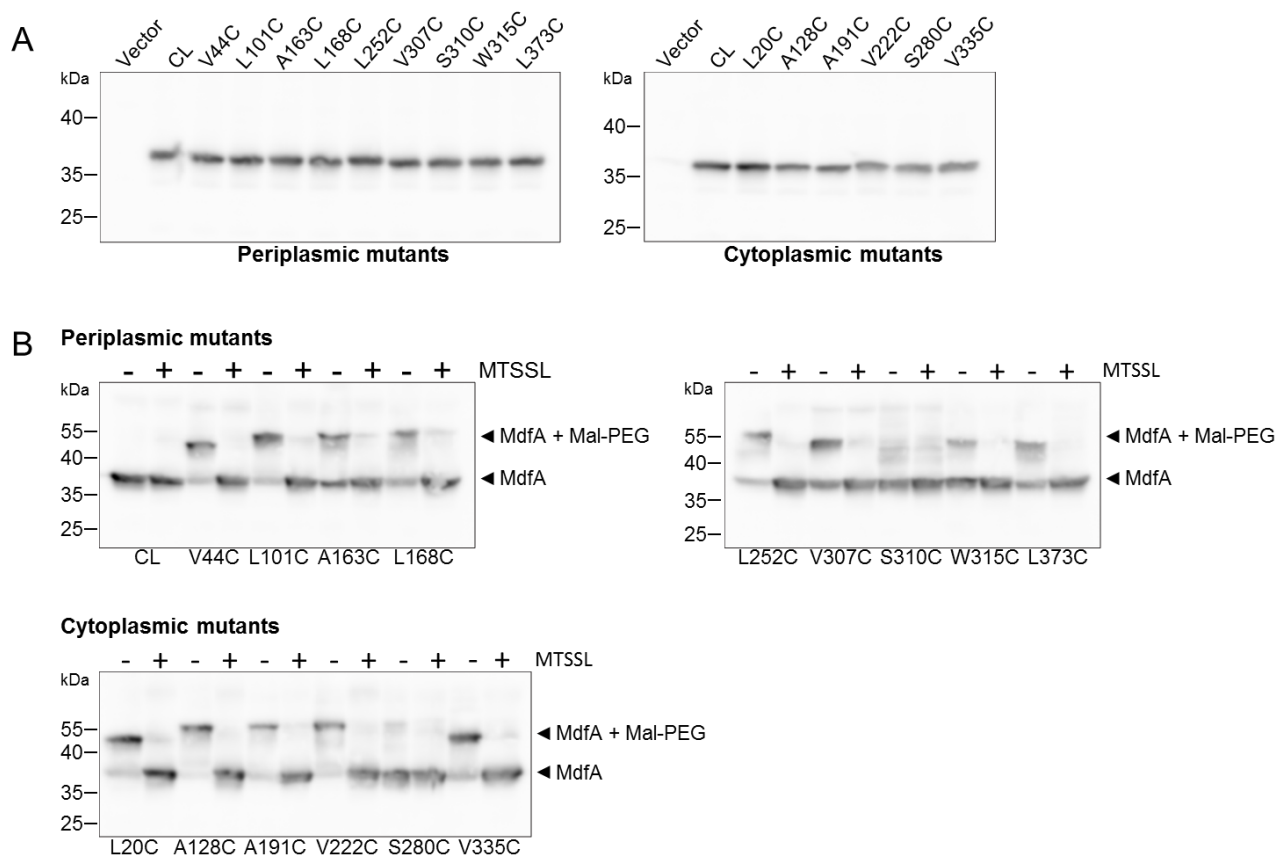


Figure S2: Expression and MTSSL-accessibility of the single cysteine mutants. **A.** Western blotting of membranes, prepared from cells expressing the indicated MdfA constructs. **B.** Cysteine accessibility to MTSSL. Solubilized, single cysteine mutants were incubated with (+) or without (-) MTSSL. After removal of non-bound label the samples were denatured with 1% SDS and incubated with Mal-PEG(5000).

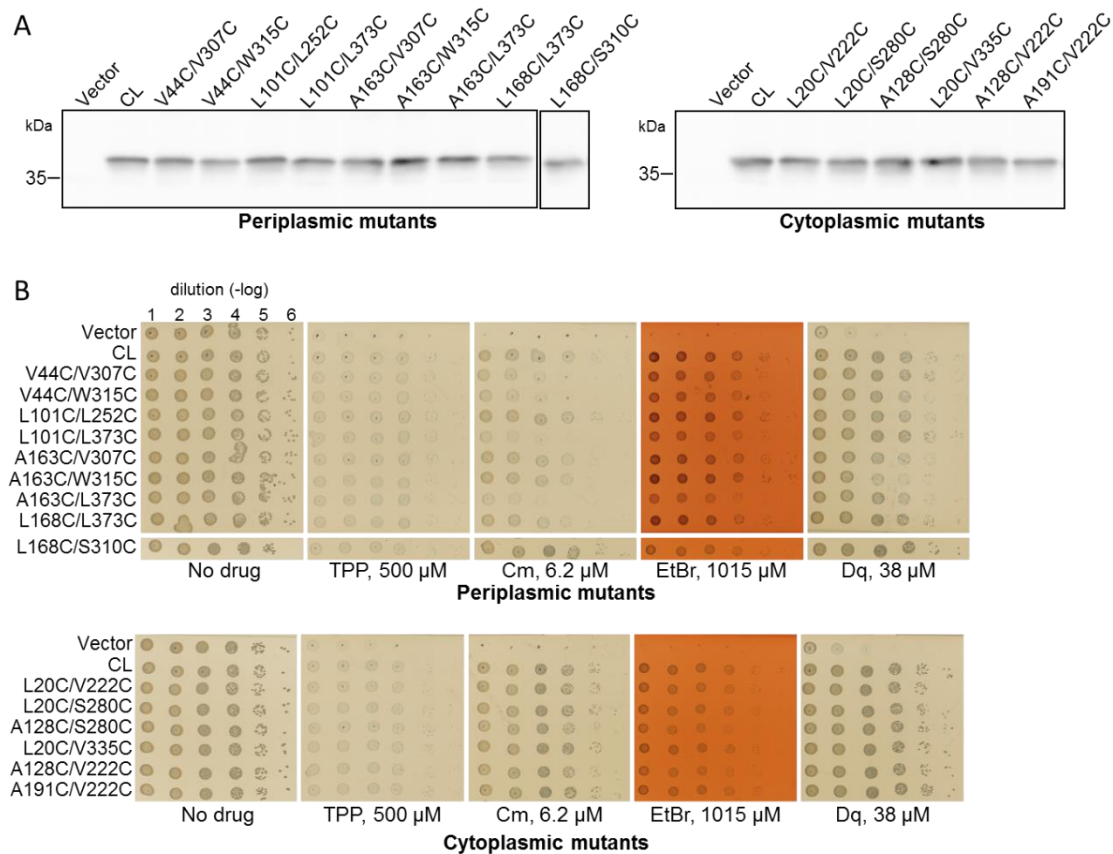


Figure S3: Characterization of double-cysteine mutants. **A.** Western blotting of membranes, prepared from cells expressing the indicated constructs. The expression of mutant L168C/S310C was analyzed in a separate gel, as shown by the separated frame. **B.** Cells expressing the indicated constructs were spotted on LB-agar plates containing the indicated drugs. Upper panel, mutant L168C/S310C was analyzed in a separate plate, as shown by the space line.

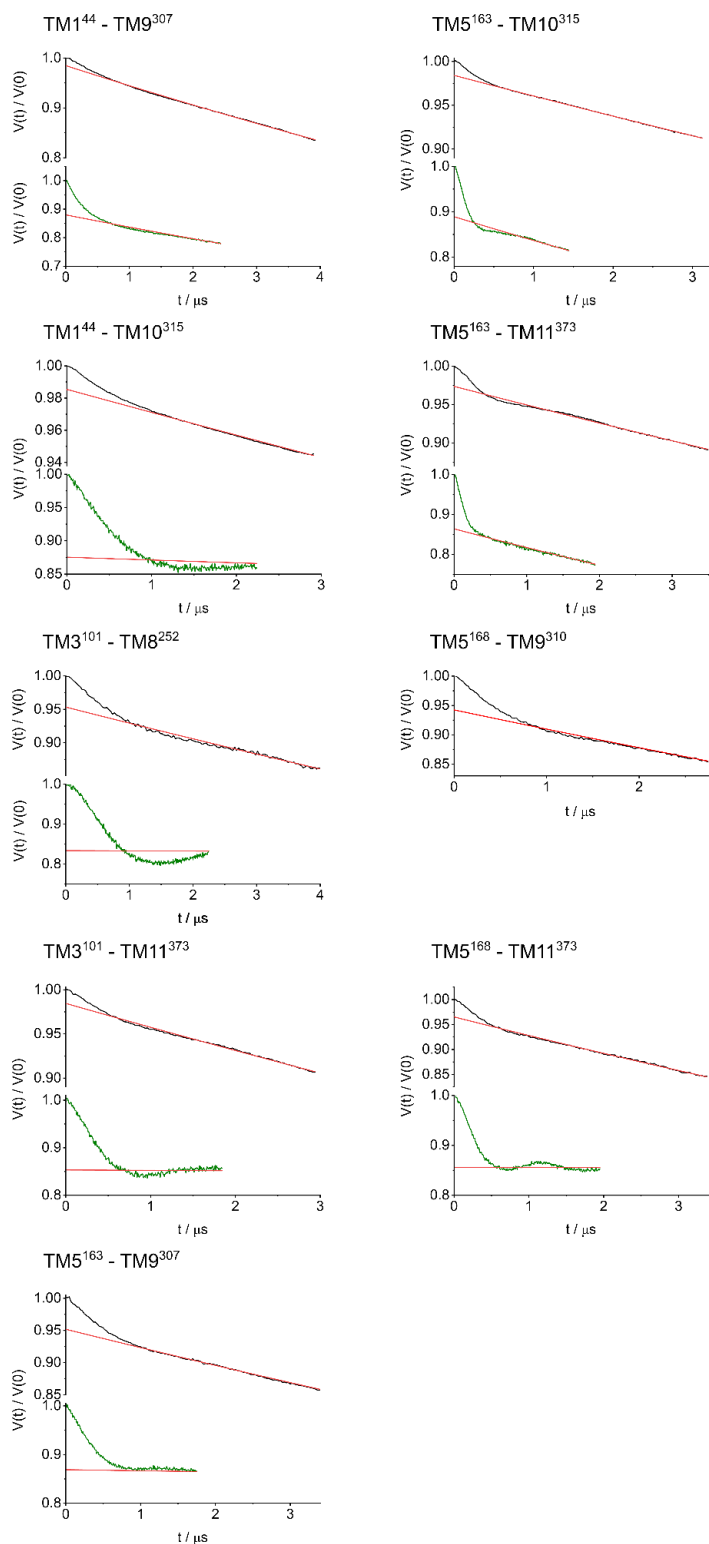


Figure S4: Raw data of all DEER experiments of periplasmic constructs shown in Fig. 4. For each mutant, the top panel shows Gd(III) data (black trace) and the bottom panel MTSSL data (green trace). The background decay **obtained by the DD software** is indicated by the red trace.

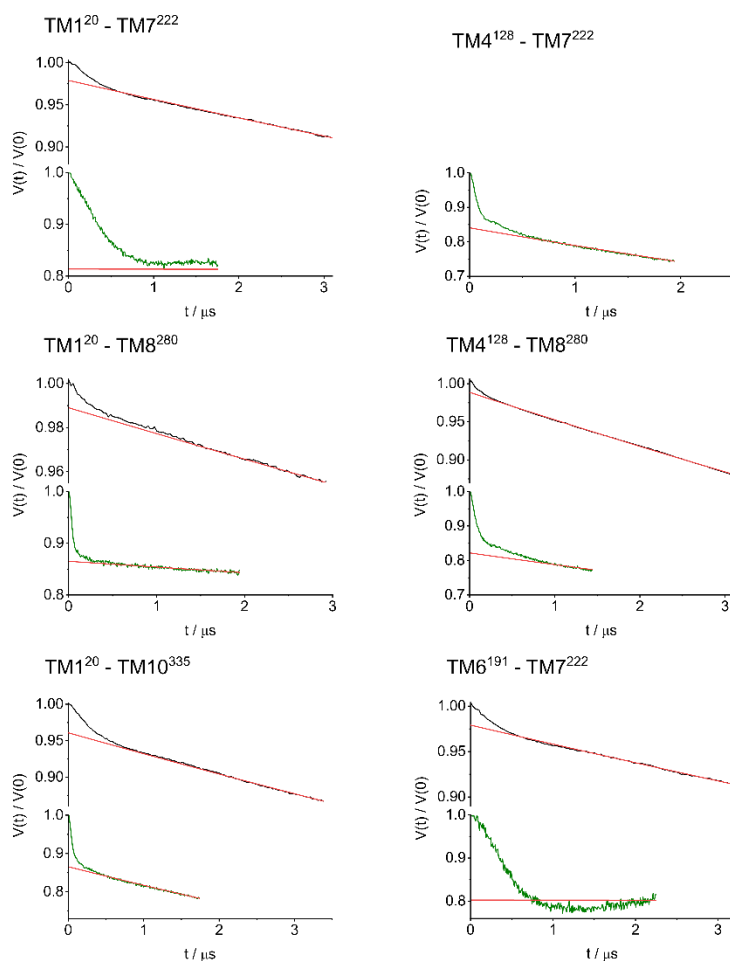
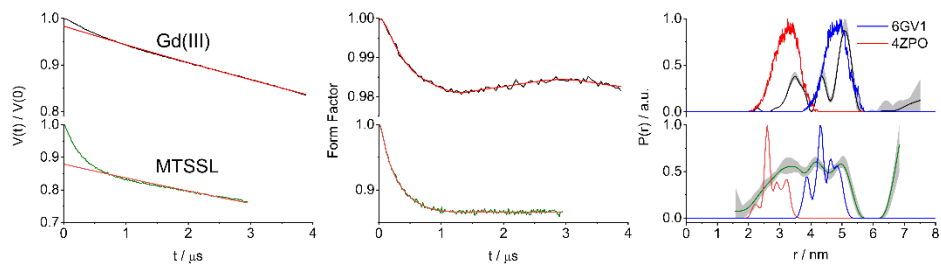
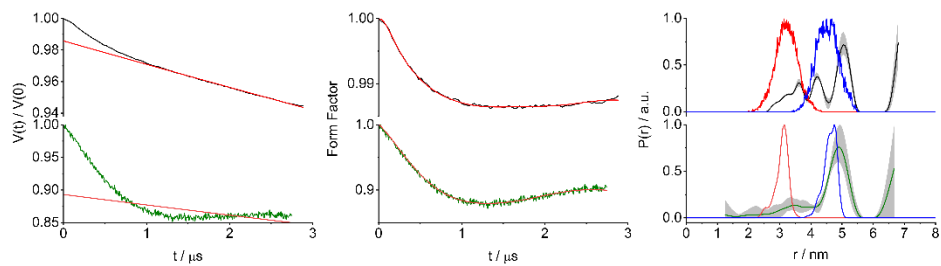


Figure S5: Raw data of all DEER experiments of cytoplasmic constructs shown in Fig. 5. The top panel of each dataset shows Gd(III) data (black trace) and the bottom panel MTSSL data (green trace). The background decay obtained by the DD software is indicated by the red trace.

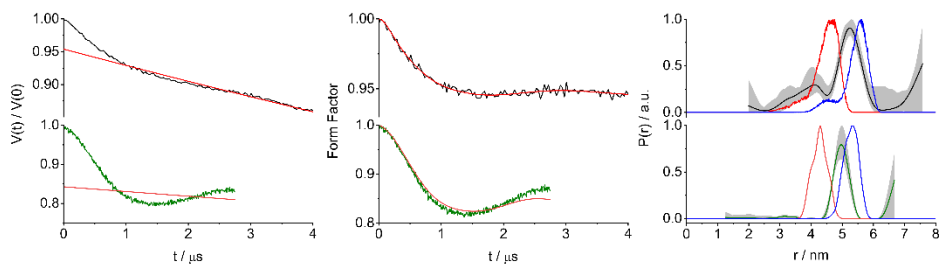
TM1⁴⁴ - TM9³⁰⁷



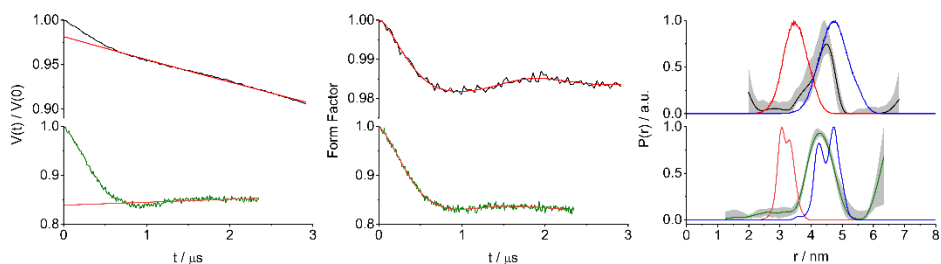
TM1⁴⁴ - TM10³¹⁵



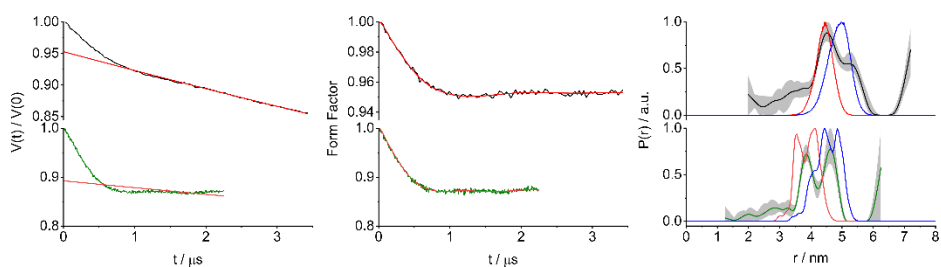
TM3¹⁰¹ - TM8²⁵²



TM3¹⁰¹ - TM11³⁷³



TM5¹⁶³ - TM9³⁰⁷



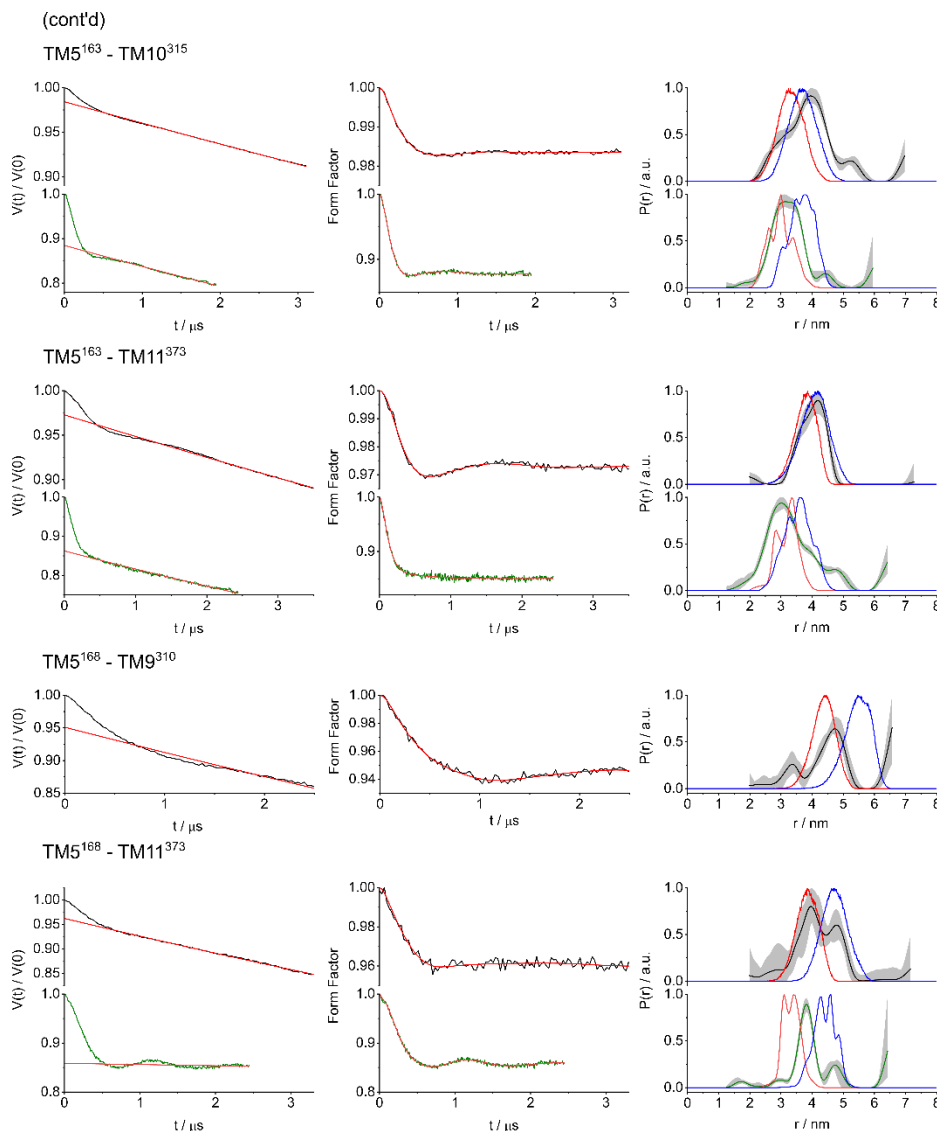


Figure S6: DEER data for all periplasmic mutants analyzed using Tikhonov regularization with DeerAnalysis 2018. The top panel of each dataset shows Gd(III) data (black trace) and the bottom panel MTSSL data (green trace, including the last 500 ns which were removed in Fig. S4 due to pulse overlapping). For every mutant, raw data with the background correction function (red, left column), form factor after background correction with the fit data (red) obtained with distance distribution on the right (middle column) and distance distribution (right column) are shown. The shaded gray area denotes the confidence bands for the distance distribution as obtained from the validation function in DeerAnalysis. The distance distribution calculated from the crystal structures 4ZP0 (I_f , red) and 6GV1 (O_o , blue) are shown as well.

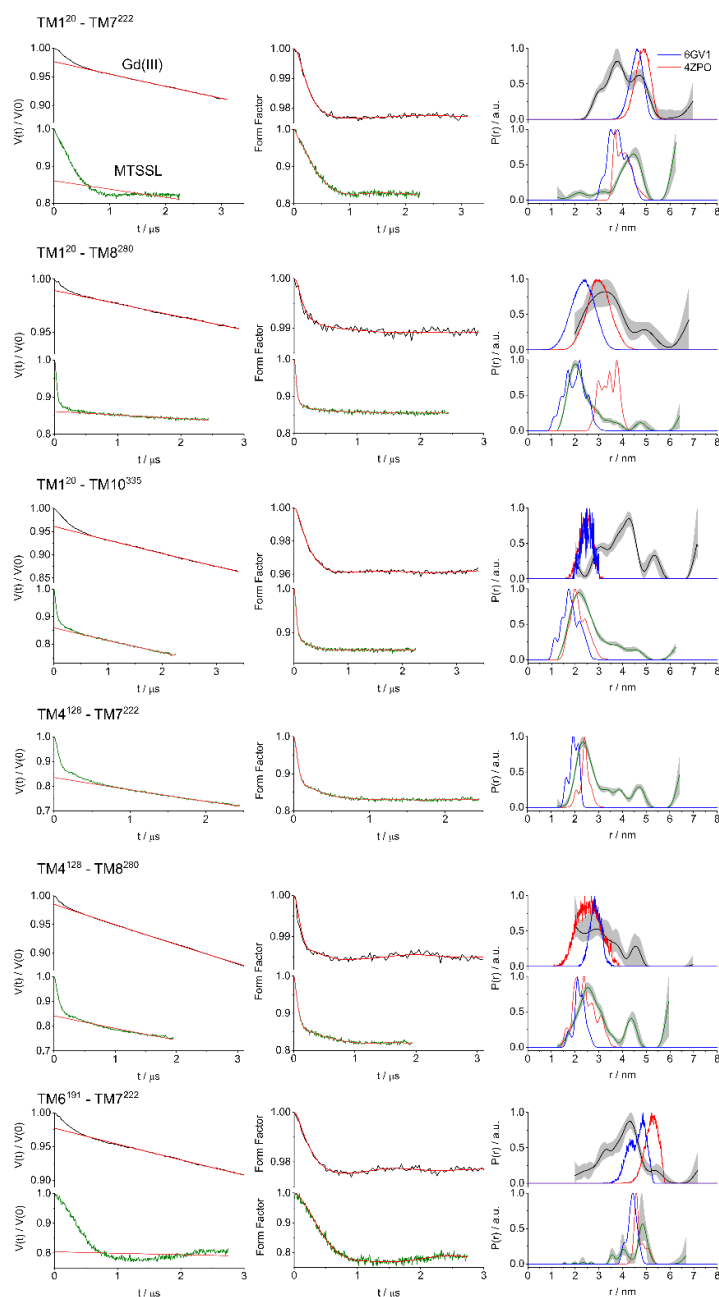


Figure S7: DEER data for all cytoplasmic mutants analyzed with Tikhonov regularization with DeerAnalysis 2018. The top panel of each dataset shows Gd(III) data (black trace) and the bottom panel MTSSL data (green trace, including the last 500 ns which were removed in Fig. S5 due to pulse overlapping). For every mutant, raw data with the background correction function (red, left column), form factor after background correction with the fit data (red) obtained with distance distribution on the right (middle column) and distance distribution (right column) are shown. The shaded gray area denotes the confidence bands for the distance distribution as obtained from the validation function in DeerAnalysis. The distance distribution calculated from the crystal structures 4ZPO (I_f , red) and 6GV1 (O_o , blue) are shown as well.

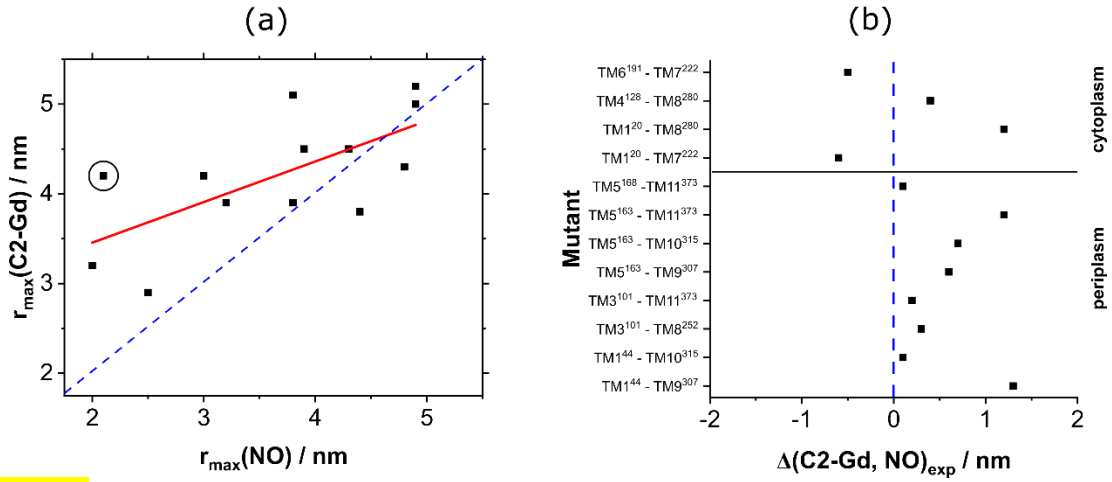


Figure S8: (a) Plot of the experimental $r_{\max}$ for the Gd-C2 labeled variants vs $r_{\max}$ for the NO labeled ones obtained from the analysis with Tikhonov regularization (see Fig.S6 and S7). When more than 1 peak appeared in the distance distribution, we considered only the major one. The blue, dotted line corresponds to a perfect match, whereas the red line is a linear fit. Mutant TM1²⁰ - TM10³³⁵, which was removed from subsequent analysis as described in the text, is indicated by a black circle; (b) Plot of $\Delta(\text{C2-Gd, NO})_{\text{exp}}$ for each mutant studied, *except for* TM1²⁰ - TM10³³⁵.

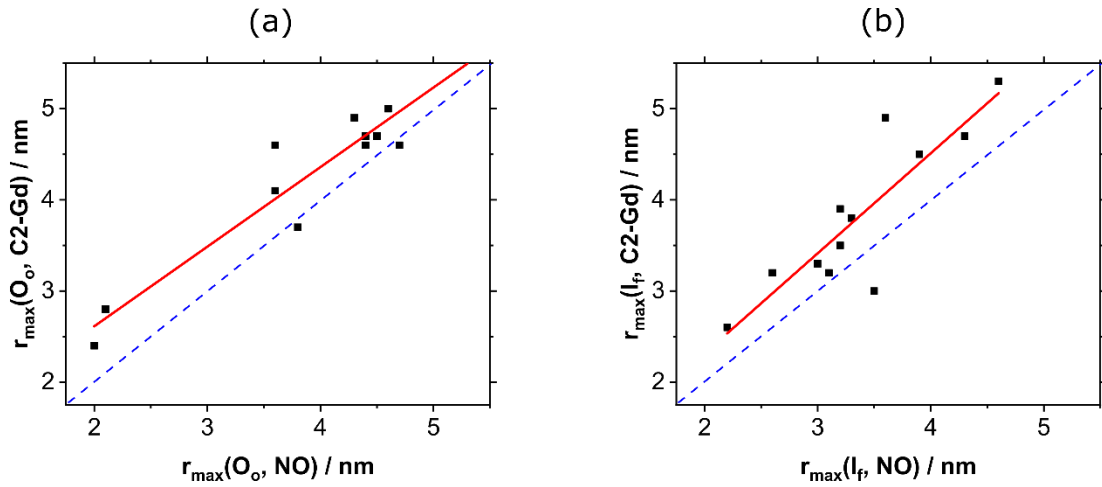


Figure S9: Plots of the calculated $r_{\max}$ (C2-Gd) vs $r_{\max}$ (NO) for the (a) O_o and (b) I_f structures. The dotted blue line corresponds to the perfect correlation and the red line is a linear fit.

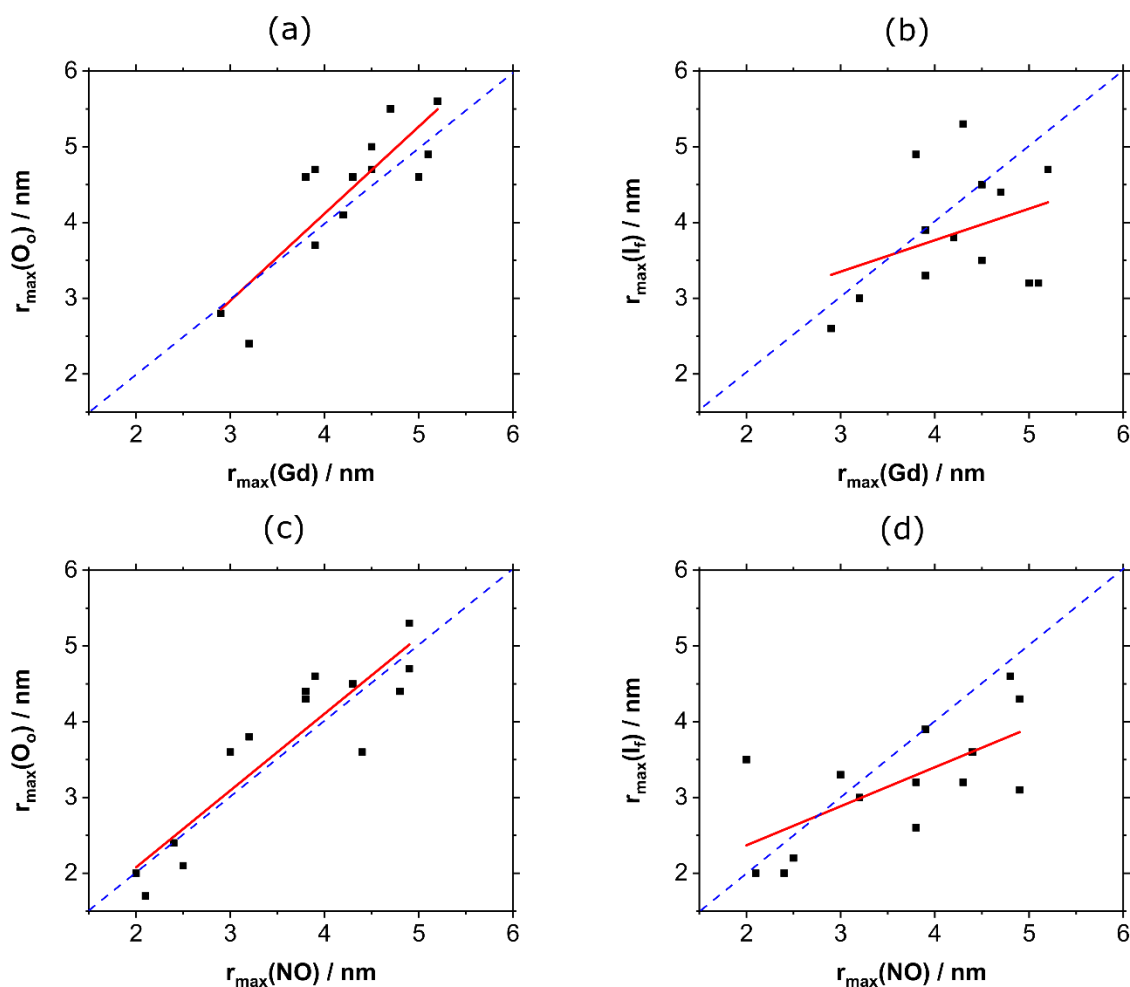


Figure S10: Comparison of the experimental and crystal structure derived $r_{\max}$ values for the Gd-C2 labelled mutants for the (a) 6GV1 (O_o) structure and (b) 4ZP0 (I_f) structure and the same for the NO labeled mutants (c,d). Experimental data were obtained using Tikhonov regularization. Blue dotted lines represent perfect correlation and the red line linear fits. Fit parameters were: slope = 1.15, $R^2 = 0.73$ (a), slope = 0.41, $R^2 = 0.12$ (b), slope = 1.01, $R^2 = 0.83$ (c), and slope = 0.51, $R^2 = 0.46$ (d).

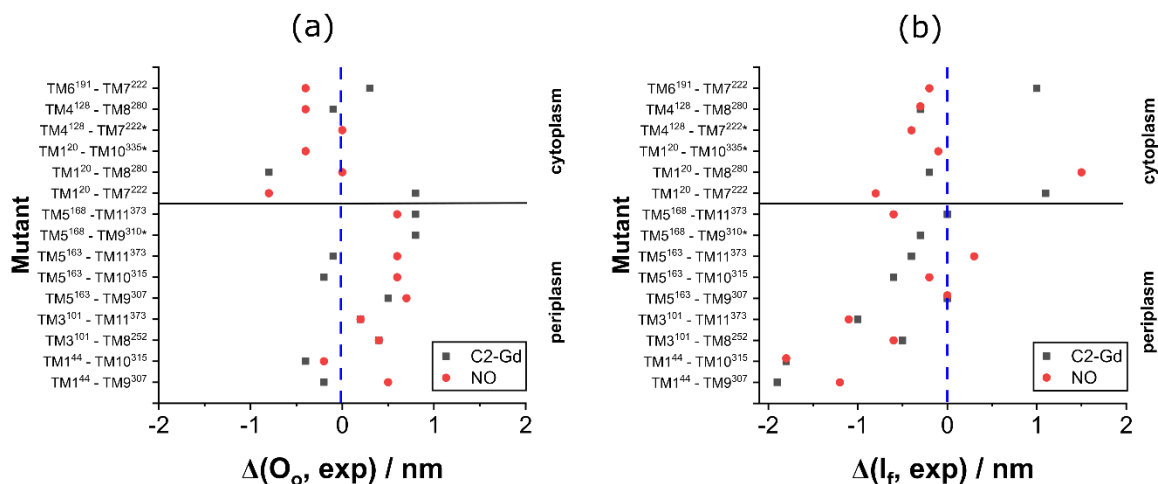
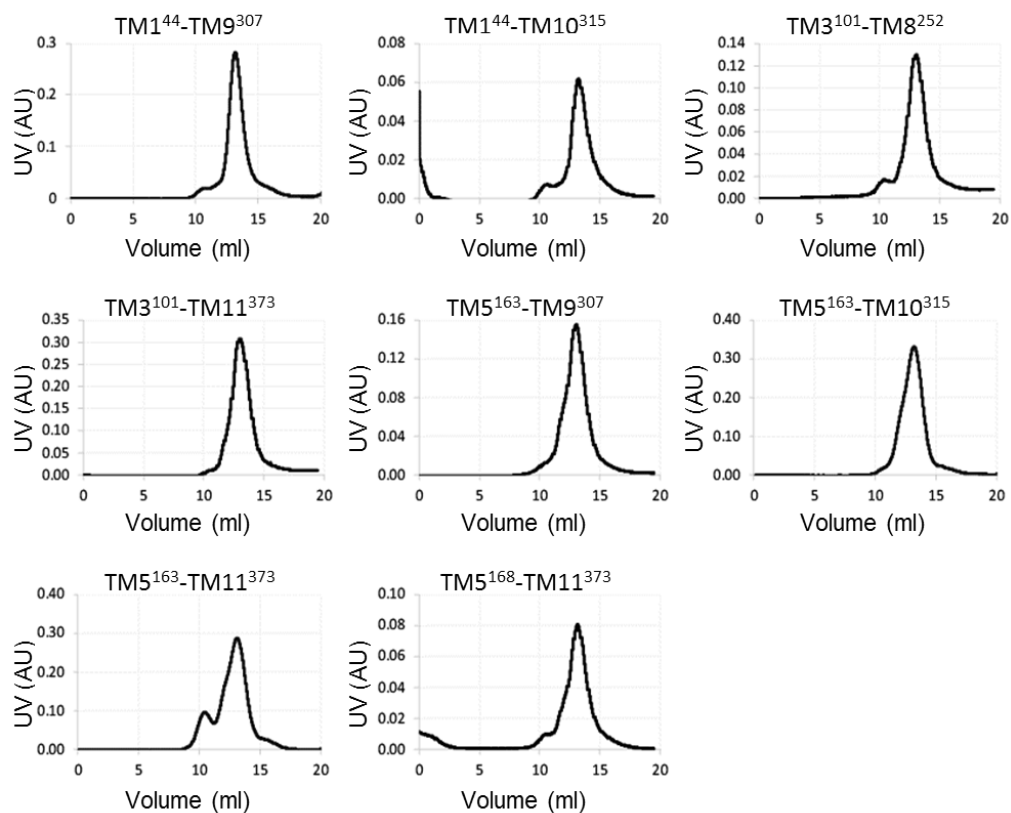


Figure S11: Plots showing the difference between the predicted and experimental $r_{\max}$ values calculated using Tikhonov regularization for the mutants studied (red symbols are for Gd-C2 and black for NO). (a) for the O_o and (b) I_f structure. The vertical blue dotted lines mark the zero lines (perfect agreement) and the black horizontal lines represent the division between periplasmic and cytoplasmic pairs.

Periplasmic mutants



Cytoplasmic mutants

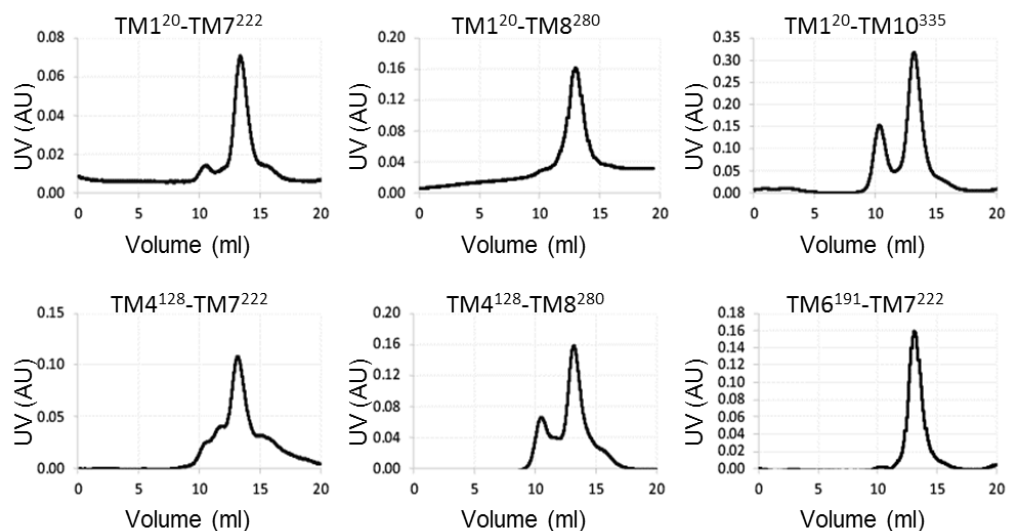


Figure S12: Size exclusion chromatography (SEC) elution profiles of all MTSSL labeled mutants. In cases where multiple peaks are visible, only the fractions in the major peak were used subsequently. In the TM1³³ – TM10³¹⁵ and TM6¹⁹¹ – TM7²²² the UV detection was not zeroed.

Table S1: Overview of all mutants in the background of a cysteine-less MdfA, their designation in the text as well as the main distance $r_{\max}$ and (where applicable) the distance maximum of a second distance (in parenthesis) present in the data. Evaluation was performed using the software DD.

Mutant	Designation	$r_{\max}$ (NO)	$r_{\max}$ (Gd)
V44C W307C	TM1 ⁴⁴ - TM9 ³⁰⁷	4.4 (3.2)	5.0 (3.5)
V44C W315C	TM1 ⁴⁴ - TM10 ³¹⁵	5.1	5.0 (4.1)
L101C L252C	TM3 ¹⁰¹ - TM8 ²⁵²	5.2	5.0
L101C L373C	TM3 ¹⁰¹ - TM11 ³⁷³	4.3	4.5 (3.6)
A163C V307C	TM5 ¹⁶³ - TM9 ³⁰⁷	4.1	4.5
A163C W315C	TM5 ¹⁶³ - TM10 ³¹⁵	3.1	4.0 (3.0)
A163C L373C	TM5 ¹⁶³ - TM11 ³⁷³	3.0	4.2 (3.6)
L168C S310C	TM5 ¹⁶⁸ - TM9 ³¹⁰	--	4.6
L168C L373C	TM5 ¹⁶⁸ - TM11 ³⁷³	3.8	4.2
L20C V222C	TM1 ²⁰ - TM7 ²²²	4.2	4.0
L20C S280C	TM1 ²⁰ - TM8 ²⁸⁰	2.0	3.3
L20C V335C	TM1 ²⁰ - TM10 ³³⁵	2.0 (2.4)	4.3 (3.4)
A128C V222C	TM4 ¹²⁸ - TM7 ²²²	2.3 (3.9)	--
A128C S280C	TM4 ¹²⁸ - TM8 ²⁸⁰	2.6 (4.5)	3.2
A191C V222C	TM6 ¹⁹¹ - TM7 ²²²	4.7	4.3 (3.6)

Table S2: Experimental conditions for all Gd(III)-Gd(III) DEER experiments. “AWG DEER” relates to a standard DEER experiment where the pump pulse is replaced by a linear chirp; “rDEER” indicates that the pump pulse is swept between the primary echo and the first π pulse. For details, refer to the experimental section in the main text. t_p and f_p are the pump pulse length and frequency, T indicates the time up to which the DEER trace was accumulated. Experiment run-time is given including hardware overhead.

Mutant	Experiment	t_p	f_p	T	Run-time
L20C V222C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.5 μ s	9:30 h
L20C S280C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3 μ s	10:00 h
L20C V335C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.5 μ s	3:20 h
V44C V307C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	4 μ s	1:40 h
V44C W315C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3 μ s	5:30 h
L101C L252C	AWG DEER	2 x 76 ns	94.55 - 94.85, 95.05 - 95.35	3 μ s	1:00 h
L101C L373C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3 μ s	3:00 h
A128C S280C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.2 μ s	10:20 h
A163C V307C	AWG DEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	4 μ s	5:40 h
A163C W315C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.2 μ s	11:00 h
A163C L373C	rDEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.6 μ s	7:30 h
L168C S310C	AWG DEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.5 μ s	2:10 h
L168C L373C	AWG DEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	4 μ s	1:10 h
A191C V222C	AWG DEER	2 x 96 ns	94.55 - 94.85, 95.05 - 95.35	3.5 μ s	8:00 h