

Supplementary Information

Proton-driven alternating access in a spinster transporter, an emerging family of broad-specificity efflux pumps

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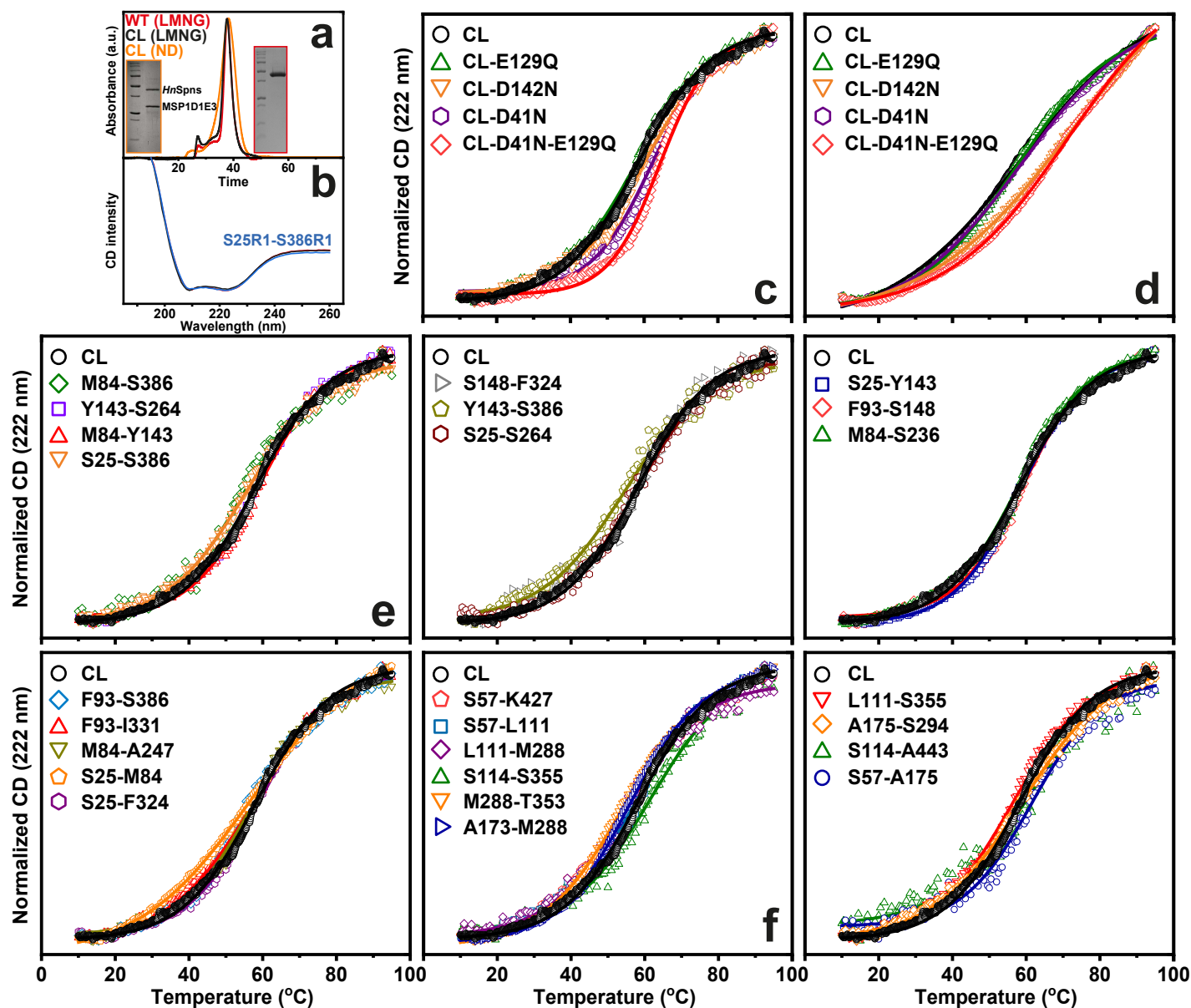
⁴ These authors contributed equally to this work.

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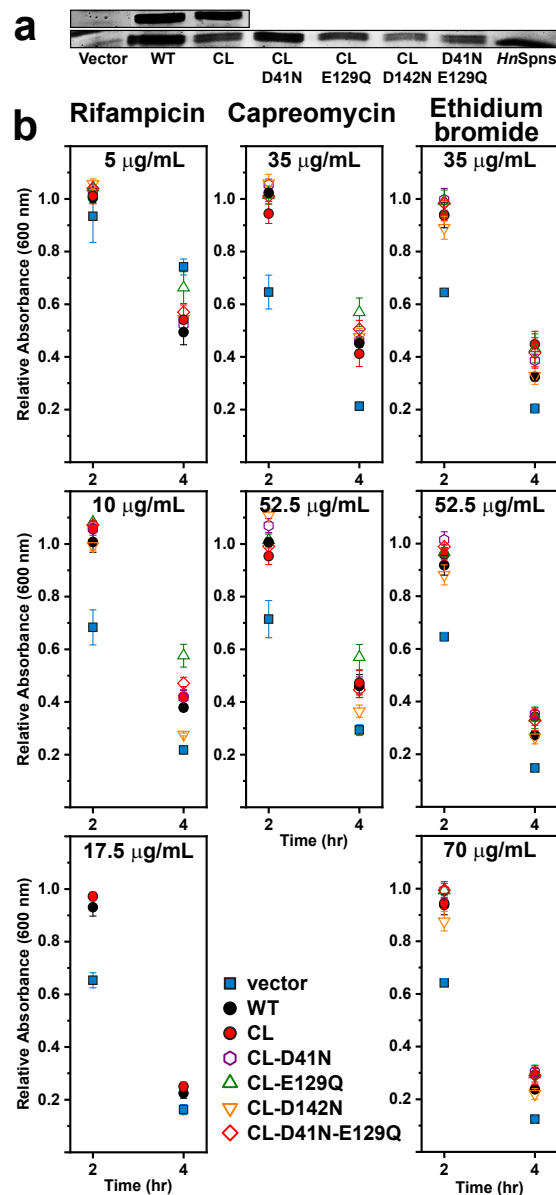
Figs. S1 to S16

Tables S1 and S2

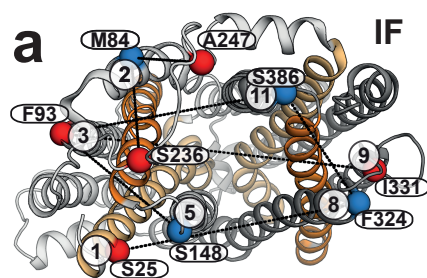
DEER Data Appendices



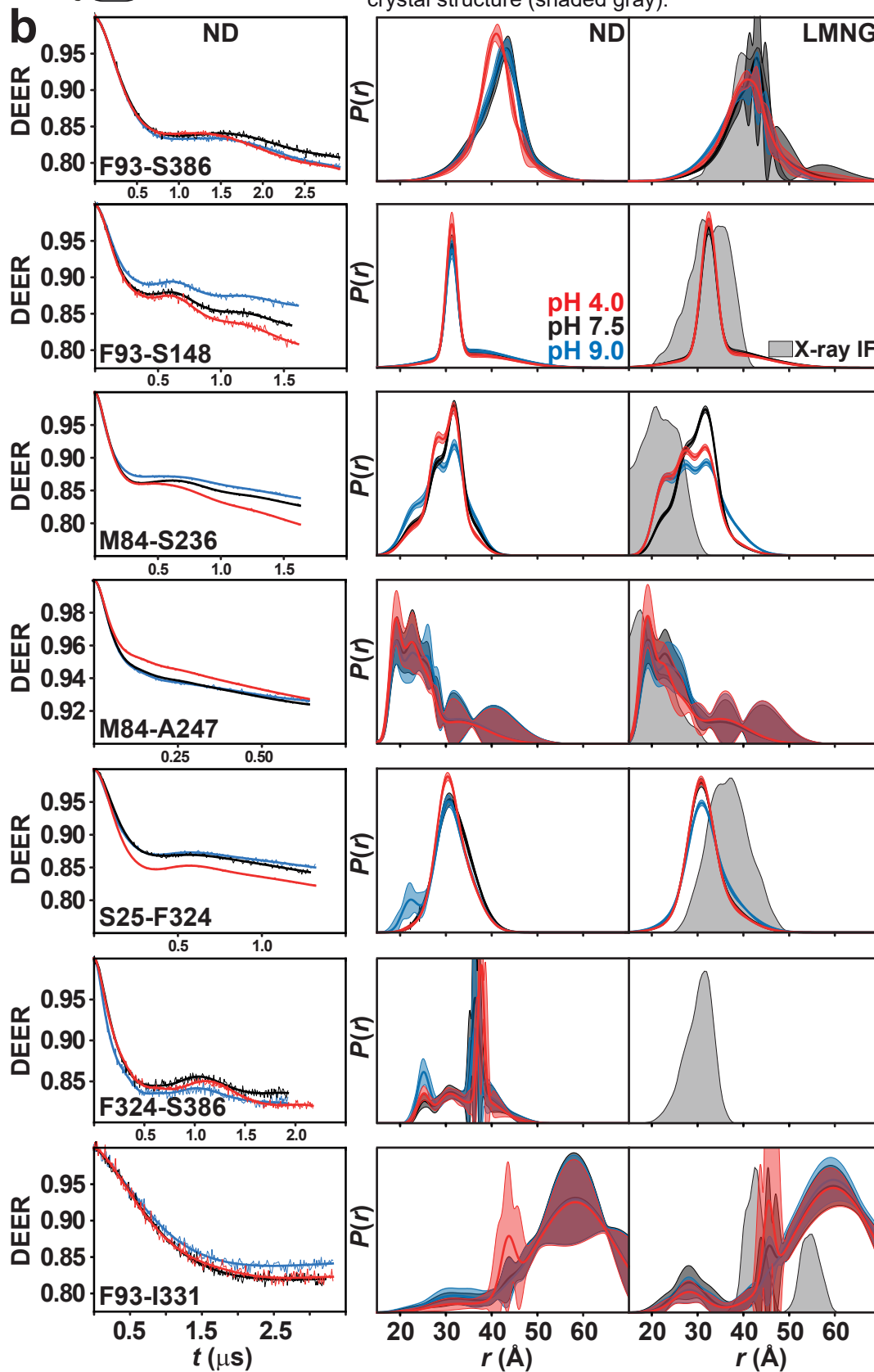
Supplementary Figure 1. Thermal stability analysis. (a) Size exclusion chromatography profiles of the wildtype (WT) and cysteine-less (CL) *HnSpns* in LMNG micelles and nanodiscs (ND), and (b) their circular dichroism spectra in micelles along with a spin-labeled DEER mutant (S25R1-S386R1). (c) Thermal stability of the protonation-mimetic mutants in LMNG micelles and (d) in lipid nanodiscs, compared with the CL protein. (e) Thermal stability of the spin-labeled DEER mutants on the intracellular and (f) extracellular side in LMNG micelles compared with CL protein.

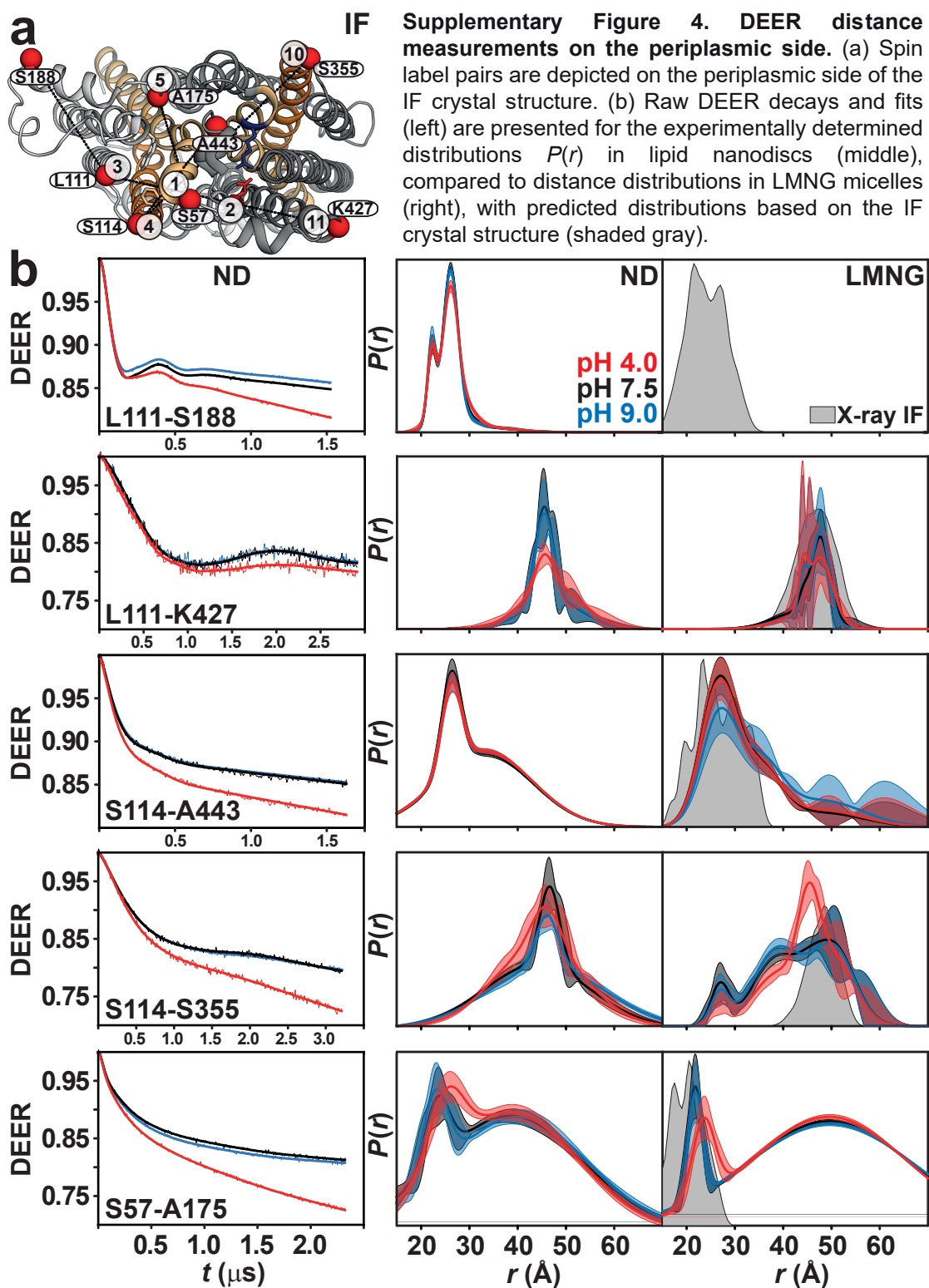


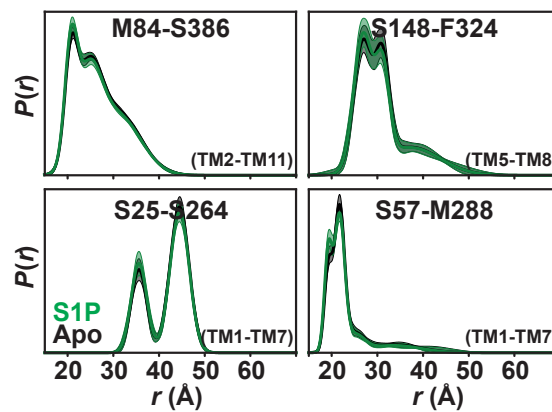
Supplementary Figure 2. Cell growth-based drug resistance assays. (a) The relative expression of WT, CL, and the protonation mimetic mutants of *HnSpns* in the cell growth assays was visualized by SDS-PAGE and staining with InVision His tag stain, confirming similar levels of expression for each construct. Purified *HnSpns* used as a standard is shown for comparison. (b) Cell growth for the WT and its variants relative to the vector at 37 °C, in the presence of different concentrations of rifampicin, capreomycin and ethidium bromide. Each data point was performed in triplicate, and the experiments were repeated. Absorbances at 600 nm in the presence of drugs were divided by the 0 µg/mL drug well. The standard deviation is shown for each data point. The *p* values were determined by an unpaired *t* test.



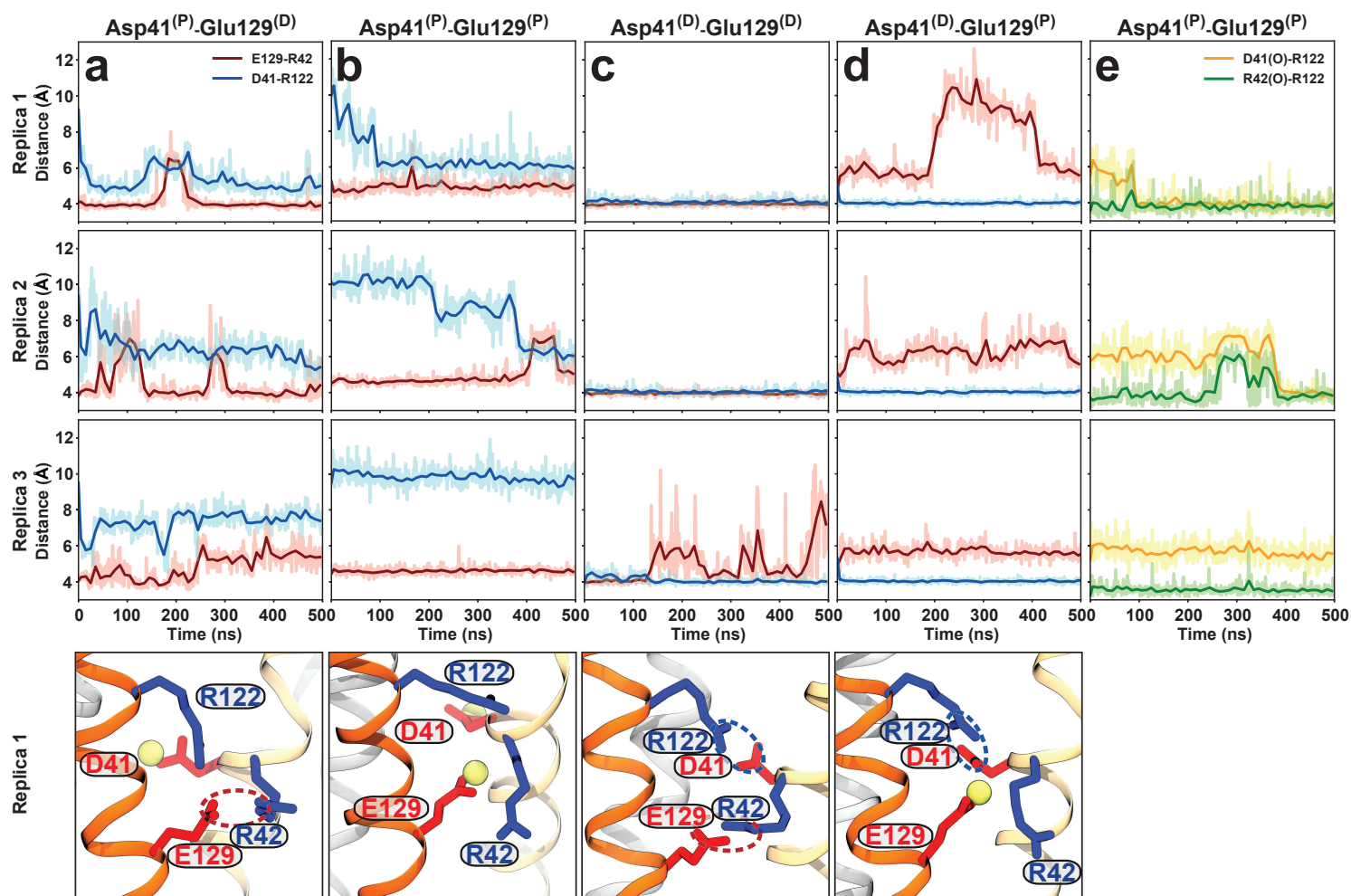
Supplementary Figure 3. DEER distance measurements on the intracellular side. (a) Spin label pairs are depicted on the intracellular side of the IF crystal structure. (b) Raw DEER decays and fits (left) are presented for the experimentally determined distributions $P(r)$ in lipid nanodiscs (middle), compared to distance distributions in LMNG micelles (right), with predicted distributions based on the IF crystal structure (shaded gray).



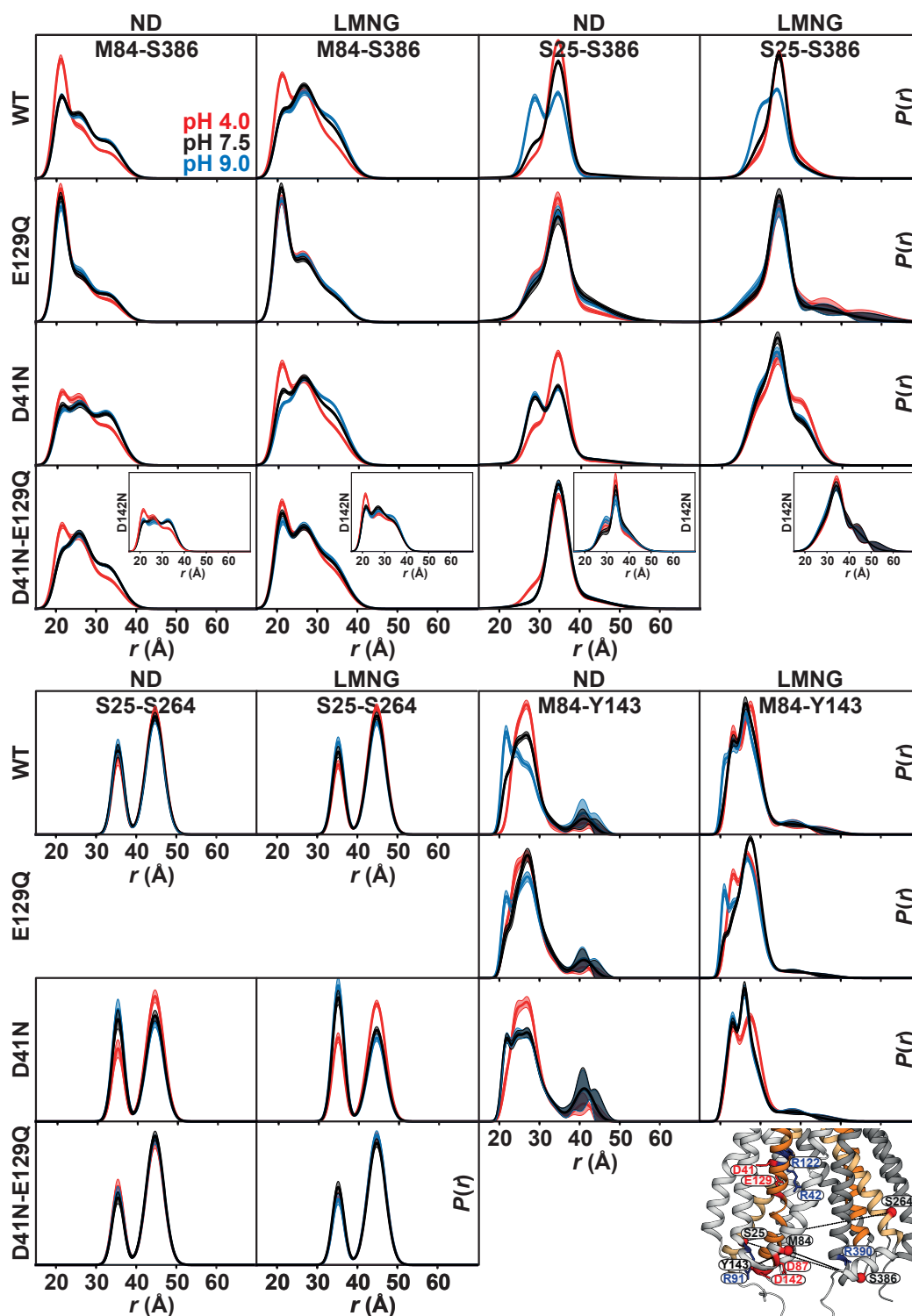




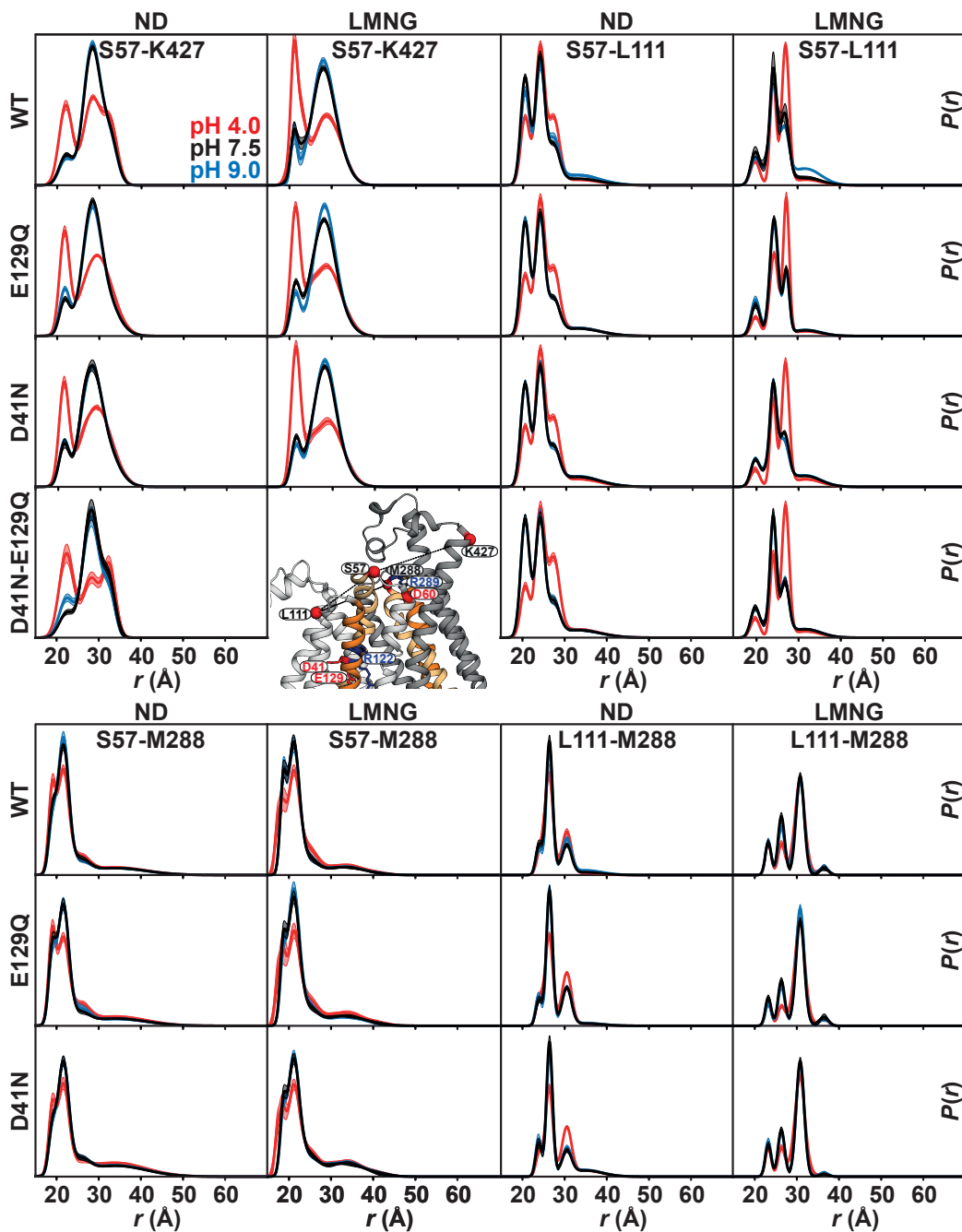
Supplementary Figure 5. S1P binding to *HnSpns* in lipid nanodiscs does not induce major conformational changes but rather a slight shift to shorter distances. The distance distributions $P(r)$ in lipid nanodiscs (pH 7.5) are shown in the apo state (black) and with S1P (green).



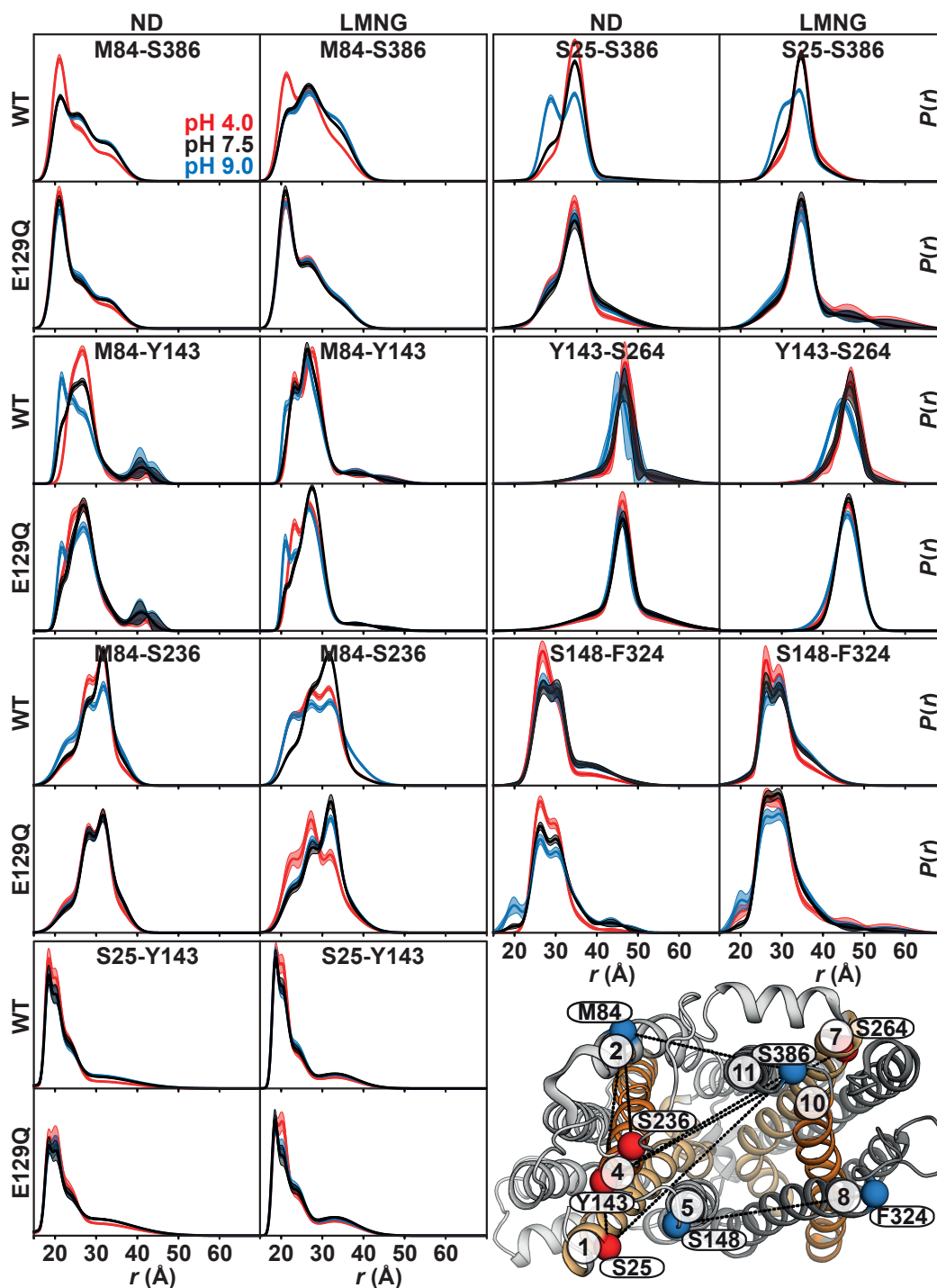
Supplementary Figure 6. Molecular dynamics simulations of IF *HnSpns* in lipid membranes. (a-d) Different permutations of protonation states of Asp41 and Glu129 and their interactions with two nearby basic residues (four different systems in three independent copies). (e) Double protonation of these acidic residues stabilizes a distinct conformation in which the side chain of Arg122 continuously interacts with the backbone oxygen of Arg42 but only transiently with Asp41. Molecular snapshots (bottom) are captured from the last frame of each trajectory of the first replica. Acidic and basic residues are shown in red and blue, respectively, and the proton is represented with a yellow sphere.



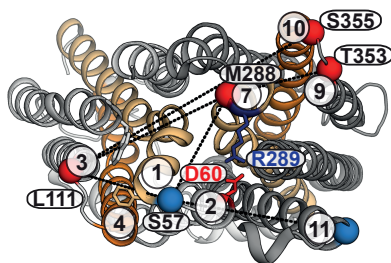
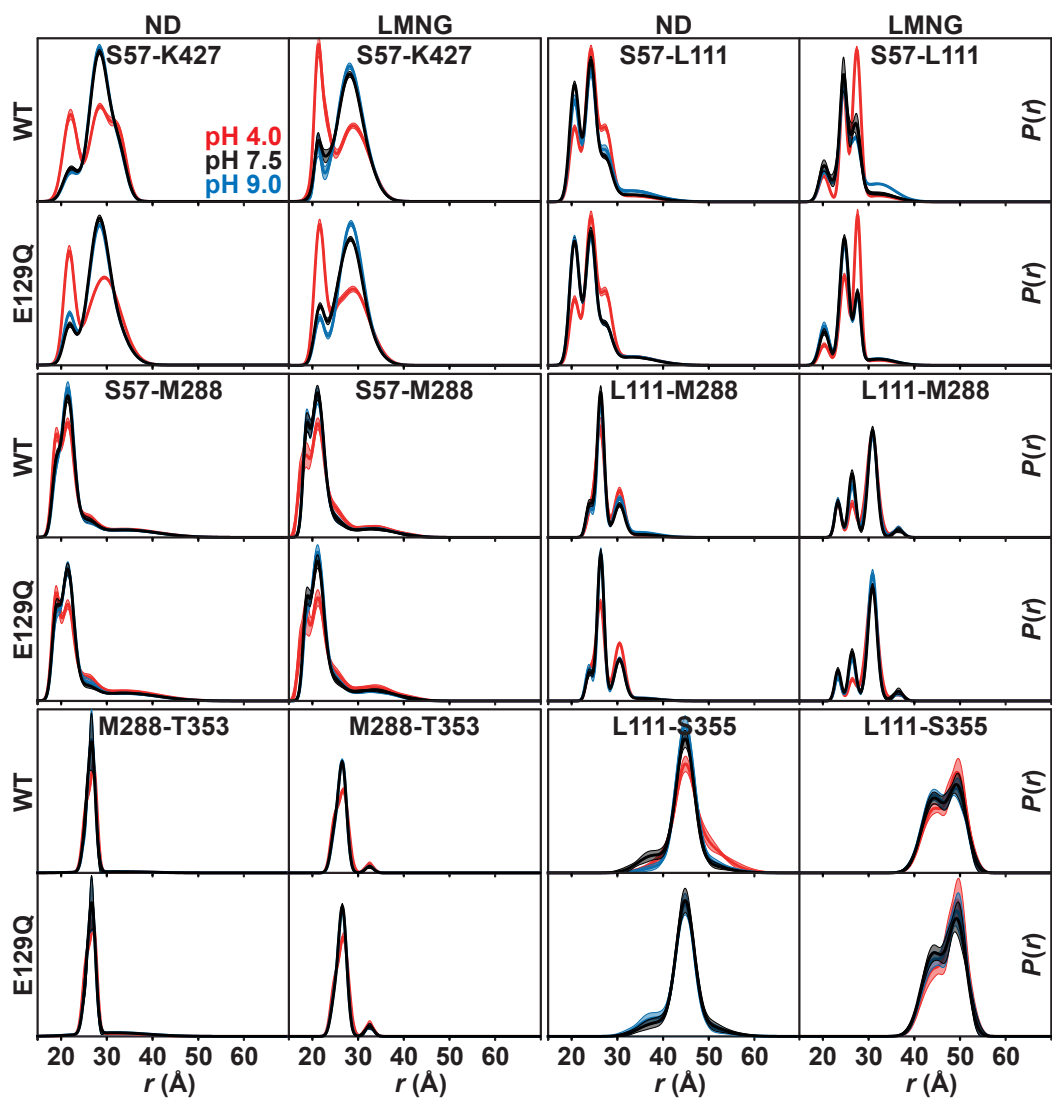
Supplementary Figure 7. Effect of protonation-mimetic mutation of acidic residues on conformational equilibrium on the intracellular side in lipid nanodiscs and detergent micelles. The single (E129Q, D41N, D142N) or double (D41N/E129Q) mutations were combined with the double-cysteine mutations. DEER experiments in the presence of these mutations support the results of the MD simulations and reveal that Asp41 protonation has the opposite effect from that of Glu129 on the conformational changes on the intracellular side.



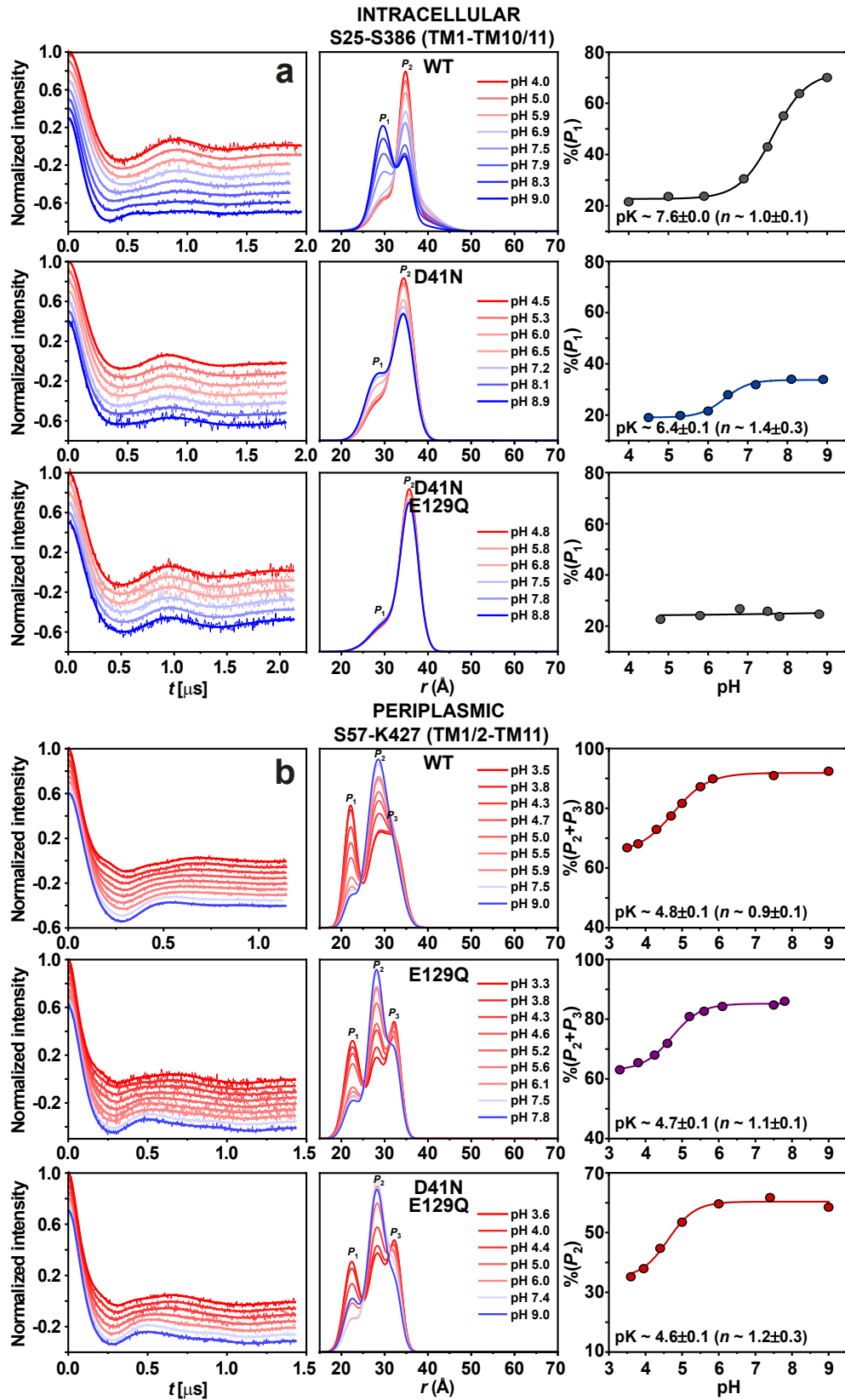
Supplementary Figure 8. Effect of protonation-mimetic mutation of acidic residues on conformational equilibrium on the periplasmic side. The single (E129Q, D41N, D142N) or double (D41N/E129Q) mutations were combined with the double-cysteine mutations. DEER experiments in the presence of these mutations reveal that the periplasmic side remains mostly invariant in response to these mutations.



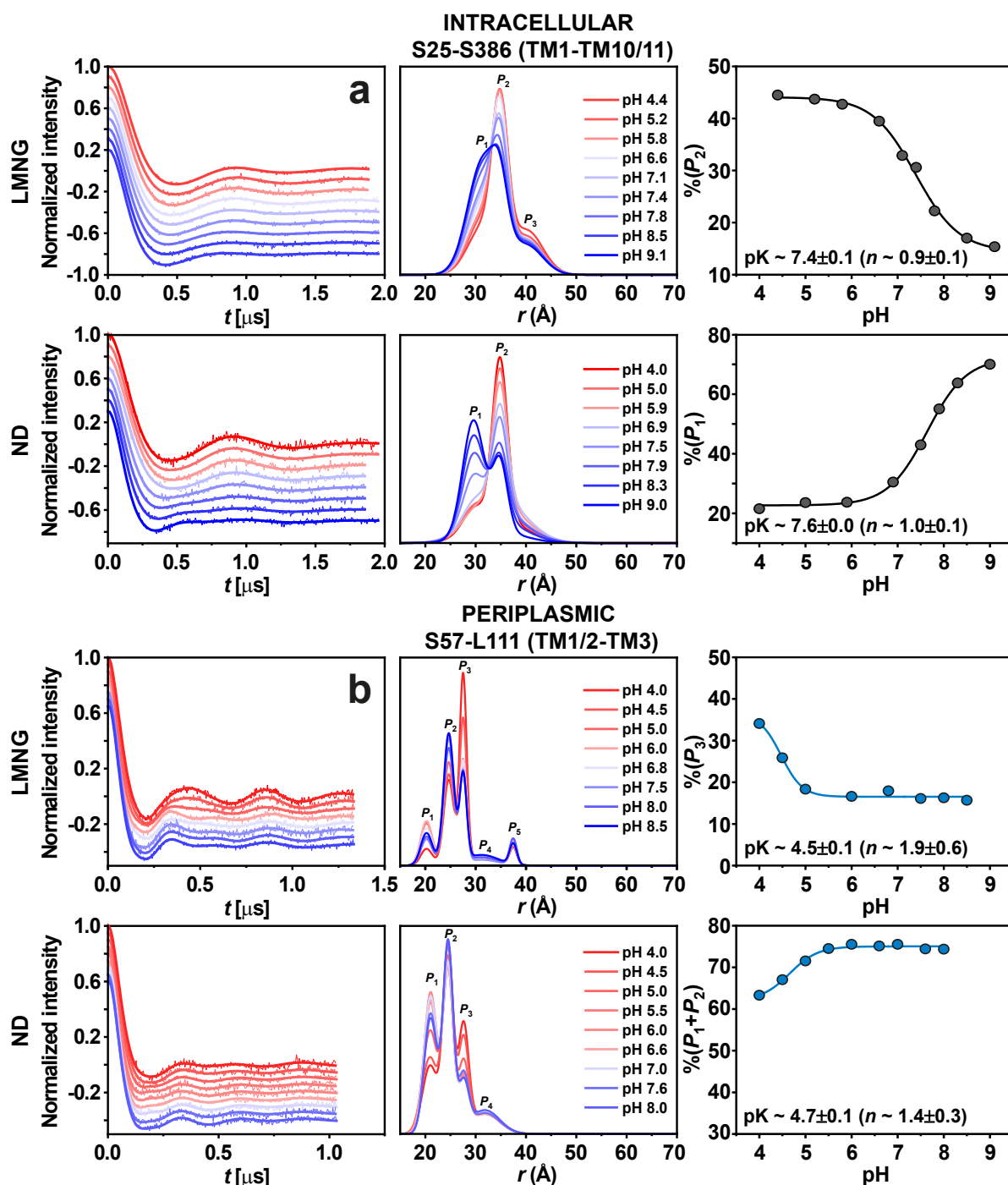
Supplementary Figure 9. Effect of protonation-mimetic mutation of the intracellular protonation master switch glutamate 129 (E129Q) on conformational equilibrium on the intracellular side. The E129Q mutation was combined with the double-cysteine mutations. The E129Q mutation captures the transporter in the pH 4 population state, i.e., the inward-closed conformation. Thus, our data suggest that E129 is the intracellular protonation master switch regulating the conformational transition between IF and inward-closed states.



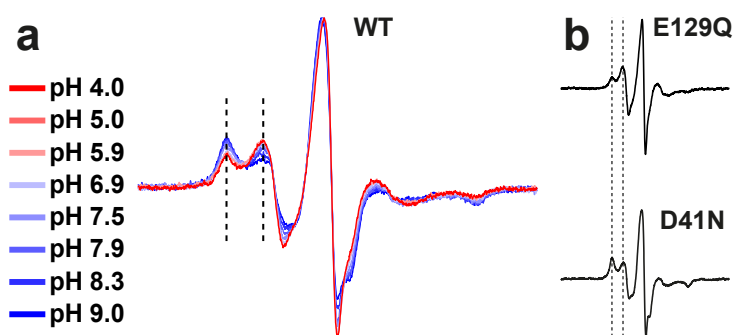
Supplementary Figure 10. Effect of protonation-mimetic mutation of the intracellular protonation master switch glutamate 129 (E129Q) on conformational equilibrium on the periplasmic side. The E129Q mutation was combined with the double-cysteine mutations. DEER experiments reveal that the periplasmic side remains mostly invariant in response to this mutation.



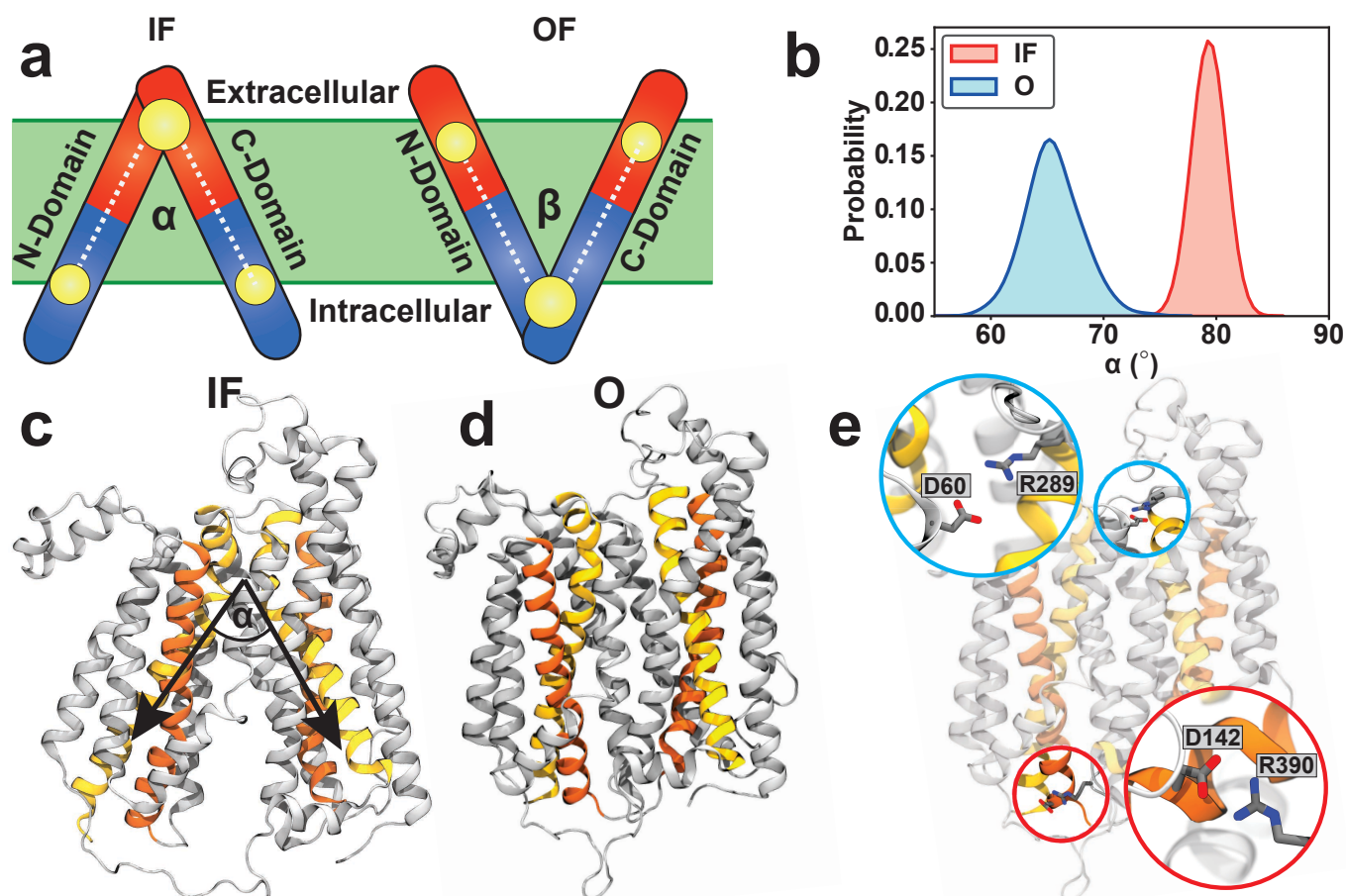
Supplementary Figure 11. Intracellular and periplasmic sides of *HnSpns* sense its protonation state differently. (a) Distance distributions of intracellular (S25-S386) and (b) periplasmic (S57-K427) pairs were obtained at different pH values in lipid nanodiscs with and without protonation-mimetic mutations of acidic residues. The variation in population of rising or decreasing distance peaks as a function of pH was used to estimate the pK value for conformational changes in *HnSpns*. The D41N-E129Q double mutation abrogates conformational changes on the intracellular side with periplasmic side mainly unaffected, demonstrating the decoupling of transmembrane protonation-dependent conformational changes.



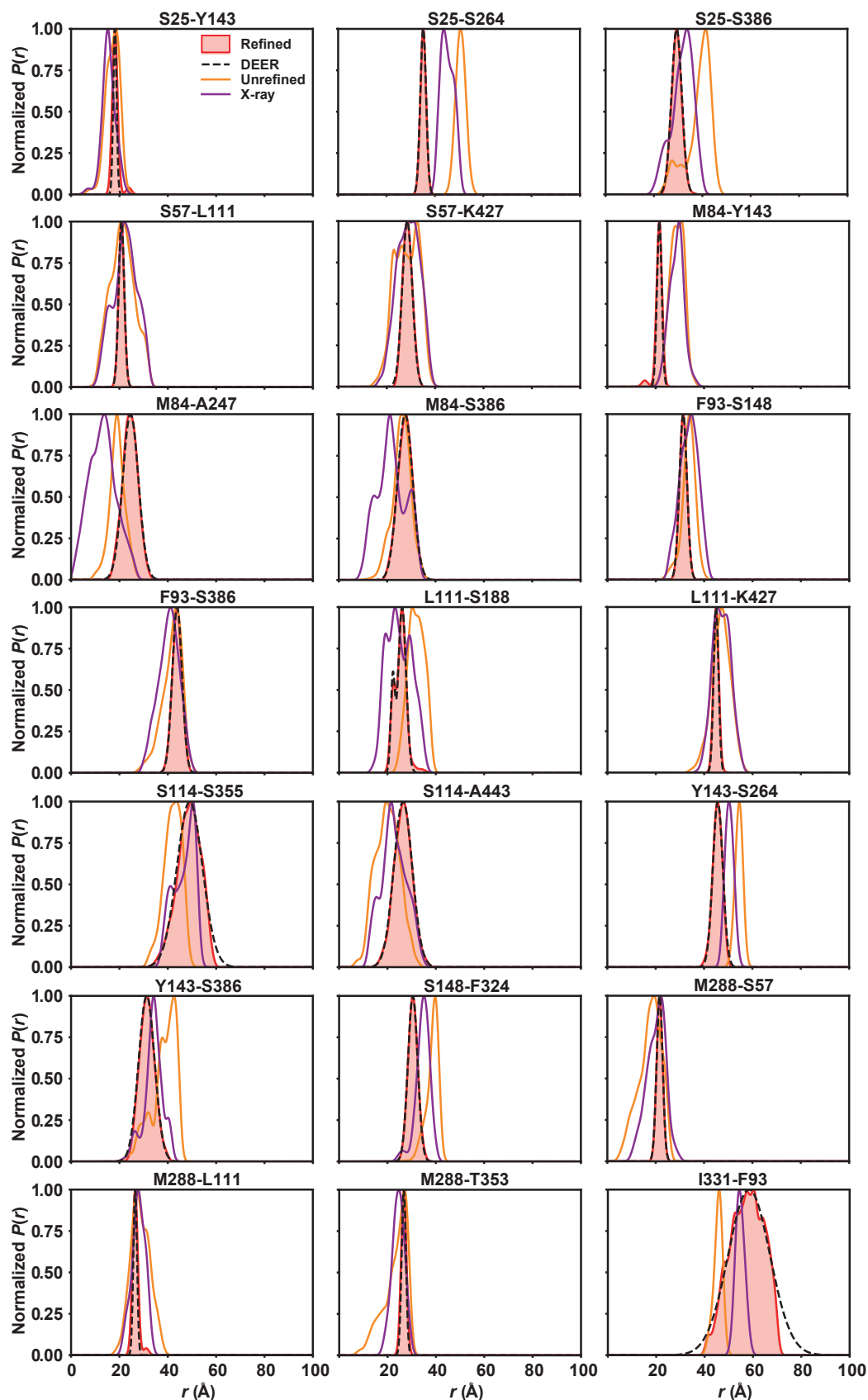
Supplementary Figure 12. Effect of environment (lipid nanodiscs vs. micelles) on sensing the protonation state of *HnSpns*. (a) Distance distributions of intracellular (S25-S386) and (b) periplasmic (S57-L111) pairs were obtained at different pH values in lipid nanodiscs and LMNG micelles. Similar pK values were obtained in LMNG micelles and lipid nanodiscs.



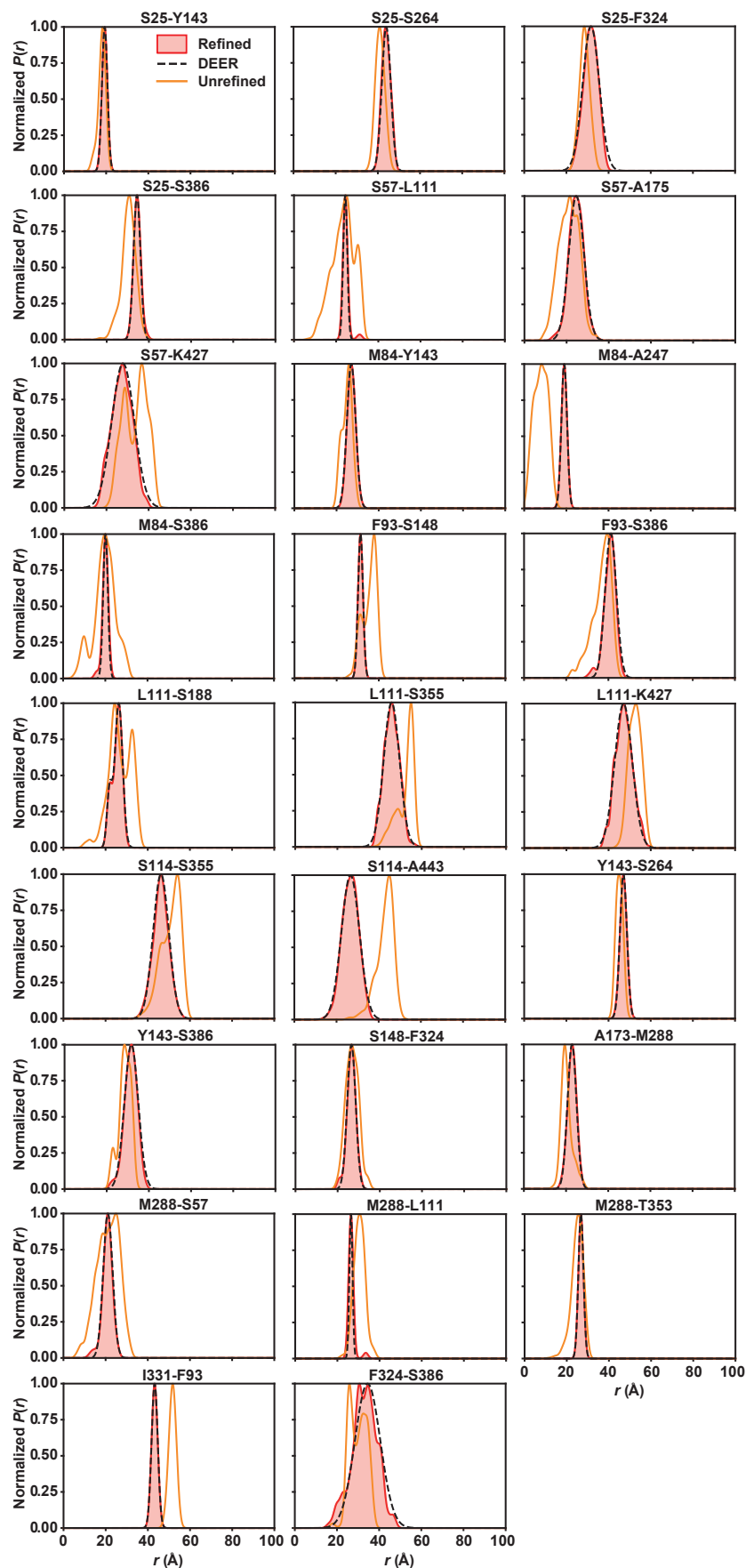
Supplementary Figure 13. The pH-dependence of the CW EPR spectra of (a) the S25-S386 (TM1-TM10/11) intracellular pair. (b) The D41N and E129Q mutations on the same cysteine pair shift the equilibrium in opposite directions towards basic and acidic pH states, respectively.



Supplementary Figure 14. Capturing a stable O conformation of *HnSpns*. (a) Two orientation-based collective variables, α and β , representing the intracellular and extracellular opening/closing of the transporter, respectively, and used to control the conformation of the transporter. N- and C-domain are split into two equal segments shown in red and blue, representing the extracellular and intracellular halves, respectively. α is defined as the angle between the centers of masses of (1) the intracellular half of N-domain, (2) the extracellular half of the protein (including both N- and C-domains), and (3) the intracellular half of C-domain. β is the angle between the centers of masses of (1) the extracellular half of N-domain, (2) the intracellular half of the protein (including both N- and C-domains), and (3) the extracellular half of C-domain. (b) Probability distribution of the intracellular opening of the transporter for the IF and stabilized O conformations through 500 ns of equilibrium simulations. (c) The IF conformation and definition of the intracellular opening angle. (d) The stable O conformation. (e) Periplasmic (Arg289^{TM7}-Asp60^{TM2}) and intracellular (Arg390^{TM11}-Asp142^{TM4}) salt bridges are formed, stabilizing the O conformation.



Supplementary Figure 15. DEER-guided refinement of IF *Hn*Spns structure in lipid membranes. Comparison of the distance distributions from the refined structure (shaded red) to the experimental pH 9 DEER distance histograms in nanodiscs (dashed black lines), the relaxed unrefined template (orange lines), and the predicted distributions on the crystal structure (purple lines). The refined model matches the experimental distance populations.



Supplementary Figure 16. Refinement of the O state of *HnSpns* in lipid membrane. Comparison of the distance distributions from the refined model (shaded red) to the experimental pH 4 DEER distance histograms in nanodiscs (dashed black lines), and the unrefined MD-generated O conformation without imposing the harmonic restraints on the probes (orange lines). The refined model matches the experimental distance populations.

Supplementary Table 1. Melting temperatures (T_m) of spin-labeled DEER and protonation-mimetic mutants in LMNG micelles and lipid nanodiscs.

Mutant	T_m (°C) $\pm$ Fitting error	
	LMNG	ND
CL	58.1 $\pm$ 0.1	58.3 $\pm$ 0.1
CL-E129Q	57.7 $\pm$ 0.1	57.3 $\pm$ 0.1
CL-D142N	60.0 $\pm$ 0.2	72.8 $\pm$ 0.4
CL-D41N	62.2 $\pm$ 0.2	58.8 $\pm$ 0.2
CL-D41N-E129Q	64.2 $\pm$ 0.1	71.3 $\pm$ 0.2
M84C-S386C	54.8 $\pm$ 0.4	56.6 $\pm$ 0.5
M84C-Y143C	58.8 $\pm$ 0.1	57.5 $\pm$ 0.2
S25C-S386C	55.6 $\pm$ 0.1	54.3 $\pm$ 0.1
S25C-S264C	58.0 $\pm$ 0.2	64.1 $\pm$ 0.9
S148C-F324C	58.4 $\pm$ 0.3	58.7 $\pm$ 0.5
Y143C-S264C	58.4 $\pm$ 0.1	71.3 $\pm$ 0.3
Y143C-S386C	54.7 $\pm$ 0.3	61.8 $\pm$ 0.2
S57C-K427C	55.7 $\pm$ 0.1	54.8 $\pm$ 0.2
S57C-L111C	58.0 $\pm$ 0.2	58.1 $\pm$ 0.1
L111C-M288C	54.7 $\pm$ 0.2	56.1 $\pm$ 0.1
S114C-S355C	61.2 $\pm$ 0.3	54.9 $\pm$ 0.1
F93C-S148C	59.2 $\pm$ 0.1	72.1 $\pm$ 0.5
S25C-Y143C	58.4 $\pm$ 0.1	58.6 $\pm$ 0.2
M84C-S236C	58.0 $\pm$ 0.1	61.8 $\pm$ 0.2
S25C-F324C	58.5 $\pm$ 0.1	
M84C-A247C	57.1 $\pm$ 0.1	59.8 $\pm$ 0.2
F93C-I331C	57.5 $\pm$ 0.1	67.7 $\pm$ 0.3
F93C-S386C	56.3 $\pm$ 0.1	77.3 $\pm$ 0.7
S25C-M84C	56.5 $\pm$ 0.1	
F324C-S386C		63.8 $\pm$ 0.1
S57C-A175C	61.5 $\pm$ 0.3	59.9 $\pm$ 0.2
S114C-A443C	60.7 $\pm$ 0.5	70.6 $\pm$ 0.7
L111C-S355C	56.2 $\pm$ 0.1	64.6 $\pm$ 0.2
A175C-S294C	58.9 $\pm$ 0.1	58.8 $\pm$ 0.7
M288C-T353C	53.9 $\pm$ 0.3	62.9 $\pm$ 0.3
A173C-M288C	55.5 $\pm$ 0.1	
L111C-K427C		79.0 $\pm$ 1.0
L111C-S188C		60.2 $\pm$ 0.2

Supplementary Table 2. *p* values determined from a two-tailed independent *t*-test comparing the drug resistance of *HnSpns* WT and variants with pET19b vector ($n_{\text{vector}} = 4$ for capreomycin, 5 and 10 $\mu\text{g/mL}$ rifampicin, $n_{\text{vector}} = 3$ for 17.5 $\mu\text{g/mL}$ rifampicin, ethidium bromide).

Capreomycin												
Concentration ($\mu\text{g/mL}$)	2 hours						4 hours					
	WT $n = 6$ $\text{df} = 8$	CL $n = 3$ $\text{df} = 5$	CL- D41N $n = 3$ $\text{df} = 5$	CL- E129Q $n = 3$ $\text{df} = 5$	CL- D142 N $n = 3$ $\text{df} = 5$	CL- D41N- E129Q $n = 3$ $\text{df} = 5$	WT $n = 6$ $\text{df} = 8$	CL $n = 3$ $\text{df} = 5$	CL- D41N $n = 3$ $\text{df} = 5$	CL- E129Q $n = 3$ $\text{df} = 5$	CL- D142N $n = 3$ $\text{df} = 5$	CL- D41N- E129Q $n = 3$ $\text{df} = 5$
35	<0.001 ***	0.016 *	0.004 **	0.007 **	0.005 **	0.006 **	<0.001 ***	0.017 *	0.003 **	0.003 **	0.002 **	0.001 ***
52.5	<0.001 ***	0.037 *	0.009 **	0.016 *	0.006 **	0.021 *	0.037 *	0.024 *	0.010 **	0.006 **	0.084 ns	0.014 *
Rifampicin												
Concentration ($\mu\text{g/mL}$)	2 hours						4 hours					
	WT $n = 6$ $\text{df} = 8$	CL $n = 3$ $\text{df} = 5$	CL- D41N $n = 3$ $\text{df} = 5$	CL- E129Q $n = 3$ $\text{df} = 5$	CL- D142 N $n = 3$ $\text{df} = 5$	CL- D41N- E129Q $n = 3$ $\text{df} = 5$	WT $n = 6$ $\text{df} = 8$	CL $n = 3$ $\text{df} = 5$	CL- D41N $n = 3$ $\text{df} = 5$	CL- E129Q $n = 3$ $\text{df} = 5$	CL- D142N $n = 3$ $\text{df} = 5$	CL- D41N- E129Q $n = 3$ $\text{df} = 5$
5	0.349 ns	0.497 ns	0.411 ns	0.311 ns	0.286 ns	0.355 ns	0.011 *	0.035 *	0.009 **	0.310 ns	0.004 **	0.036 *
10	0.003 **	0.006 **	0.006 **	0.004 **	0.041 *	0.005 **	<0.001 ***	0.006 **	0.002 **	0.001 ***	0.004 *	<0.001 *
17.5 [‡]	0.011 *	0.002 **	-	-	-	-	0.369 ns	0.043 *	-	-	-	-
Ethidium bromide												
Concentration ($\mu\text{g/mL}$)	2 hours						4 hours					
	WT $n = 6$ $\text{df} = 7$	CL $n = 4$ $\text{df} = 5$	CL- D41N $n = 3$ $\text{df} = 4$	CL- E129Q $n = 3$ $\text{df} = 4$	CL- D142 N $n = 3$ $\text{df} = 4$	CL- D41N- E129Q $n = 3$ $\text{df} = 4$	WT $n = 6$ $\text{df} = 7$	CL $n = 4$ $\text{df} = 5$	CL- D41N $n = 3$ $\text{df} = 4$	CL- E129Q $n = 3$ $\text{df} = 4$	CL- D142N $n = 3$ $\text{df} = 4$	CL- D41N- E129Q $n = 3$ $\text{df} = 4$
35	0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.001 ***	<0.001 ***
52.5	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***
70	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***

* *p* value ≤ 0.05

** *p* value ≤ 0.01

*** *p* value ≤ 0.001

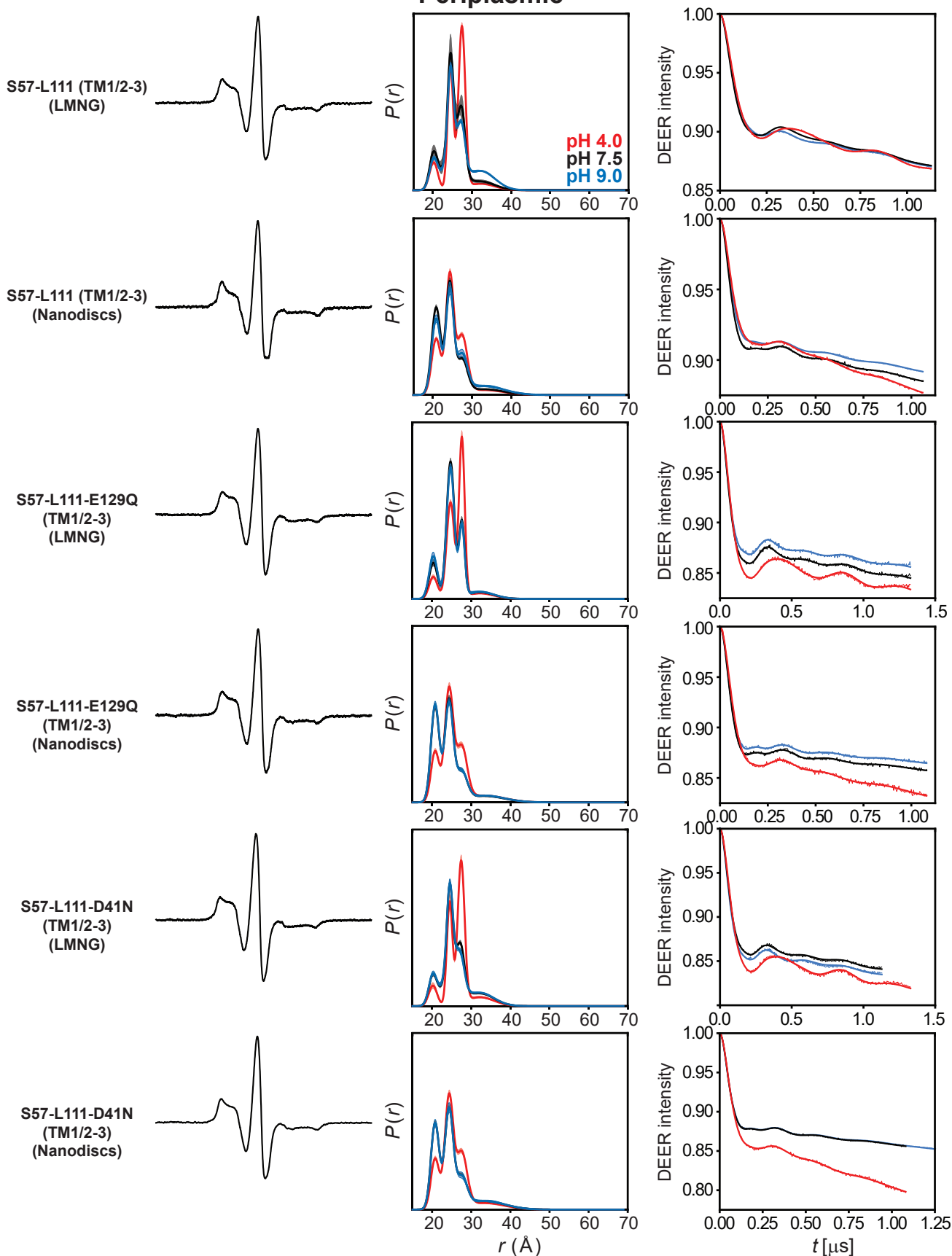
ns: not significant

n: number of technical replicates

df: degrees of freedom

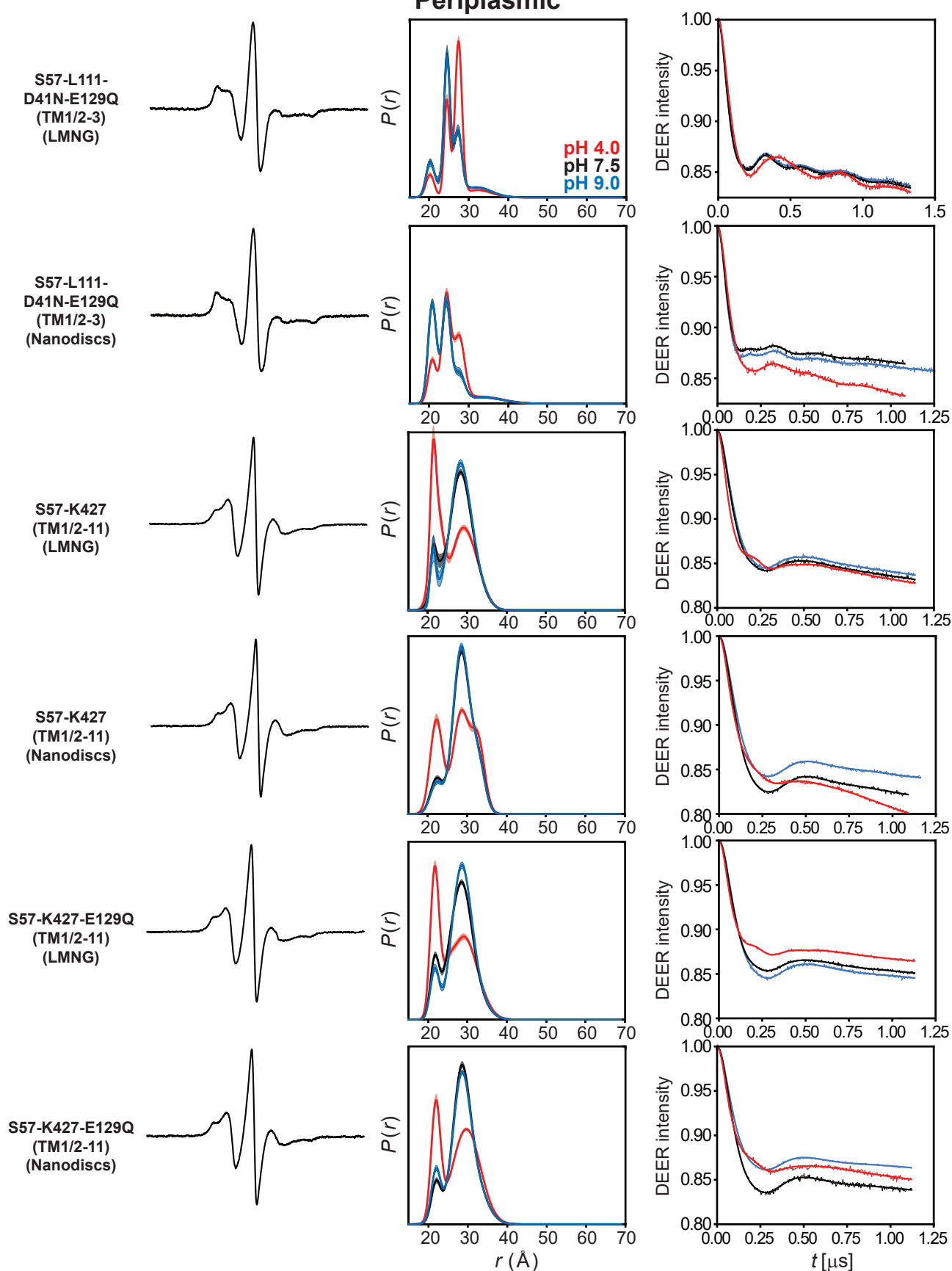
[‡] *n* = 3 and df = 3

Periplasmic



Data Appendix 1. DEER data analysis for the periplasmic side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

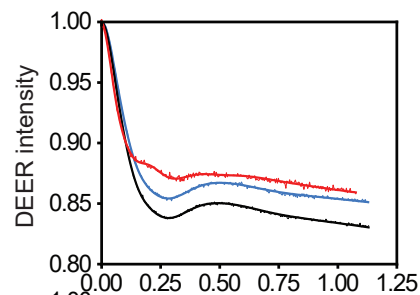
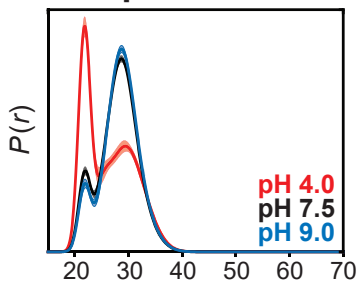
Periplasmic



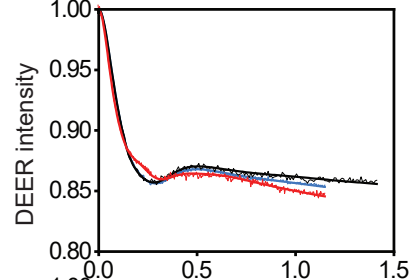
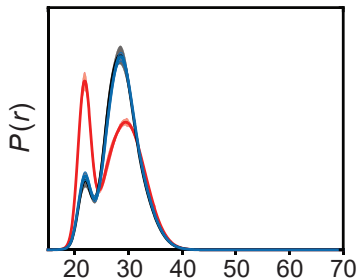
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Periplasmic

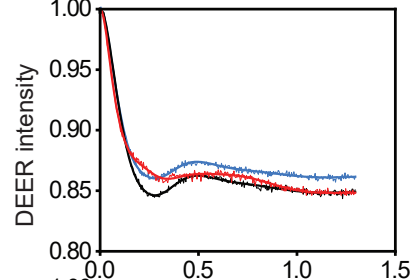
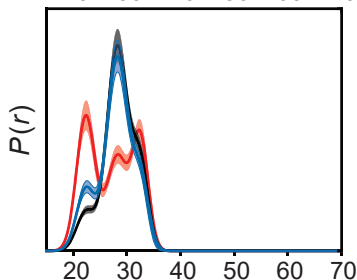
S57-K427-D41N
(TM1/2-11)
(LMNG)



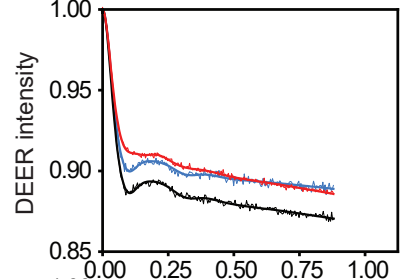
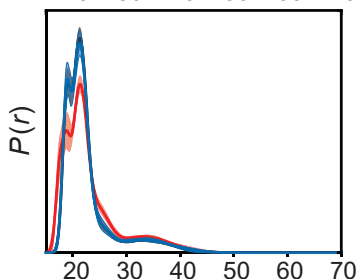
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(TM1/2-11)
(Nanodiscs)



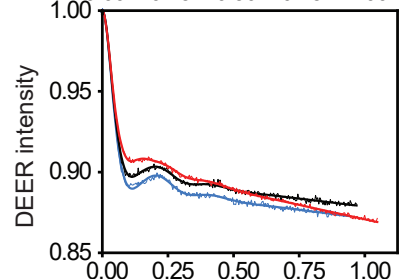
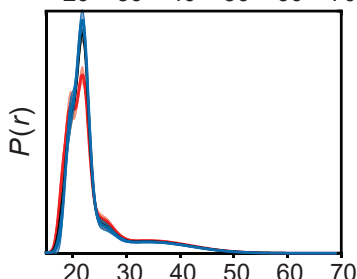
S57-K427-
D41N-E129Q
(TM1/2-11)
(Nanodiscs)



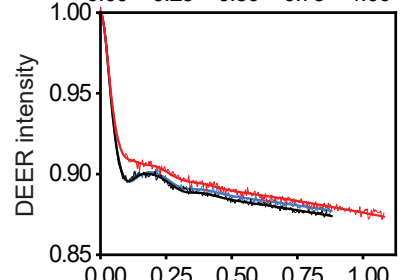
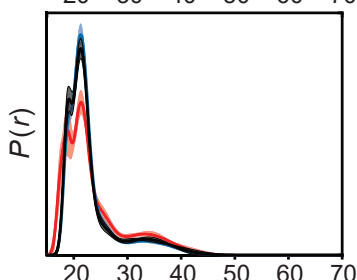
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(LMNG)



S57-M288
(TM1/2-7)
(Nanodiscs)

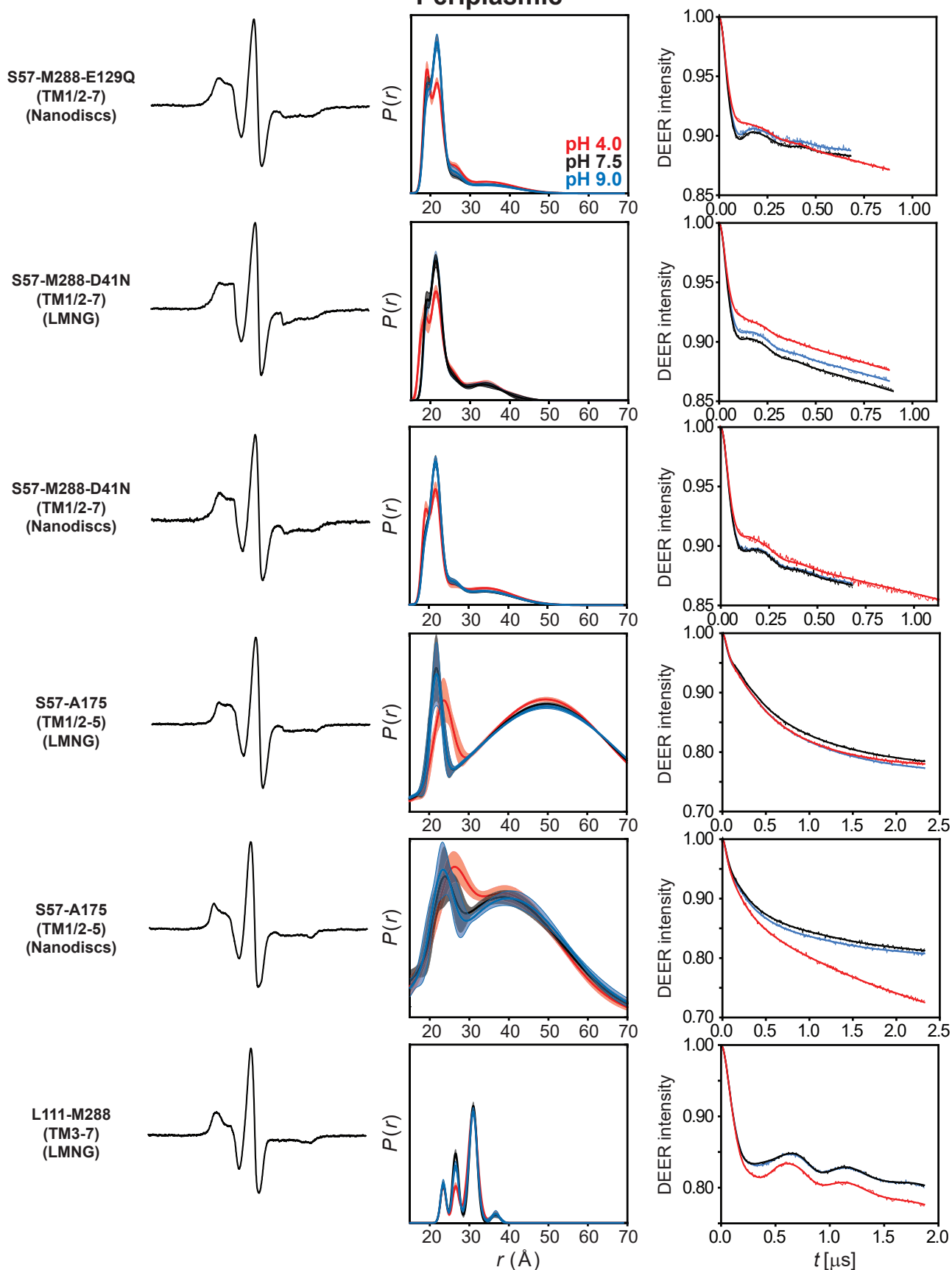


S57-M288-E129Q
(TM1/2-7)
(LMNG)



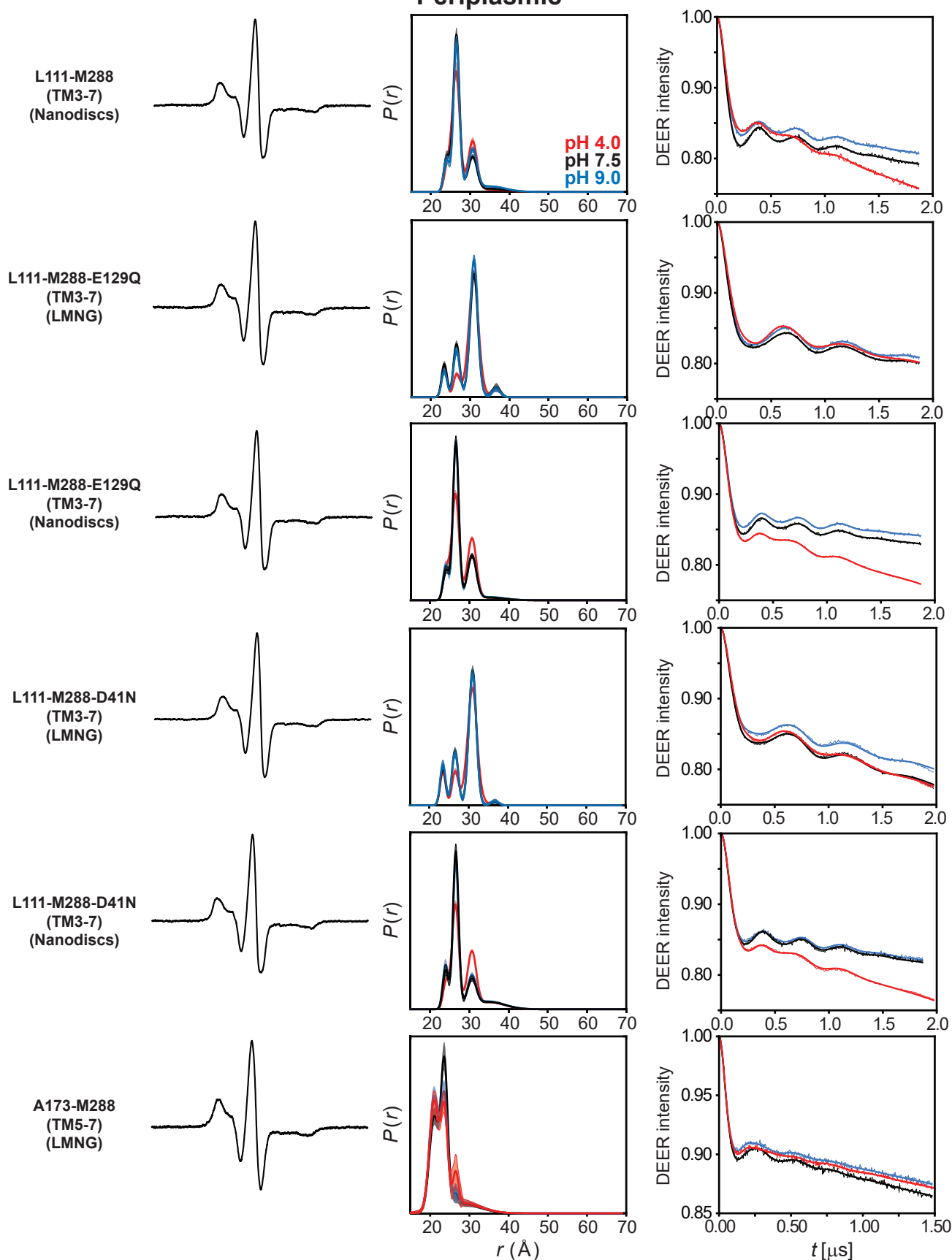
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Periplasmic



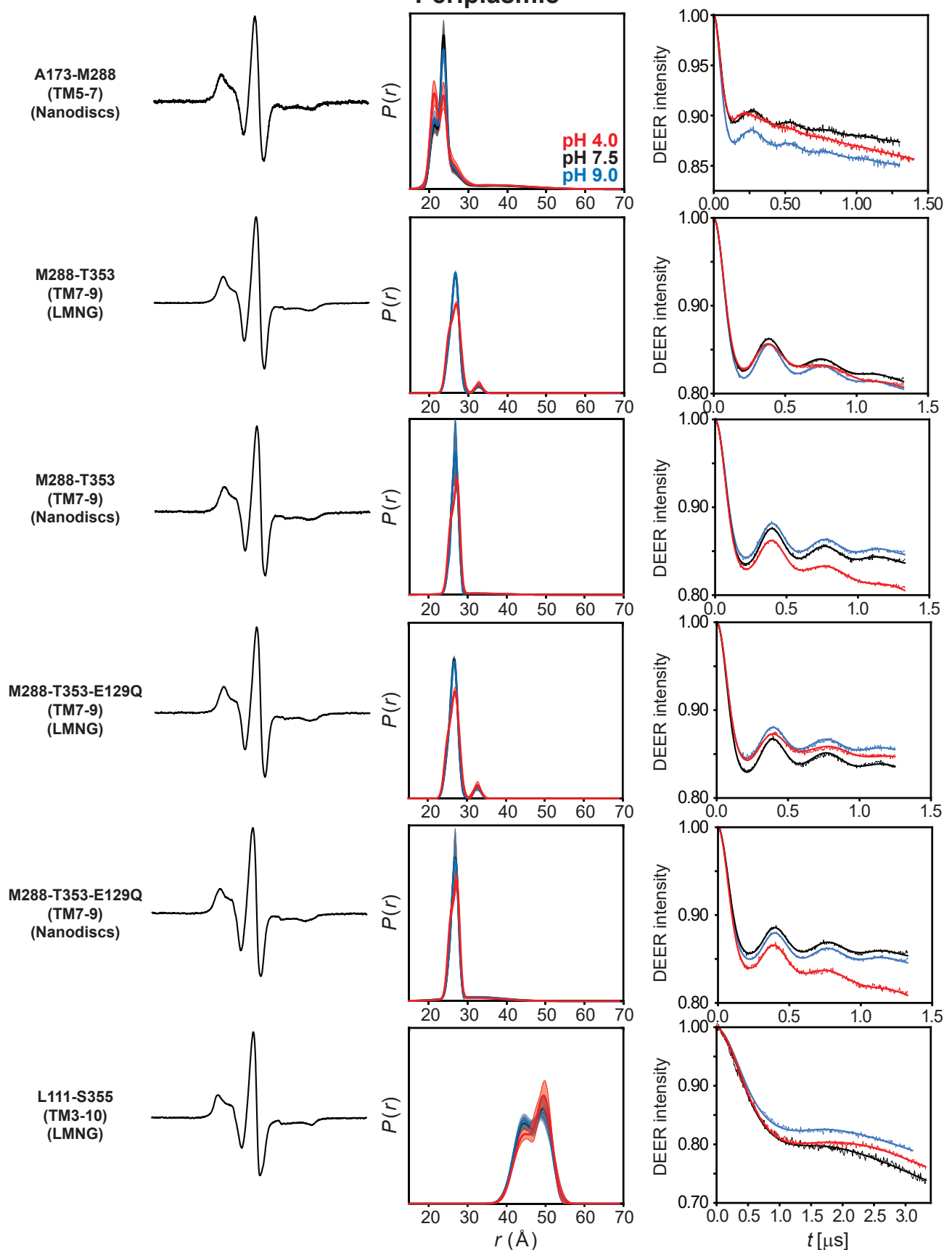
Data Appendix 4. DEER data analysis for the periplasmic side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Periplasmic



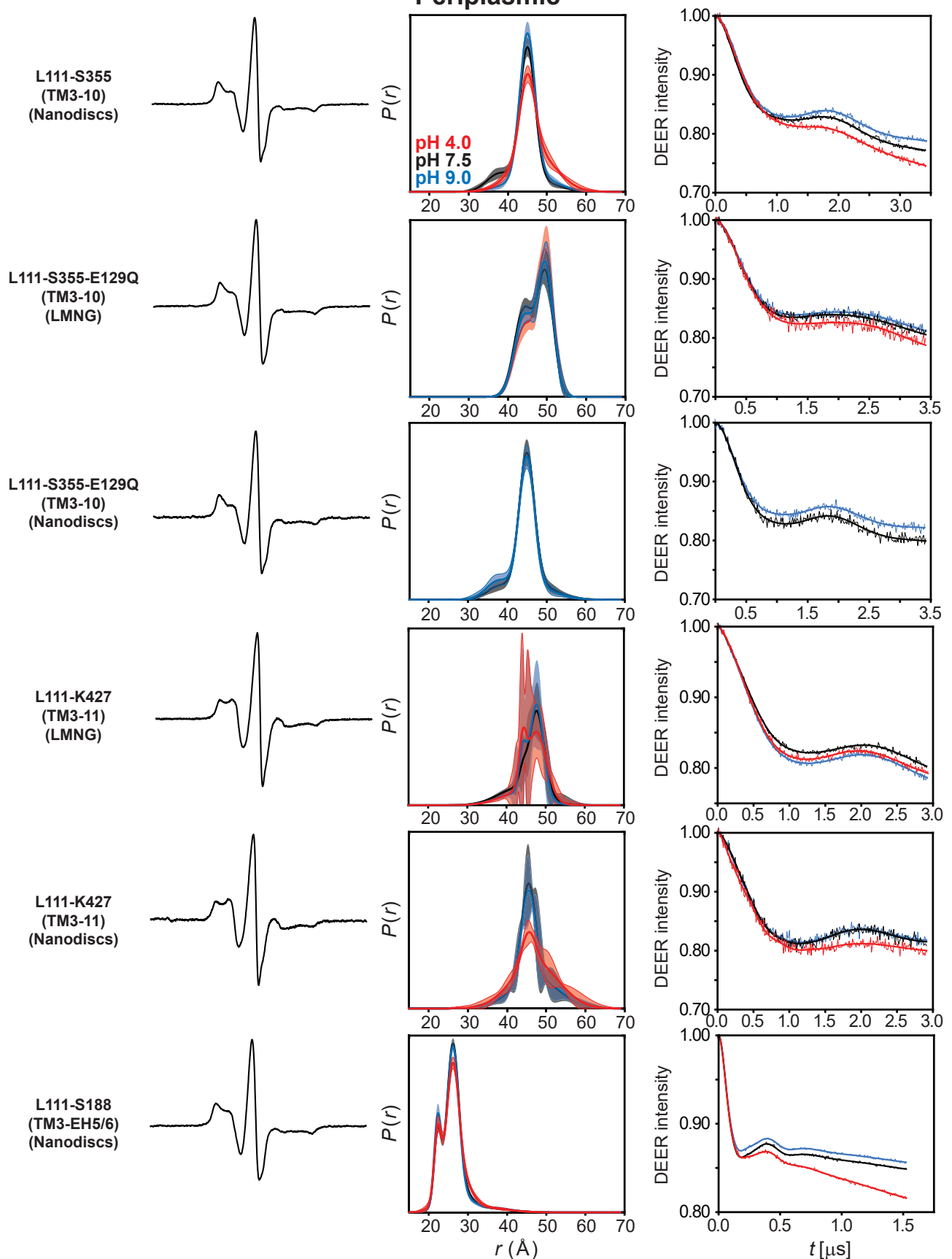
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Periplasmic

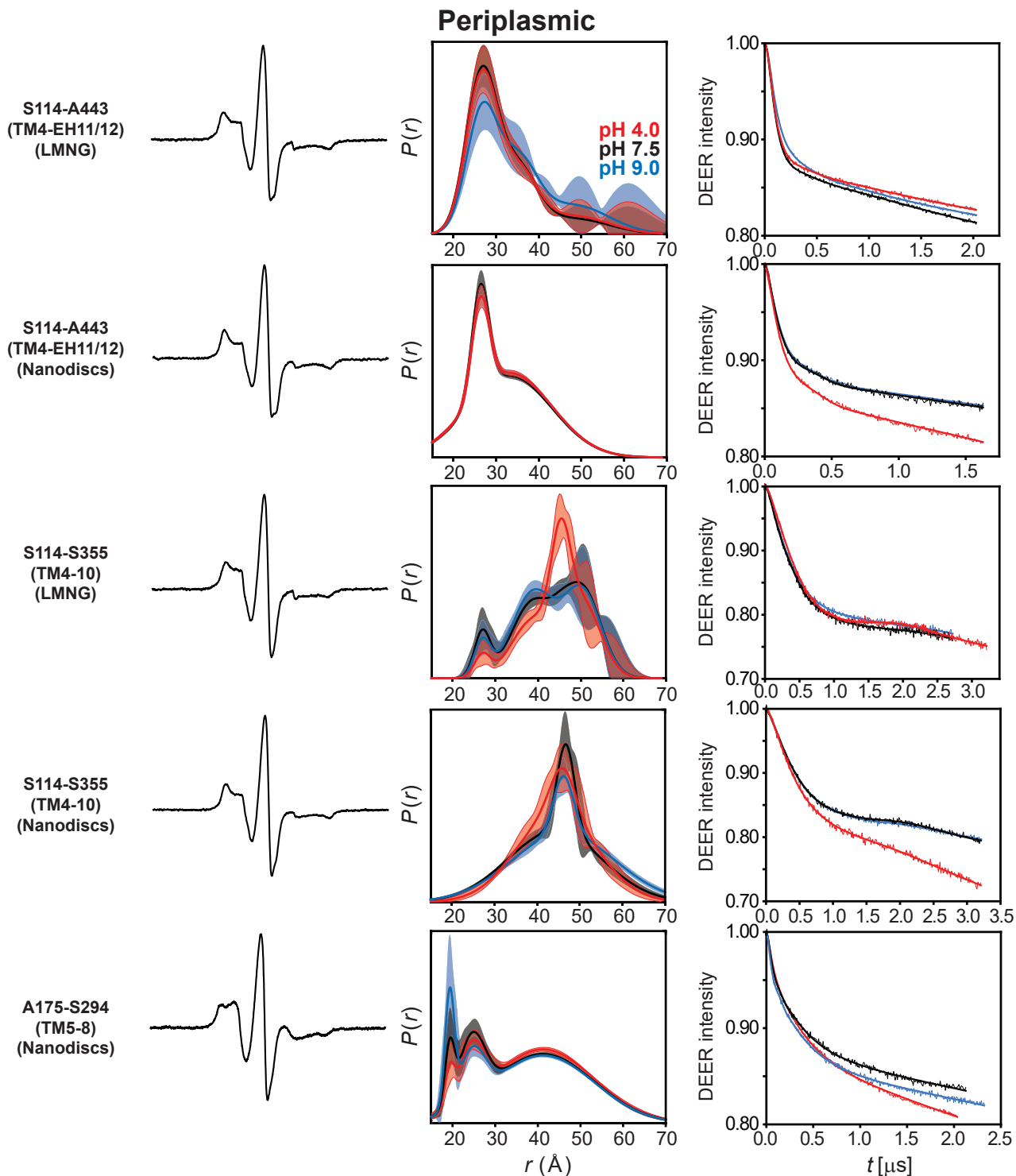


Data Appendix 6. DEER data analysis for the periplasmic side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Periplasmic

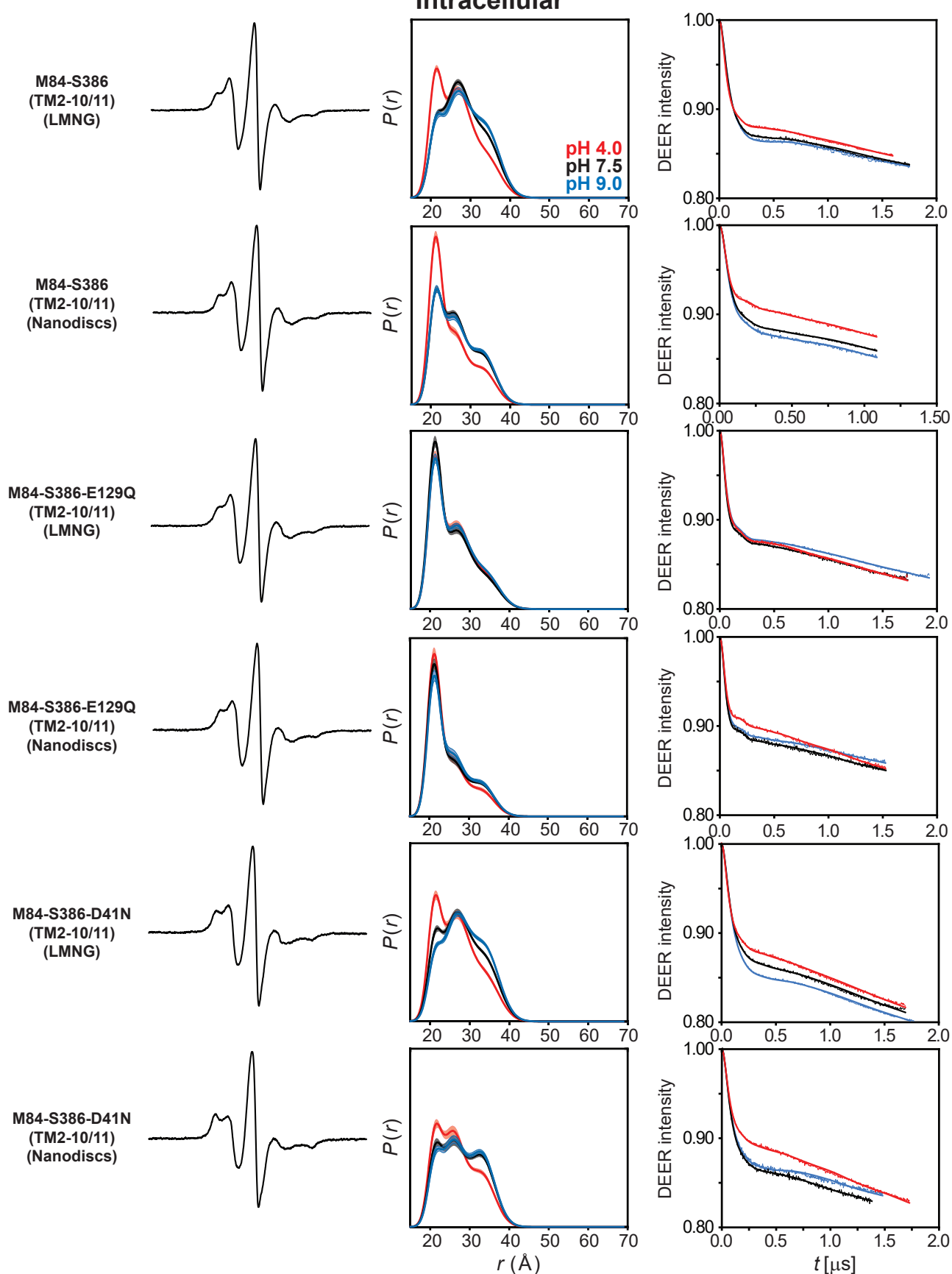


Data Appendix 7. DEER data analysis for the periplasmic side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.



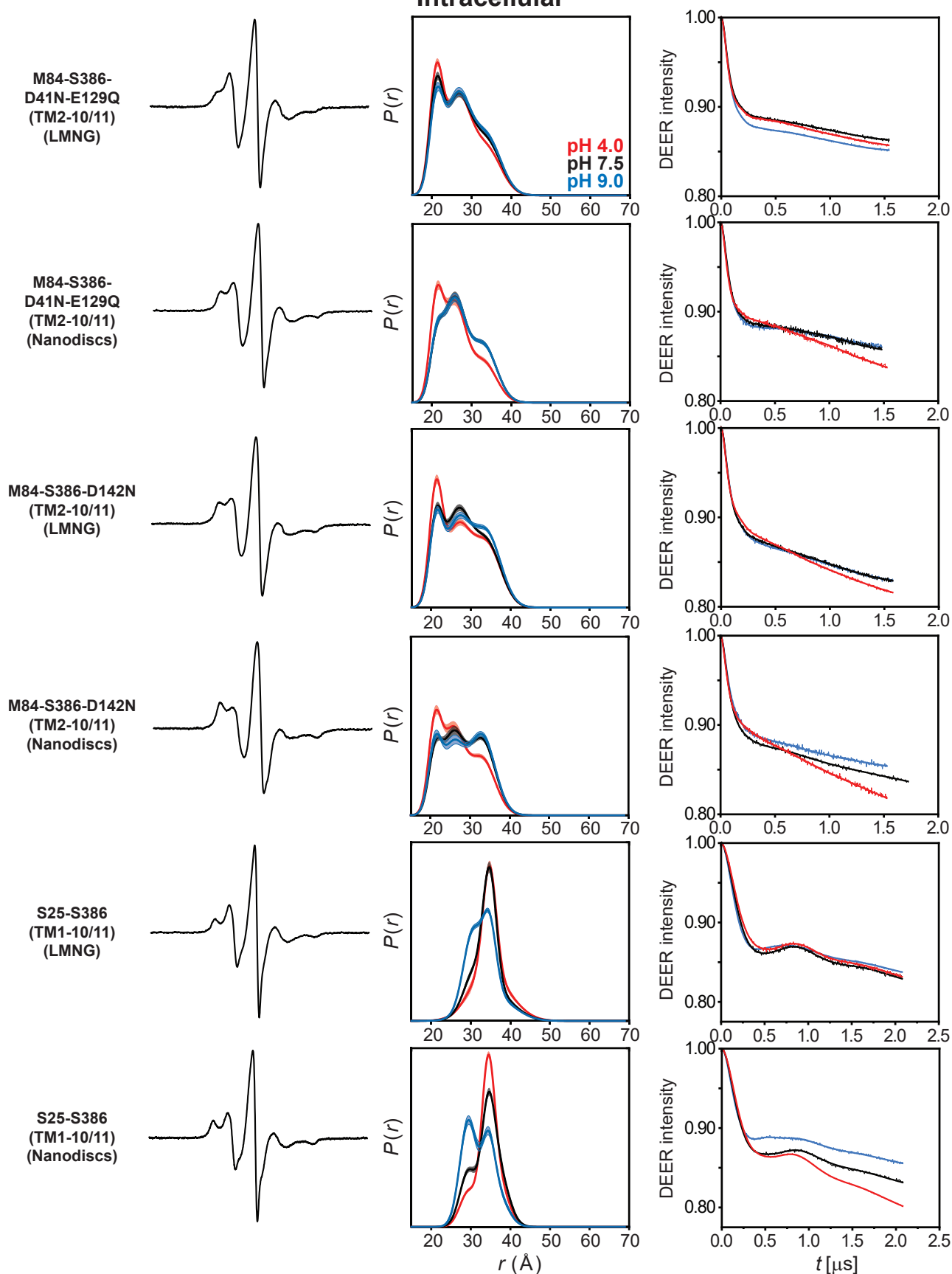
Data Appendix 8. DEER data analysis for the periplasmic side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular



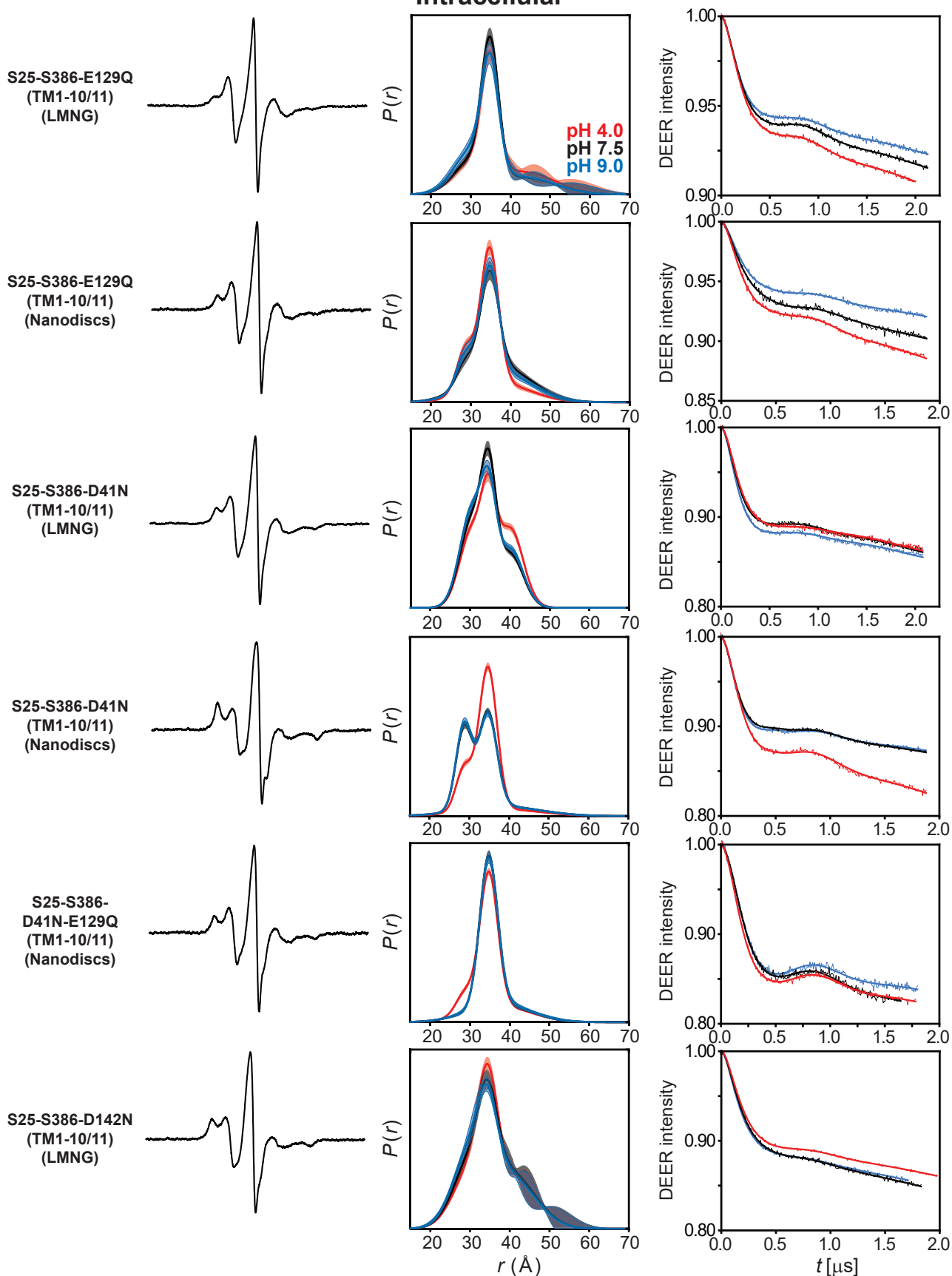
Data Appendix 9. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular



Data Appendix 10. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

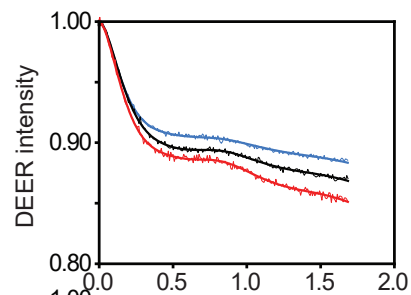
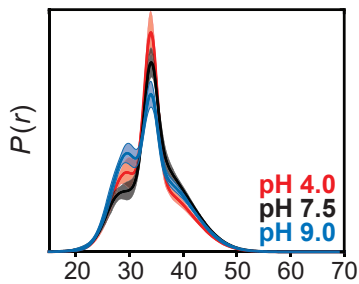
Intracellular



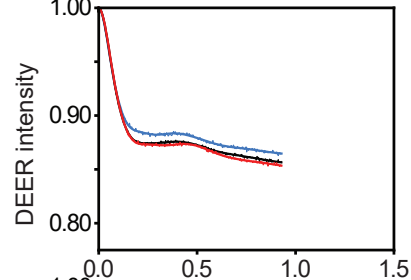
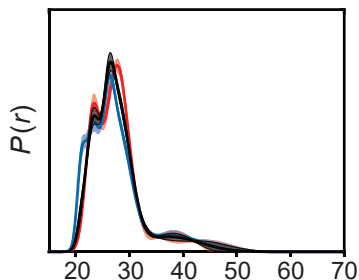
Data Appendix 11. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular

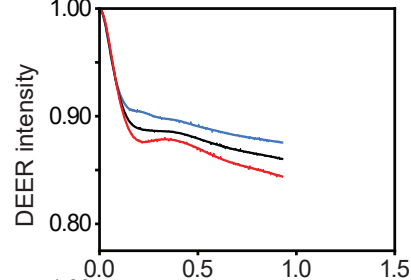
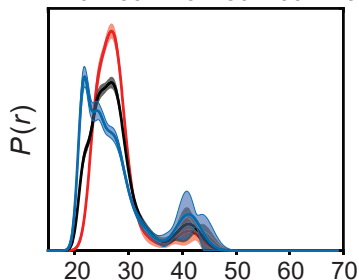
S25-S386-D142N
(TM1-10/11)
(Nanodiscs)



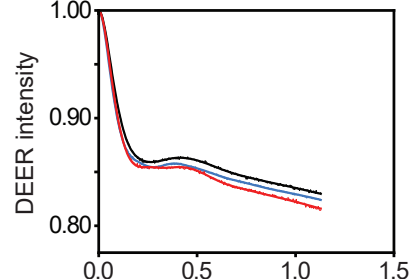
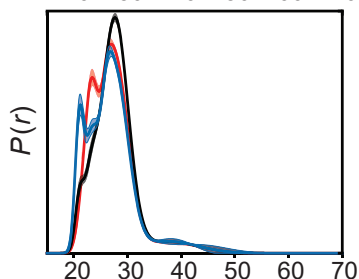
M84-Y143
(TM2-4)
(LMNG)



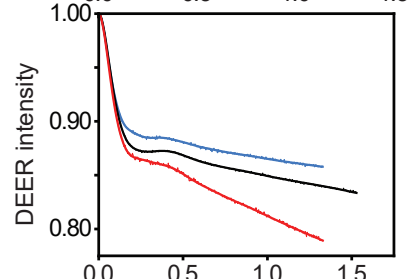
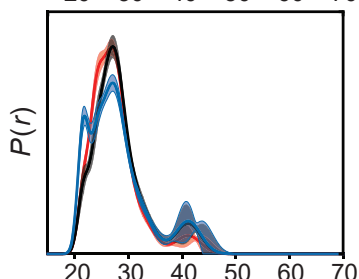
M84-Y143
(TM2-4)
(Nanodiscs)



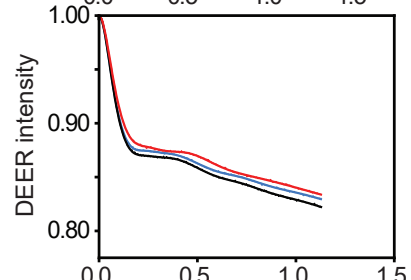
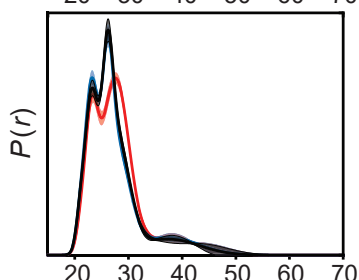
M84-Y143-E129Q
(TM2-4)
(LMNG)



M84-Y143-E129Q
(TM2-4)
(Nanodiscs)

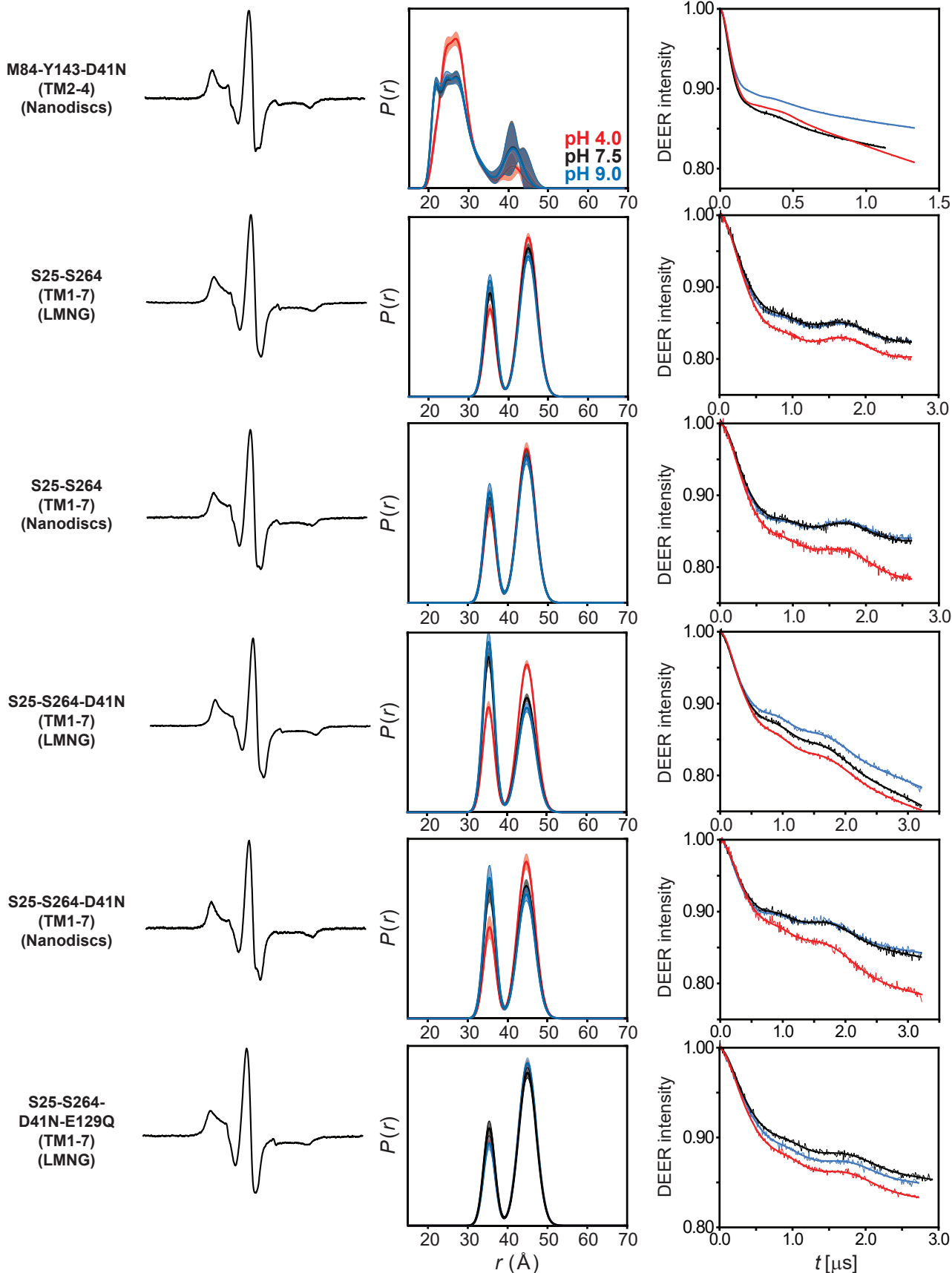


M84-Y143-D41N
(TM2-4)
(LMNG)



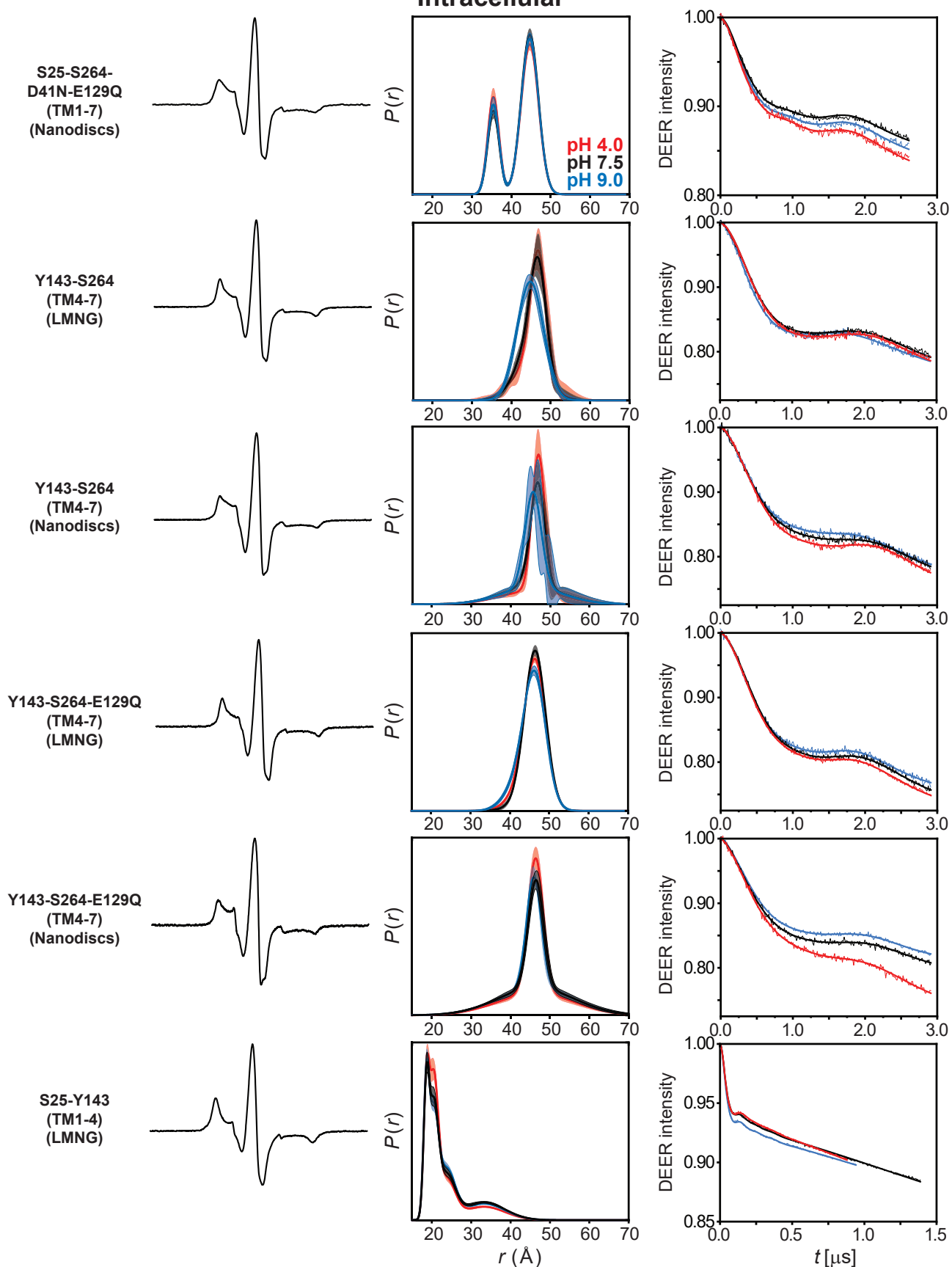
Data Appendix 12. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular



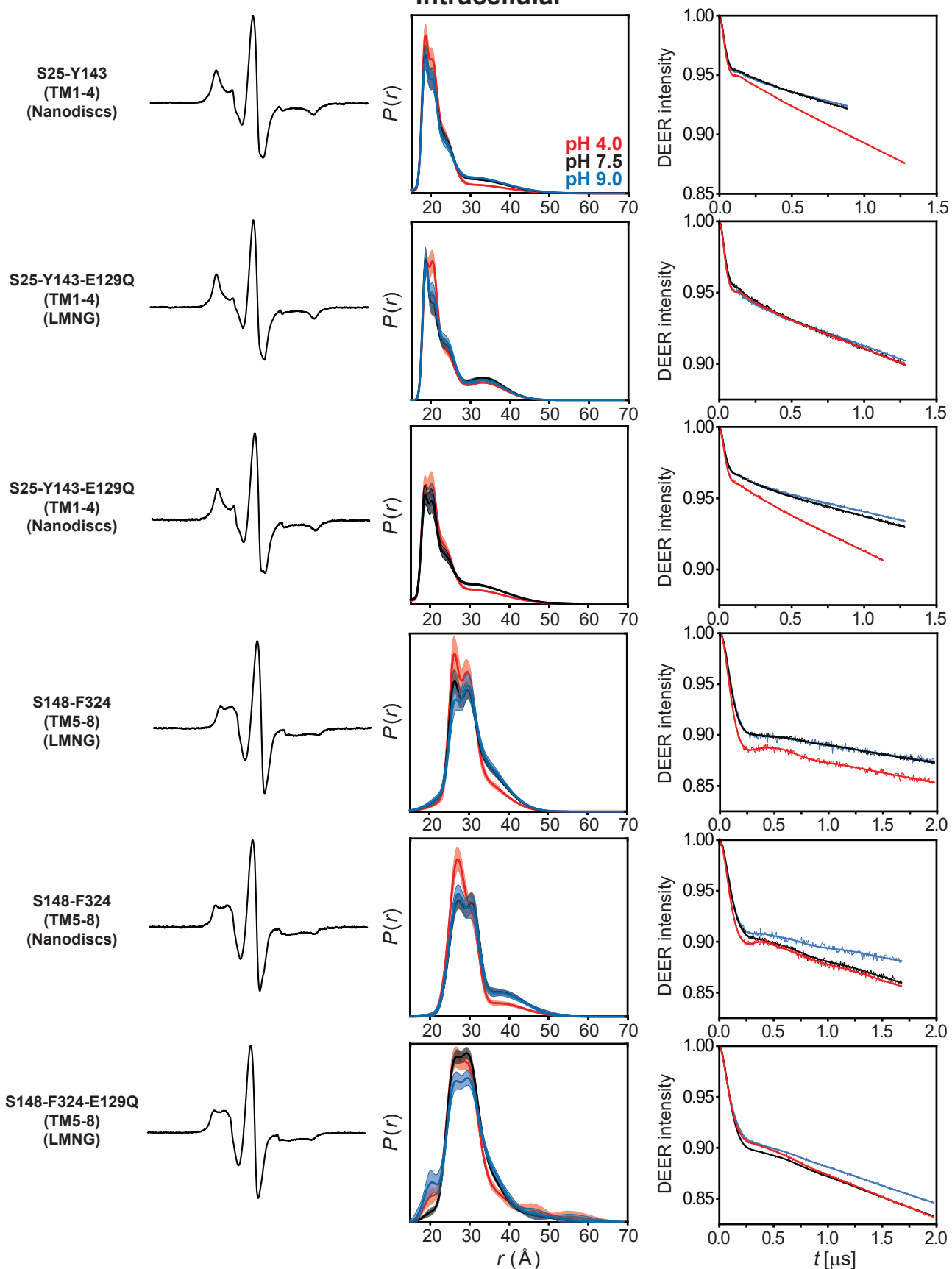
Data Appendix 13. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular



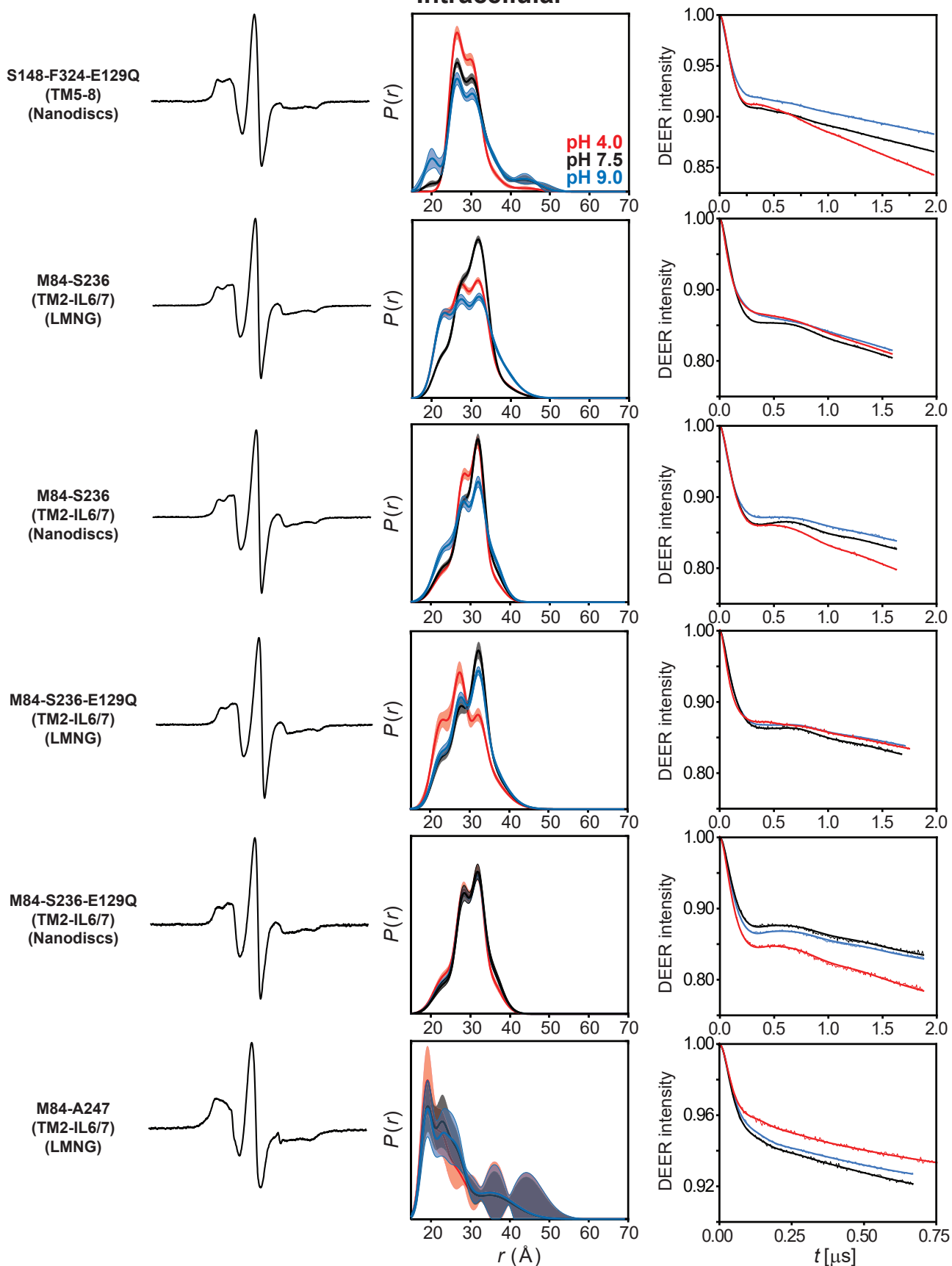
Data Appendix 14. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular



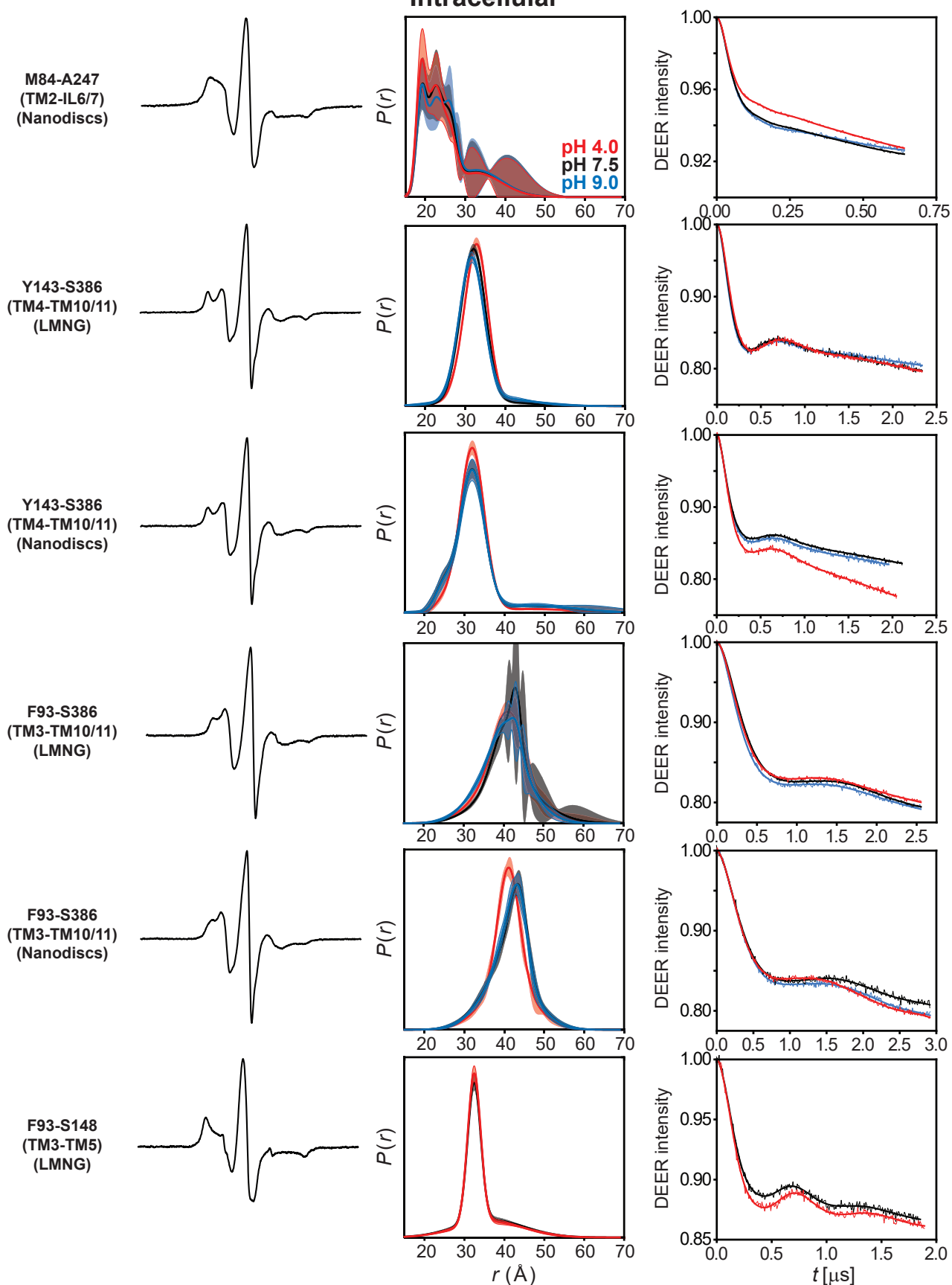
Data Appendix 15. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular



Data Appendix 16. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

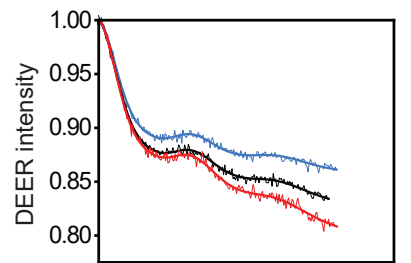
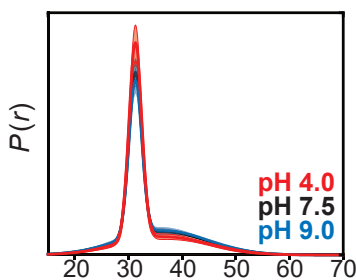
Intracellular



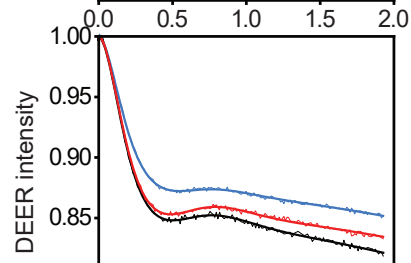
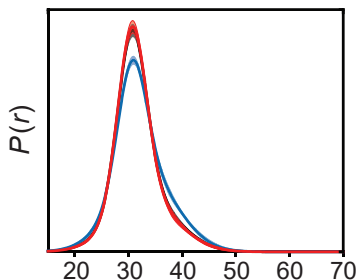
Data Appendix 17. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.

Intracellular

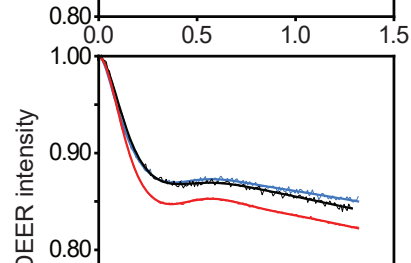
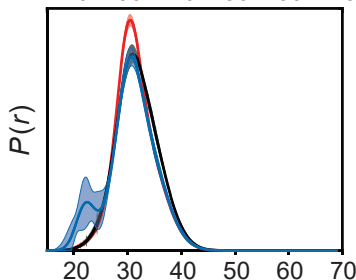
F93-S148
(TM3-TM5)
(Nanodiscs)



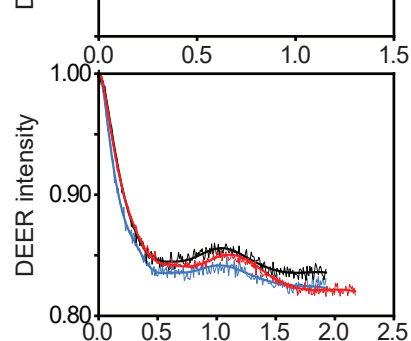
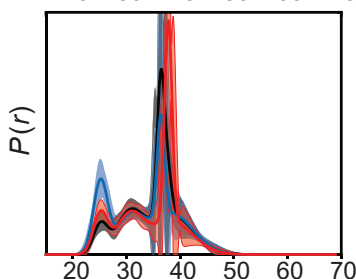
S25-F324
(TM1-TM8)
(LMNG)



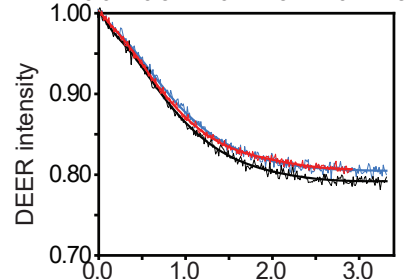
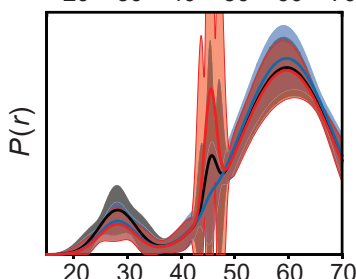
S25-F324
(TM1-TM8)
(Nanodiscs)



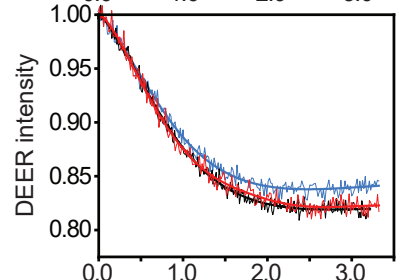
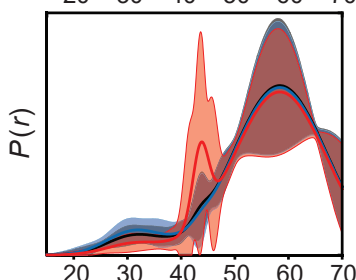
F324-S386
(TM8-TM10/11)
(Nanodiscs)



F93-I331
(TM3-TM9)
(LMNG)



F93-I331
(TM3-TM9)
(Nanodiscs)



Data Appendix 18. DEER data analysis for the intracellular side. For each mutant, from left to right, CW EPR, distance distributions, and the primary DEER traces along with the fits are shown.