Supporting Information

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SI Methods.

EPR Spectroscopy of Protective Antigen (PA) F427R1 and PA N306R1 in Liposomes and in Solution. For spectra recorded in the presence of liposomes, 500 μL of ∼20 mg/mL liposomes [containing 8% DOGS-NTA: 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]-Ni/92% DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), prepared in 20 mM Tris, pH 8.5, 400 mM NaCl (EPR buffer), as described previously (1)] were mixed with 1.5 mg of C-terminally His-tagged PA F427R1 or N306R1 prepore, and the mixture was incubated at room temperature for 30 min. The mixture was then centrifuged in a Sorvall Biofuge Fresco tabletop centrifuge for 30 min at 13,000 rpm to pellet the liposomes along with any attached protein. The supernatant was removed, and the liposomes were resuspended in 1 mL of EPR buffer and centrifuged again to wash and remove any unbound protein. The pellet was resuspended in 100 μL of EPR buffer. Fifty microliters of this sample was transferred to another tube, and 10 μL of 1 M acetate buffer, pH 5.5, was added to drop the pH to 6 (facilitating membrane insertion/pore formation). For EPR spectra recorded in solution, in the absence of liposomes, PA F427R1 or N306R1 prepore was diluted in EPR buffer to a final concentration of 225 μM PA_{63} in a volume of 20 μL. To record spectra of the prepore, this sample was used directly without pH adjustment. To record spectra of the pore, 5 μL of 1 M acetate buffer, pH 5.5 was added to these samples to drop the pH to 6 (facilitating pore formation). Immediately before EPR analysis, the samples were mixed in a 1:1 ratio with 80% glycerol as cryoprotectant. The EPR spectra were then recorded at 233 K on a Bruker EMX spectrometer at a microwave power of 2 mW sweeping the magnetic field from 3,260 to 3,460 G at frequency of 9.45 GHz. To assess spin–spin distances, the EPR spectrum of each heptameric sample was compared to the corresponding spectrum of monomeric PA_{63} F427R1 or N306R1. The monomeric samples were prepared either bound to liposomes or in solution in the same way as the heptameric samples, as described above.

Gel Shift Assays. Five micrograms of PA WT or PA $[R178A/K214E]_7$ heptamer was mixed with either 0, 0.5, 1, 2, 3, or 10 μg of lethal factor (LF) WT in a volume of 12.5 μL to make a molar ratio of 1:0, 1:0.5, 1:1, 1:2, 1:3, or 1:10 PA:LF, respectively. As a control, 5 μg of LF WT alone was used. To each sample, 12.5 μL of 2× Novex native Tris-Glycine sample buffer (Invitrogen) was added, and the mixtures were incubated for 30 min at room temperature. Twenty microliters of each sample was then loaded onto a 6% Tris-Glycine gel (Invitrogen), and the gels were run under native conditions at 100 V on ice.

EPR Spectroscopy of LF_N G2R1 and G5R1 with PA S429R1 in Liposomes. Three hundred microliters of ∼20 mg/mL liposomes [containing 8% DOGS-NTA-Ni/92% DOPC, prepared in 50 mM Bis-Tris, pH 6.0, 150 mM NaCl, as described previously (1)] were centrifuged for 30 min at 100,000 × *g* at 4 °C in a Beckman Airfuge ultracentrifuge to pellet the liposomes. The liposomes were resuspended in 350 μL of 20 mM Tris, pH 8.5, 150 mM NaCl, and either buffer alone or buffer containing 2 nmol $PA[S429R1]_1[R178A/K214E]_6$ (containing a His-tag on the C terminus of the S429R1 subunit) was added; the samples were allowed to sit at room temperature for 25 min to allow for binding of His-tagged PA to the Ni lipids. The liposomes were then pelleted for 30 min at 100,000 × *g* and resuspended in 400 μL of 50 mM Bis-Tris, pH 6.0, 150 mM NaCl buffer. The samples were allowed to sit for 30 min at room temperature to allow for PA insertion into the liposomes. Liposomes were again pelleted for 30 min at 100,000 × *g* and resuspended in 15 μL of 50 mM Bis-Tris, pH 6.0, 150 mM NaCl buffer. Either buffer alone or 2 nmol LF_N G2R1 or LF_N G5R1 was added, so that the samples generated contained either PA S429R1 alone, LF_N G2R1 or G5R1 alone, or PA S429R1 + LF_N G2R1, or PA S429R1 + LF_N G5R1. An equal volume of 80% glycerol was added as a cryoprotectant, and the EPR spectra were recorded at 233 K on a Bruker EMX spectrometer at a microwave power of 2 mW sweeping the magnetic field from 3,260 to 3,460 G at a frequency of 9.45 GHz.

1. Sun J, Vernier G, Wigelsworth DJ, Collier RJ (2007) Insertion of anthrax protective antigen into liposomal membranes: effects of a receptor. *J Biol Chem* 282:1059–1065.

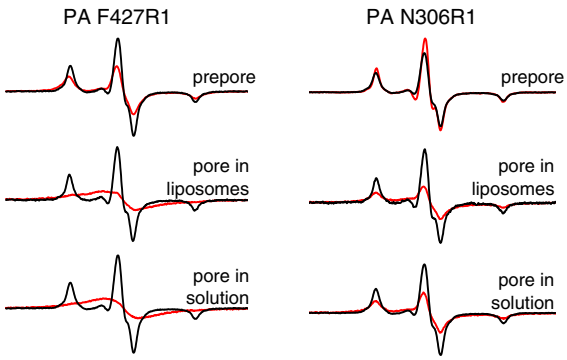

Fig. S1. *EPR analysis shows that PA pores formed in solution are indistinguishable from pores formed in liposomes*. Shown in red are the EPR spectra of PA F427R1 or N306R1 (homogeneously spin-labeled on all seven subunits) either in the prepore conformation (pH 8.5) or the pore conformation (pH 6, either inserted into liposomes or aggregated in solution). Shown in black are the spectra of monomeric PA_{83} F427R1 or N306R1 either in solution (*Top* and *Bottom*) or bound to liposomes (*Middle*). All spectra were recorded at low temperature (233 K). Shown are representative spectra; the spectrum of each condition was recorded a minimum of three times with little to no variation between trials. Calculated spin–spin interaction distances are PA F427R1 prepore = 20–25 Å, PA F427R1 pore in liposomes =< 8 Å, PA F427R1 pore in solution =< 8 Å, PA N306R1 prepore =< 25 Å, PA N306R1 pore in liposomes =< 10 Å, PA N306R1 pore in solution =< 10 Å.

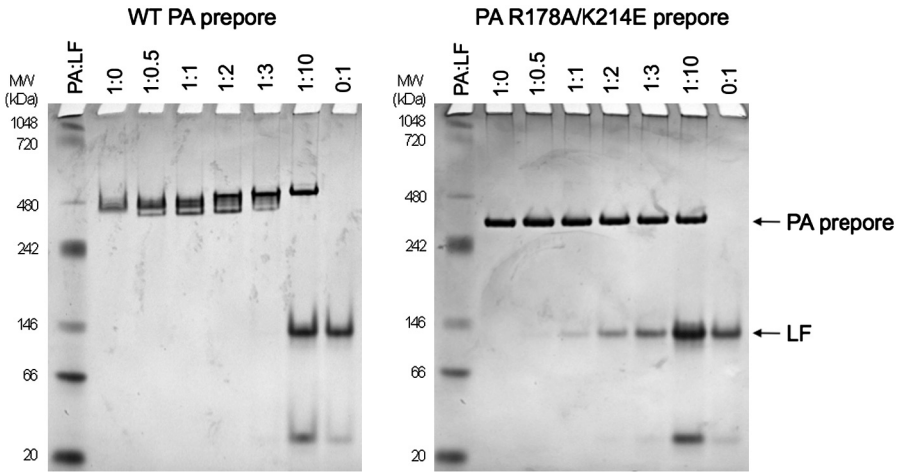

Fig. S2. *Gel shift assay shows PA [R178A/K214E]$_7$ is deficient in LF binding*. WT PA prepore or PA[R178A/K214E]$_7$ prepore were mixed in a molar ratio of 1:0, 1:0.5, 1:1, 1:2, 1:3, 1:10, or 0:1 PA heptamer:LF and assayed for binding by native polyacrylamide gel electrophoresis. The band corresponding to WT PA prepore was shifted to a higher molecular weight upon addition of LF; no shift was observed with PA [R178A/K214E]$_7$ prepore.

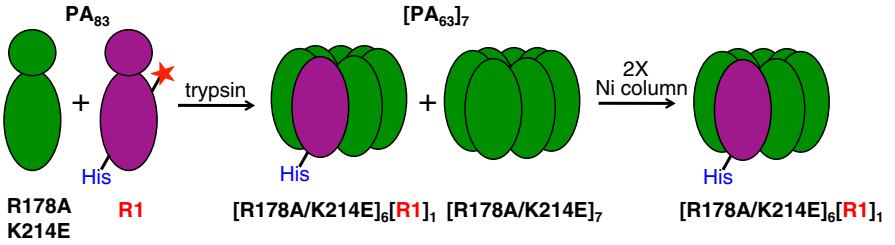

Fig. S3. *Schematic showing formation of heteroheptamers*. Spin label (R1) is represented as a red star. His-tagged, spin-labeled PA$_{83}$ subunits are mixed in a 1:20 ratio with PA$_{83}$ R178A/K214E subunits. The mixture is trypsinized to allow for heptamerization, and heptamers are separated from uncleaved PA$_{83}$ and PA$_{20}$ by anion exchange chromatography. Heteroheptamers containing a His-tagged, spin-labeled subunit are separated from PA [R178A/K214E]$_7$ heptamers by two subsequent rounds of Ni affinity chromatography.

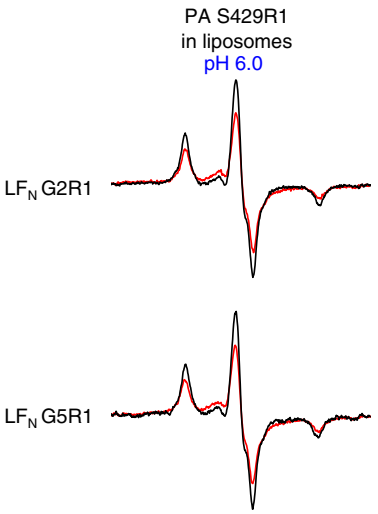

Fig. S4. *A spin–spin interaction is observed between LF$_N$ G2R1 or G5R1 and liposome-inserted PA S429R1 pore*. Black—additive EPR spectra of LF$_N$ G2R1 or G5R1 alone + liposome-inserted PA S429R1 pore alone [spectra collected in the presence of liposomes (LF$_N$) or inserted into liposomes (PA)]. Red—EPR spectra of 1:1 molar ratio LF$_N$ G2R1 or G5R1:liposome-inserted PA S429R1 pore. Spectra were collected at 233 K at pH 6.0 and are normalized to the same number of spins.

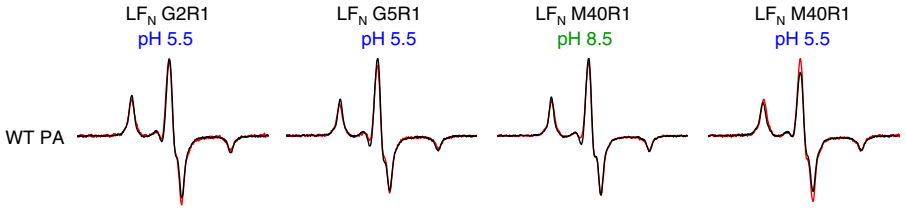

Fig. S5. *Spin–spin interaction is not observed with spin-labeled LF$_N$ variants bound to WT PA.* As a control to ensure that spin–spin interaction observed with spin-labeled LF$_N$ bound to spin-labeled PA is not due to background LF$_N$-LF$_N$ spin–spin interaction, spin-labeled LF$_N$ variants were mixed in a 1:1 molar ratio with PA WT heptamer at either pH 5.5 or pH 8.5. Black—EPR spectra of spin-labeled LF$_N$ alone. Red—EPR spectra of 1:1 molar ratio spin-labeled LF$_N$:WT PA heptamer. No spin–spin interaction was observed. Spectra were collected at 233 K and are normalized to the same number of spins.

Table S1. Binding ability of PA and LF$_N$ mutants assayed in planar lipid bilayers

PA variant	LF$_N$ variant	% initial block	% block after perfusion
PA WT	WT LF$_N$	98 ± 2	95 ± 4
PA F427W homoheptamer	WT LF$_N$	86 ± 3	77 ± 5
PA F427S homoheptamer	WT LF$_N$	60 ± 10	30 ± 20
PA F427H homoheptamer	WT LF$_N$	6 ± 2	N/A
PA [R178A/K214E]$_7$	WT LF$_N$	74 ± 7	9.5 ± 0.3
PA [WT]$_1$[R178A/K214E]$_6$	WT LF$_N$	90 ± 5	67 ± 1
PA [S429R1]$_1$[R178A/K214E]$_6$	WT LF$_N$	80 ± 6	40 ± 10
PA [F427S/S429R1]$_1$[F427S/R178A/K214E]$_6$	WT LF$_N$	46 ± 3	14 ± 6
PA [F427H/S429R1]$_1$[F427H/R178A/K214E]$_6$	WT LF$_N$	4 ± 5	N/A
PA [N180R1]$_1$[R178A/K214E]$_6$	WT LF$_N$	92 ± 4	60 ± 20
PA [F427H/N180R1]$_1$[F427H/R178A/K214E]$_6$	WT LF$_N$	1 ± 2	N/A
WT PA	LF$_N$ G2R1	97.9 ± 0.3	94 ± 3
WT PA	LF$_N$ G5R1	91 ± 6	71 ± 5
WT PA	LF$_N$ H10R1	96 ± 2	89 ± 4
WT PA	LF$_N$ E20R1	93 ± 3	86 ± 9
WT PA	LF$_N$ K30R1	94 ± 6	92 ± 5
WT PA	LF$_N$ M40R1	95 ± 3	95 ± 2
WT PA	LF$_N$ M40R1/E60R1	98 ± 1	99.2 ± 0.1
WT PA	LF$_N$ Y108R1	95 ± 2	87 ± 2
WT PA	LF$_N$ Q262R1	87 ± 2	85 ± 5

All PA mutants were tested for their ability to form channels in planar lipid bilayers and allow current block by WT LF$_N$. All LF$_N$ mutants were tested for their ability to block current through WT PA. All PA variants formed channels. % initial block = % of current blocked after the addition of 30 nM LF$_N$. % block after perfusion = % of current blocked after perfusion (in which 10× volume was exchanged).

Table S2. Calculated spin–spin interaction distances

PA variant	LF$_N$ variant	pH	Spin–spin distance	% interacting
None	LF$_N$ M40R1/E60R1	pH 8.5	<10 Å	77%
PA WT	LF$_N$ M40R1/E60R1	pH 8.5	<10 Å	24%
PA WT	LF$_N$ G2R1	pH 5.5	>25 Å	N/A
PA WT	LF$_N$ G5R1	pH 5.5	>25 Å	N/A
PA WT	LF$_N$ M40R1	pH 8.5	>25 Å	N/A
PA WT	LF$_N$ M40R1	pH 5.5	>25 Å	N/A
PA [N180R1]$_1$[R178A/K214E]$_6$	LF$_N$ M40R1	pH 8.5	16 Å	85%
PA [N180R1]$_1$[R178A/K214E]$_6$	LF$_N$ M40R1	pH 5.5	>25 Å	N/A
PA [F427H/N180R1]$_1$[F427H/R178A/K214E]$_6$	LF$_N$ M40R1	pH 8.5	16 Å	67%
PA [F427H/N180R1]$_1$[F427H/R178A/K214E]$_6$	LF$_N$ M40R1	pH 5.5	15 Å	23%
PA [S429R1]$_1$[R178A/K214E]$_6$	LF$_N$ G2R1	pH 8.5	>25 Å	N/A
PA [S429R1]$_1$[R178A/K214E]$_6$	LF$_N$ G2R1	pH 5.5	13 Å	50%
PA [S429R1]$_1$[R178A/K214E]$_6$	LF$_N$ G5R1	pH 8.5	>25 Å	N/A
PA [S429R1]$_1$[R178A/K214E]$_6$	LF$_N$ G5R1	pH 5.5	12 Å	49%
PA [S429R1]$_1$[R178A/K214E]$_6$ in liposomes	LF$_N$ G2R1	pH 6.0	12 Å	32%
PA [S429R1]$_1$[R178A/K214E]$_6$ in liposomes	LF$_N$ G5R1	pH 6.0	13 Å	32%
PA [F427H/S429R1]$_1$[F427H/R178A/K214E]$_6$	LF$_N$ G2R1	pH 5.5	>25 Å	N/A
PA [F427H/S429R1]$_1$[F427H/R178A/K214E]$_6$	LF$_N$ G5R1	pH 5.5	>25 Å	N/A
PA [F427S/S429R1]$_1$[F427S/R178A/K214E]$_6$	LF$_N$ G2R1	pH 5.5	>25 Å	N/A
PA [F427S/S429R1]$_1$[F427S/R178A/K214E]$_6$	LF$_N$ G5R1	pH 5.5	>25 Å	N/A

The dynamic range of the technique is ~10–25 Å (1). Shown are the calculated spin–spin distances and the percentage of spins in each sample exhibiting detectable spin–spin interaction

1. Rabenstein MD, Shin YK (1995) Determination of the distance between two spin labels attached to a macromolecule. *Proc Natl Acad Sci USA* 92:8239–8243.