Supporting Information

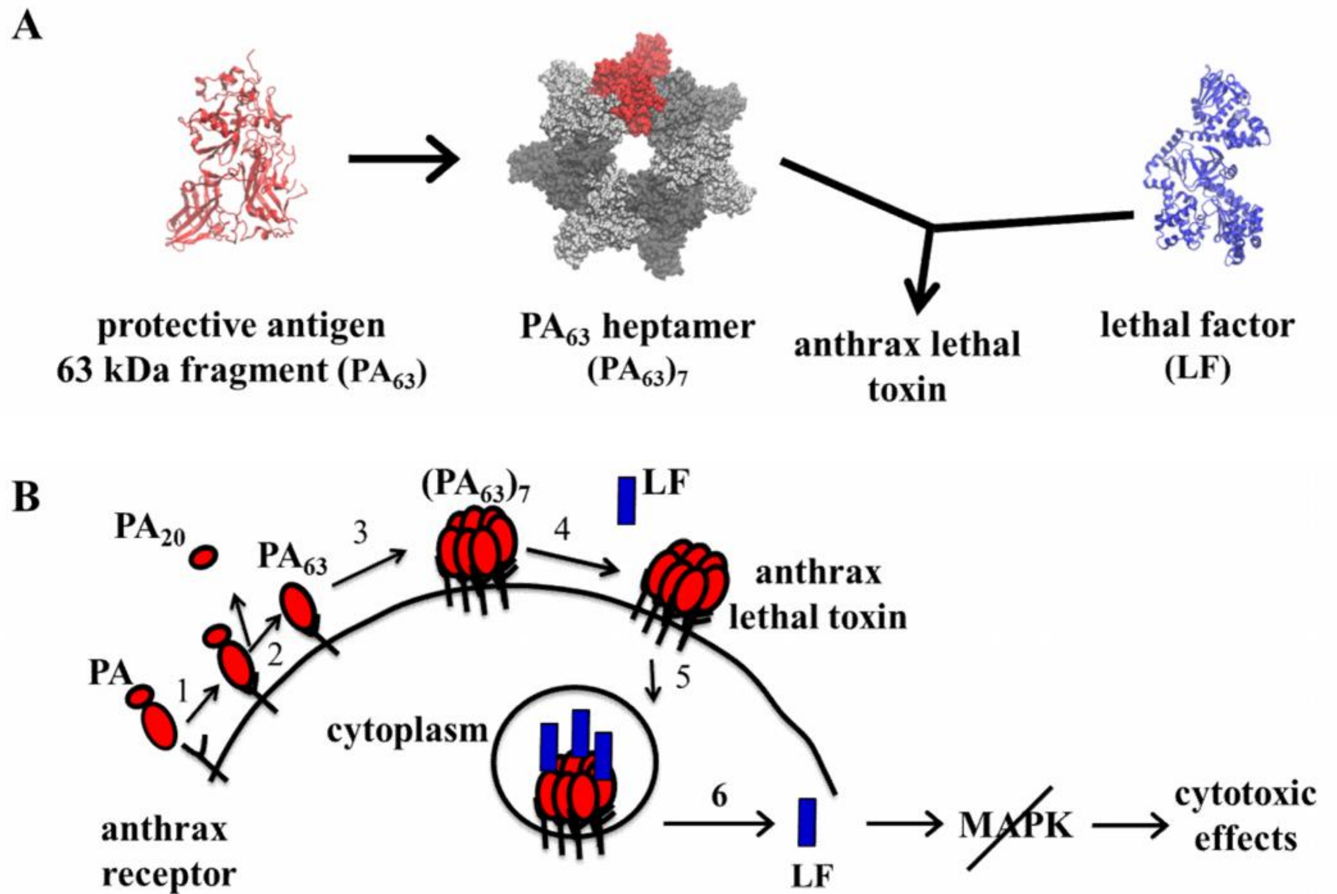

***Figure S1*. Anthrax toxin assembly and intoxication pathway.** (A) Toxin assembly involves the binding of the toxic enzyme, lethal factor (LF) to the heptameric receptor-binding subunit, $(PA_{63})_7$. (B) Steps involved in the intoxication pathway are: (1) Binding of protective antigen (PA) to its receptor (2) Proteolytic cleavage of PA into PA_{20} and PA_{63} (3) Oligomerization of PA_{63} to form a heptameric "prepore" (4) Binding of LF to $(PA_{63})_7$ (5) Endocytosis of the receptor – prepore – LF complex. (6) pH-mediated activation of the PA_{63} prepore and translocation of LF.

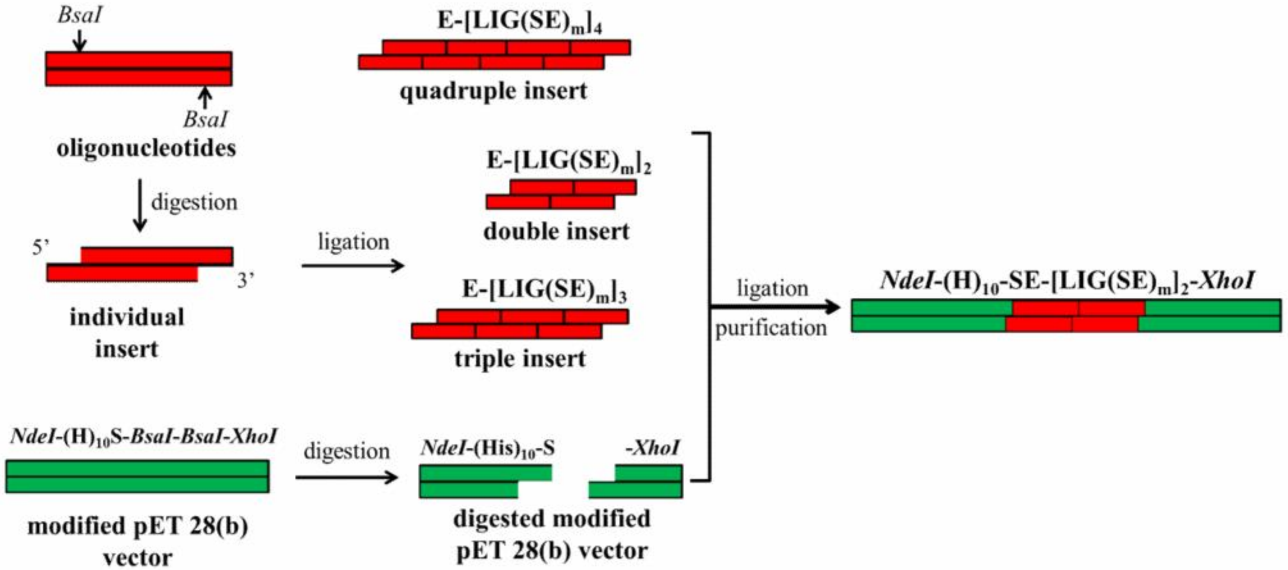

***Figure S2*. Schematic showing the process used for generation of oligonucleotide sequence encoding $(H)_{10}$-SE[LIG$(SE)_m$]$_2$ polypeptides.**

Amino acids	Distance between amino acids on adjacent PA_{63} monomers of $(PA_{63})_7$ (Å)
P184	25.8 ± 0.3
L187	30.5 ± 0.2
K197	34.2 ± 0.3
R200	30.2 ± 0.2

***Table 1*. Distance between the predicted ligand binding sites on adjacent PA_{63} monomers of $(PA_{63})_7$ is in the range of 25 - 35 Å**

Value of ‘m’	RMS end-to-end distance of “GAP-$(SE)_m$-HTS” linker (Å)
1	15.5
2	18.1
3	21.1
4	23.9
5	**26.9**
6	**30.2**
7	**33.0**
8	36.3
13	50.3

Table S2. **RMS end-to-end distances for GAP-$(SE)_m$-HTS linkers calculated using implicit solvent REMD simulations.** The RMS end-to end distances for linkers with linker lengths “matching” the distance between the predicted binding sites on the target PA_{63} heptamer are shown in bold.

Experimental Section

Construction of Expression Plasmids for Polypeptide Inhibitors:

Oligonucleotides encoding sequences [LIG$(SE)_m$] with terminal restriction sites (*BsaI*-[LIG$(SE)_m$]-*BsaI*) were obtained from Genemed Synthesis Inc. (San Antonio, TX). Here, LIG represents the inhibitory peptide ligand (LIG - HTSTYWWLDGAP) and 'm' represents the number of sequential repeats of serine and glutamic acid. The *BsaI*-[LIG$(SE)_m$]-*BsaI* oligonucleotides were digested using the restriction endonuclease to generate individual inserts encoding [LIG$(SE)_m$] (Figure S2) with complementary 5' and 3' overhangs. Ligation resulted in assembly into a mixture of repetitive sequences of the form *BsaI*-E$[LIG(SE)_m]_n$-*BsaI*, where 'n' represents the number of tandem repeats of individual inserts encoded in the sequence. The mixture of ligation products was separated by agarose gel electrophoresis, and the individual bands were excised and recovered using a gel extraction kit. In a separate experiment, we modified a pET28(b) expression vector so that the expression domain read *NdeI* - $(H)_{10}$ – S – *BsaI* – *BsaI* – *XhoI*, which allowed for the introduction of *BsaI* - E$[LIG(SE)_m]_n$ – *BsaI* tandem repeats between the *BsaI* restriction sites and added a sequence encoding a decahistidine tag at the N-terminus of the polypeptide. The modified pET28(b) was digested using *BsaI* and dephosphorylated using calf intestinal alkaline phosphatase (CIP). After agarose gel electrophoresis and subsequent gel extraction, the digested DNA fragments were ligated with the *BsaI*-E$[LIG(SE)_m]_n$-*BsaI* inserts, yielding plasmids encoding for various $(H)_{10}$-SE$[LIG(SE)_m]_n$ polypeptides, which were sequenced by MCLAB (San Francisco, CA). Plasmids encoding $(H)_{10}$-SE$[LIG(SE)_m]_n$ polypeptides were transformed in *E. coli*, expressed, and purified as described below. Restriction enzymes required for the digestion reactions were obtained from New

England Biolabs (Ipswich, MA). Standard molecular biology procedures were used based on manufacturer's instructions.

Expression and Purification of Polypeptides:

The plasmids encoding the designed polypeptides were transformed into *E. coli* expression strain BL21(DE3)pLysS. The transformation step was followed by the expression of polypeptides in *E. coli*. Urea buffer (8 M urea, 100 mM monosodium phosphate, 10 mM Tris-HCl, pH 8.3) was used to lyse the cells following the expression of the polypeptides. The polypeptides were purified by immobilized metal affinity chromatography using PerfectPro resin (5 Prime) and using the protocol recommended by the resin manufacturer. Elution buffer (8 M urea, 100 mM monosodium phosphate, 10 mM Tris-HCl, pH 4.3) was used to elute the polypeptides of interest. The purity of the eluted polypeptides was characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. Purified protein was dialyzed against phosphate buffered saline buffer (PBS) (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) with several rounds of buffer exchange. The concentrations of the purified proteins were determined on a per peptide basis using a NanoDrop 2000 UV-Vis Spectrometer (Thermo Scientific) using calculated values for the molecular weight (MW) and extinction coefficients estimated by the amino acid profile of the designed sequences. The control polypeptide $(H)_{10}$-SE$[CTRL(SE)_5]_7$ was expressed and purified using the same procedure as for $(H)_{10}$-SE$[LIG(SE)_5]_7$.

Replica Exchange Molecular Dynamics (REMD) simulations:

Conformational sampling of various peptide linkers (i.e., GAP–$(SE)_m$–HTS) using REMD[1] simulations was used to characterize the root mean-squared (RMS) end-to-end distances. REMD simulations were carried out for linkers with 'm' values of 1, 2, 3, 4, 5, 6, 7, 8, and 13. In each of

the simulations, the starting point was an extended chain built via the LEaP program of AMBER (www.ambermd.org). Implicit solvent simulations were performed using AMBER11[2] and the amber force field – *ff99SB*[3] was used along with the condition of 150 mM salt concentration. For each linker, REMD simulations with a total of 16 replicas in the temperature range of 260 – 600 K were performed with each replica simulated for a total of 24 ns. Using the data between 5 and 24 nanoseconds for each replica, snapshots of the linker conformation that were 20 picoseconds apart were extracted leading to a total of ca. 15000 possible conformations. The distance between the alpha carbon atom of the first and last residue of the linker (the end-to-end distance) was calculated for each of the simulated conformations for a given linker, and used to compute the RMS end-to-end distance for the linker.

Cytotoxicity assay:

Monovalent peptide Ac-HTSTYWWLDGAPK-Am was synthesized by Genemed Synthesis Inc. (San Antonio, TX). RAW264.7 cells were seeded in 96-well plates and incubated overnight. The cells were treated with 3×10^{-9} M PA and 3×10^{-10} M LF in the absence or presence of various concentrations of inhibitors. After an incubation period of 4 h, cell viability was quantified by performing the MTS assay according to the manufacturer's instructions (Promega, Madison, WI). Each sample was tested in triplicate, and half maximal inhibitory potency (IC_{50}) values were determined from the dose response curve for individual inhibitors on a per-peptide basis.

PEGylation of polypeptides:

PEGylation of polypeptides was performed by using a protocol described elsewhere.[4] Briefly, a cysteine residue was introduced at the N-terminus of the protein by replacing the N-terminal histidine residue from the decahistidine tag using site directed mutagenesis. Methoxy PEG maleimide (MWs 20,000 kDa) was purchased from JenKem Technologies USA (Allen, TX).

The PEGylation reaction was carried out in 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, 2 mM EDTA, at 0.5 – 1 mg/mL polypeptide inhibitor, and five-fold higher molar concentrations of *tris*(2-carboxyethyl)phosphine (TCEP) and polyethylene glycol (PEG) relative to inhibitor concentrations. The reaction mixture was incubated under a nitrogen atmosphere at room temperature overnight. PEGylated polypeptides were separated from non-PEGylated polypeptides and unreacted PEG by size exclusion chromatography using a Superdex 200 10/300 GL analytical column (GE Healthcare).

Size Exclusion Chromatography (SEC):

All SEC experiments were performed using a protocol described elsewhere.[5] SEC experiments were performed on a Superdex 200 10/300 GL analytical grade column pre-calibrated with standard MW proteins using an AKTA Explorer purification system (GE Healthcare). The column was equilibrated with the dialysis buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) prior to loading each sample. Approximately 30 – 50 µL of each protein sample at 0.5 – 1 mg/mL polypeptide were loaded onto the column and the absorbance of the eluent was monitored at 280 nm.

Circular Dichroism (CD) Spectroscopy:

Far UV-CD experiments were performed using a J-815 CD spectrometer (Jasco Systems, MD). Approximately 300 µL of protein samples at 0.5 mg/mL were used for the measurement of CD spectra and also for thermal denaturation experiments. Experimental settings were identical to those described previously.[6]

References

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