

## **A DARPIn Promotes Faster Onset of Botulinum Neurotoxin A1 Action**

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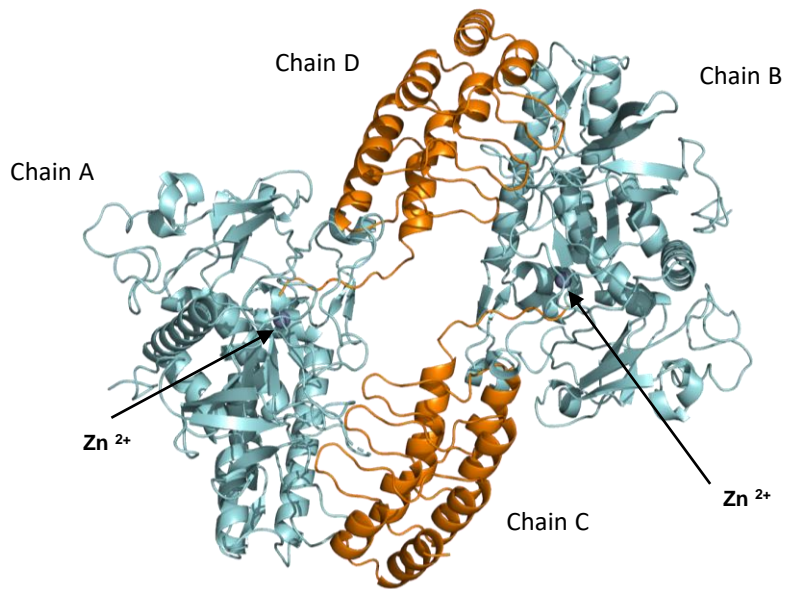
\* These authors contributed equally to the study.

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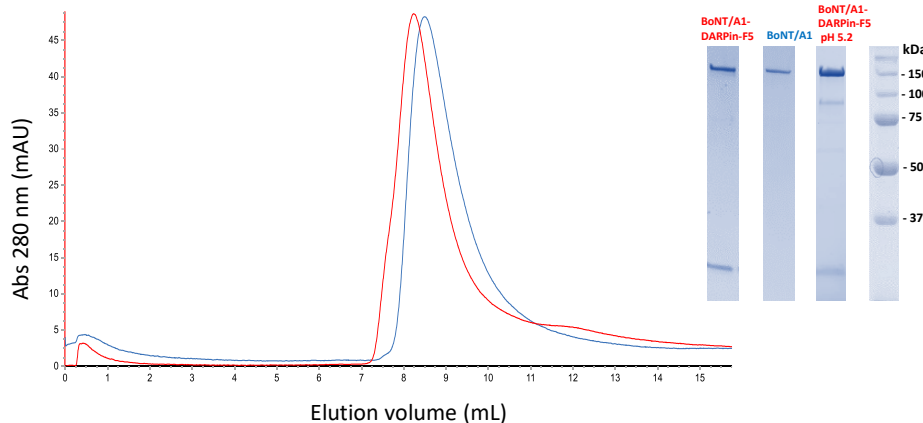
Richard A. Kammerer, Laboratory of Biomolecular Research, Division of Biology, Paul Scherrer Institut, CH-5232 Villigen PSI, Switzerland.

8xHis-tagged DARPin-F5	MRGSHHHHHHHGSDLGKKLLEAARAGQDDEVIRILMANGADVNAVDMHG YTPLHLAAAVGHLEIVEVLLKAGADVNAWDQVGKTPHLAAKWGHEIVEVLL KHGADVNAQDWMGRTPFDLAIDNGNEDIAEVLQKAAKLN <del>DKDDDD</del>
Truncated DARPin-F5 with cleavable 8xHis tag	MRGSHHHHHHHGSGLVPRDLGKKLLEAARAGQDDEVIRILMANGADVNAV DMHG <del>YTPLHLAAAVGHLEIVEVLLKAGADVNAWDQVGKTPHLAAKWGHEI</del> IVEVLLKHGADVNAQDWMGRTPFDLAIDNGNEDIAEVLQKAAKLN <del>DKDDDD</del> <del>DK</del> LVPR GS- Thrombin cleavage site

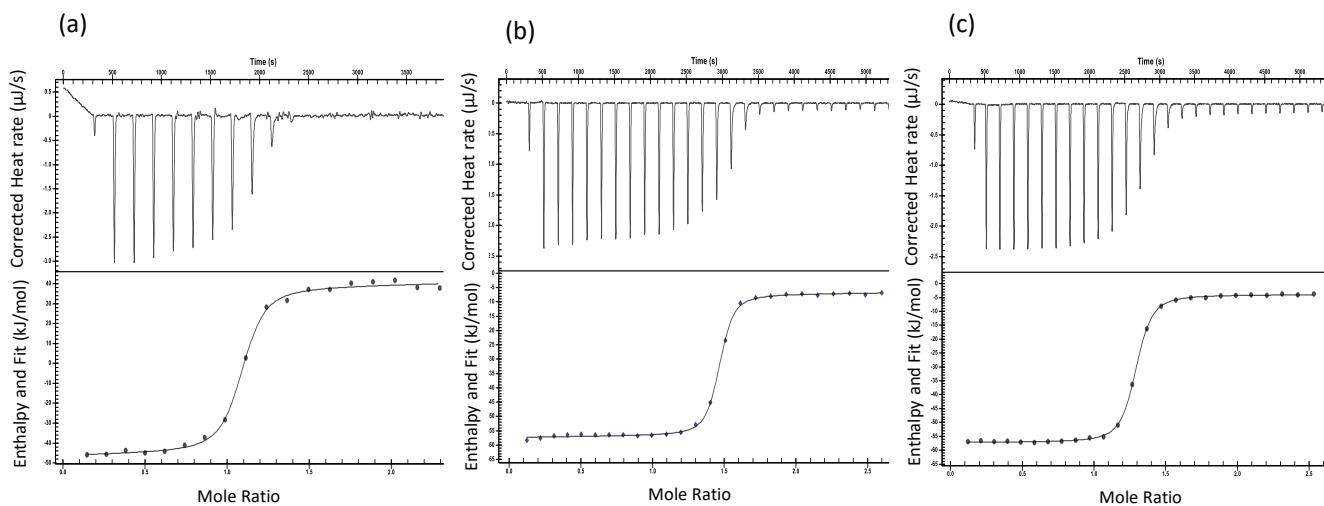
**Supplementary Figure S1.** Amino-acid sequence of DARPin-F5 and its truncated version with a cleavable 8xHis tag.



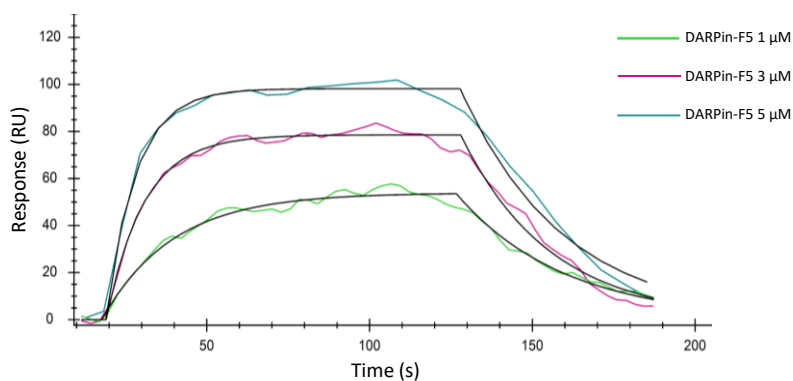
**Supplementary Figure S2. Crystal structure of the LC/A1-DARPin-F5 complex.** Cartoon representation of the four polypeptide chains in the asymmetric unit. (PDB code 8HKH). LC/A1 is shown in cyan (chain A and B) and DARPin-F5 (chain C and D) in orange. The zinc ion is shown as grey sphere.



**Supplementary Figure S3. Size exclusion chromatography (SEC) and SDS-PAGE analysis of BoNT/A1-DARPin-F5 complex.** BoNT/A1 (blue trace), BoNT/A1-DARPin-F5 (red trace). SEC analysis was performed with a Superdex 75 10/300 column (GE Healthcare) in 20 mM Tris-HCl pH 7.4, 100 mM NaCl (or in 20 mM Sodium Acetate pH 5.2, 100 mM NaCl) and fractions from respective peaks were analyzed on a gradient SDS-PAGE gel (BioRad). Left side gel fraction from peak complex BoNT/A1-DARPin-F5 at pH 7.4, middle gel BoNT/A1 alone (153 kDa), right side gel fraction from peak complex BoNT/A1-DARPin-F5 at pH 5.2. The experiments were repeated twice independently and yielded similar results.

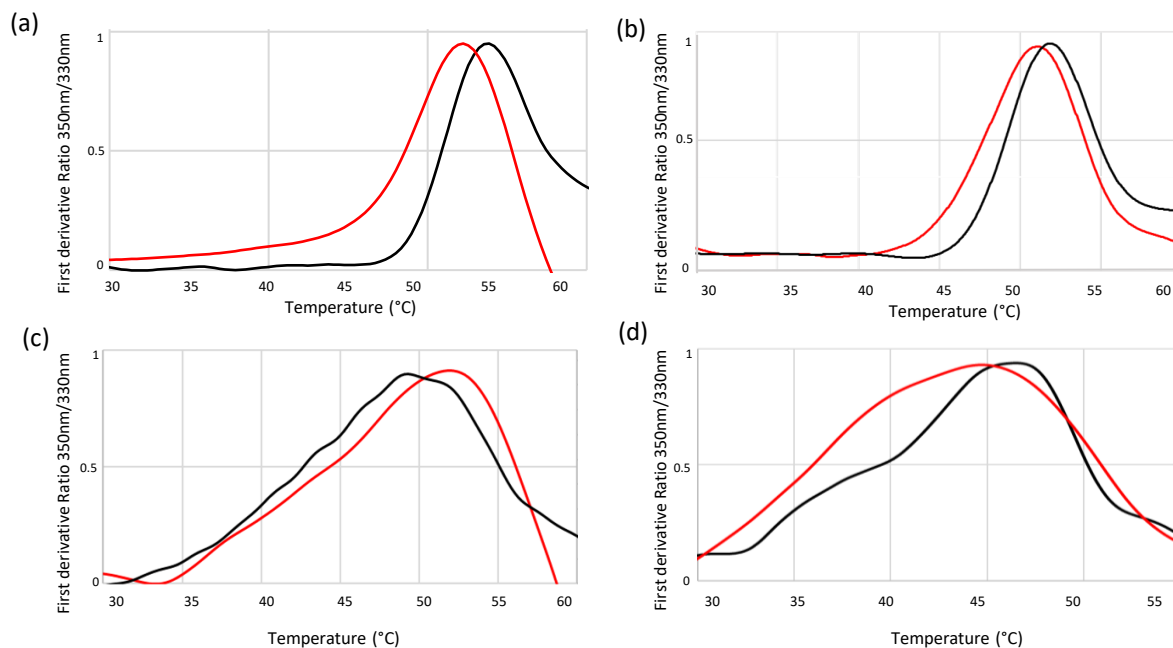


**Supplementary Figure S4. ITC titrations of DARPin-F5 variants to LC/A1.** Representative ITC titrations of DARPin-F5 to LC/A1 at pH 7.5 with (a) and without (b) the N-terminal 8xHis tag on DARPin-F5 and (c) without the N-terminal 8xHis tag on DARPin-F5 at pH 5.5. (a)  $K_D = 9,61 \times 10^{-8} \pm 0.02$  M, (b)  $K_D = 4,43 \times 10^{-8} \pm 0.01$  M, (c)  $K_D = 7,01 \times 10^{-8} \pm 0.01$  M. The error is the standard deviation on at least two independent measurements.

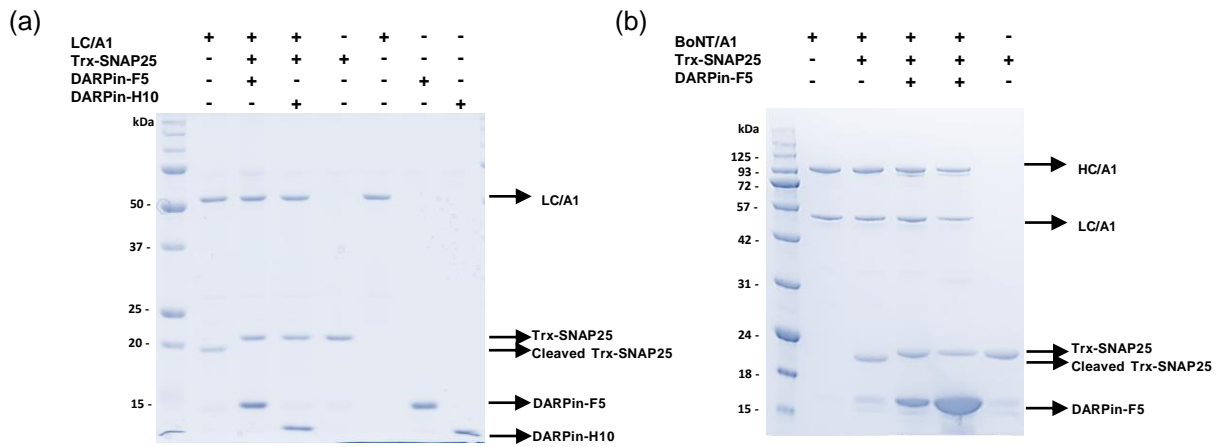


**Supplementary Figure S5. Binding affinity and kinetic parameters of the interaction of DARPin-F5 with LC/A3.**

SPR analysis of DARPin-F5 binding to the LC/A3, performed on the Nicoya OpenSPR™; overlaid with a fit of 1:1 binding model (black line). Increasing DARPin-F5 concentrations ranging from 1 to 5  $\mu\text{M}$  were applied. The  $K_D$  value of  $1.68 \pm 0.58 \mu\text{M}$  was calculated from the raw data using the TraceDrawer Software (Ridgeview Instruments AB).



**Supplementary Figure S6. Thermal stability of the BoNT/A1-DARPin-F5 and LC/A1-DARPin-F5 complexes.** First derivatives of the fluorescence ratio (350nm/330nm) are shown as a function of temperature for BoNT/A1-DARPin-F5 (red) and for BoNT/A1 alone (black) at (a) pH 5.5 and (b) pH 7.4. (c) and (d) Same experiment shown for LC/A1-DARPin-F5 (red) and for LC/A1 alone (black) at pH 5.5 and pH 7.4. Each curve is the average of three independent measurements.



**Supplementary Figure S7.** Uncropped gels of data shown in figure 1a and b. The experiments were repeated twice independently and yielded similar results.

Figure 3c

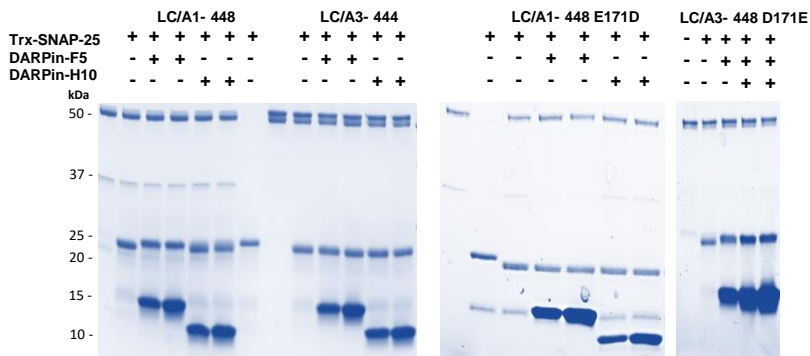
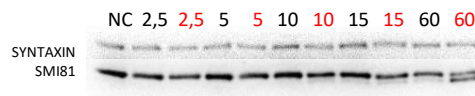


Figure 5b



**Supplementary Figure S8.** Above, uncropped gels of data shown in figure 3c. Below, supplementary western blot of data shown in figure 5b. In both cases, experiments were repeated twice independently and yielded similar results.

<i>Data collection</i>	
Resolution range (Å)	49.19 - 2.55 (2.62 - 2.55)
Space group	<i>P1</i>
Polypeptide chains/AU	4
Unit cell parameters	
a, b, c (Å)	59.56, 59.63, 102.18
$\alpha, \beta, \gamma$ (°)	74.976, 89.904, 73.154
Observed reflections	147652 (10839)
Unique reflections	39460 (2962)
Multiplicity	3.7 (3.8)
Completeness (%)	93.4 (94.2)
Mean I/sigma(I)	4.49 (1.17)
CC(1/2)	0.976 (0.452)
Wilson B-factor	47.03
<i>Refinement</i>	
Resolution range (Å)	49.19 - 2.7 (2.797 - 2.7)
$R_{\text{work}}$ (%)	26.17
$R_{\text{free}}$ (%)	30.61
Protein atoms	8840
rmsd of bond lengths	0.003
rmsd of bond angles	0.558
Average <i>B</i> -factor (Å <sup>2</sup> )	
Total Protein Complex	47.75
LC/A1 (chain A/B)	47.80
DARPin-F5 (chain C/D)	51.71
Ligands	42.38
Solvent	31.97
Ramachandran plot (%)	
Favored	97.45
Allowed	2.55
Outliers	0
Crystallization condition	0.1 M HEPES pH 7.5 28 % w/v Jeffamine ED-2003
Values in parentheses refer to the outermost resolution shell.	

**Supplementary Table S1.** Values in parentheses refer to the outermost resolution shell.

Residue (LC/A1 chain A)	Distance (Å)	Residue (DARPin-F5 chain C)
Glu 171 [O]	3.5	Arg 25 [NE]
Glu 171 [OE1]	3.2	Tyr 50 [OH]
Glu 171 [OE2]	2.5	Lys 91 [NZ]
Lys 128 [NZ]	2.9	Asp 124 [O]
Asp 131 [OD1]	3.2	Trp 113 [NE1]
Asp 131 [OD1]	3.4	Arg 116 [NH1]

**Supplementary Table S2.** Table summarizing interacting residues of the interface between chain A and chain C.

Primer Name	Sequence
LC/A1 5'	CTC GTC GGG ATC CGC TGG AAG TGC TGT TTC AGG GCC CGT TTG TGA ACA AAC AGT TCA AC
LC/A1 3'	CAG GTC CTC GAG TTA TTA TTT GTT GTA GCC TTT GTC CAG AC
LC/A3 5'	CTC GTC GGG ATC CGC TGG AAG TGC TGT TTC AGG GCC CGT TTG TGA ACA AAC AGT TCA ATT ATC
LC/A3 3'	CAG GTC CTC GAG TTA TTA TTT GTT ATA GCC CTC ATC CAG AC
LC/A1 E171D 5'	GCT TTG GCC ACG ATG TTC TGA ATC TGA CCC GTA ATG G
LC/A1 E171D 3'	GTC AGA TTC AGA ACA TCG TGG CCA AAG CTT TTA CAT TC
LC/A3 D171E 5'	GCT TTG GCC ACG AAG TGT TTA ATC TGA CCC GTA ATG G
LC/A3 D171E 3'	GTC AGA TTA AAC ACT TCG TGG CCA AAG CTT TTA CAT TC
Avi tag-LC/A 5' (LC/A1 and LC/A3)	CTC GTC GGA TCC GGC CTG AAC GAT ATT TTT GAA GCG CAG AAA ATT GAA TGG CAT GAA GGT TCA ATG CCG TTT GTG AAC AAA CAG TTC
DARPin-F5 5'	CTC GTC GGA TCC GGC CTG GTG CCG CGT GGT AGC GAC CTG GGT AAG AAA CTG CTG GAA GC
DARPin-F5 3'	CTC GTC AAG CTT ATT AAT TAA GTT TAG CAG CTT TCT GCA GAA CTT CAG C

**Supplementary Table S3.** Table showing sequences of oligonucleotides.