Definition of a saxitoxin (STX) binding code enables discovery and characterization of the anuran saxiphilin family

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Edited by Nieng Yan, Princeton University, Princeton, NJ; received June 12, 2022; accepted September 19, 2022

American bullfrog (Rana catesbeiana) saxiphilin (Rsxph) is a high-affinity “toxin sponge” protein thought to prevent intoxication by saxitoxin (STX), a lethal bis-guaindinium neurotoxin that causes paralytic shellfish poisoning (PSP) by blocking voltage-gated sodium channels (Navs). How specific Rsxph interactions contribute to STX binding has not been defined and whether other organisms have similar proteins is unclear. Here, we use mutagenesis, ligand binding, and structural studies to define the energetic basis of Sxph:STX recognition. The resultant STX “recognition code” enabled engineering of Rsxph to improve its ability to rescue Navs from STX and facilitated discovery of 10 new frog and toad Sxphs. Definition of the STX binding code and Sxph family expansion among diverse anurans separated by ∼140 My of evolution provides a molecular basis for understanding the roles of toxin sponge proteins in toxin resistance and for developing novel proteins to sense or neutralize STX and related PSP toxins.

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Significance

Saxitoxin (STX) is a lethal neurotoxin made by cyanobacteria and dinoflagellates associated with oceanic red tides. Its accumulation in seafood causes paralytic shellfish poisoning (PSP), a public health hazard that is increasing due to climate change. Bullfrog saxiphilin (RfSxph) is a soluble STX binding protein that acts as a ‘toxin sponge’ involved in toxin resistance. Studying Rsxph:STX interaction energetics defines a STX ‘recognition code’ that enabled discovery of ten Sxphs in frogs and toads separated by ∼140 million years of evolution. These findings provide a molecular basis for understanding the roles of toxin sponge proteins in toxin resistance and should enable STX binding site identification in natural proteins and design of proteins to sense or neutralize STX and related toxins.
positions. Comparison of the new Sxph family members further identifies dramatic differences in the number of thyroglobulin (Thy1) domains inserted into the modified transferrin fold upon which the Sxph family is built. Biochemical characterization of NpSxph, Oophaga sylvatica Sxph (OsSxph) (24), Mantella aurantia Sxph (MaSxph), and Ranitomeya imitator Sxph (RiSxph), together with structural determination of NpSxph, alone and as STX complexes, shows that the different Sxphs share the capacity to form high-affinity STX complexes and that binding site preorganization (10) is a critical factor for tight STX association. Together, these studies establish an STX molecular recognition code that provides a template for understanding how diverse STX binding proteins engage the toxin and its congeners and uncover that Sxph family members are abundantly found in the most varied and widespread group of amphibians, the anurans. This knowledge and suite of diverse Sxphs, conserved among anuran families separated by at least 140 My of evolution (25), provide a starting point for defining the physiological roles of Sxph in toxin resistance (9, 24, 26), should facilitate identification or design of other STX binding proteins, and may enable the development of new biologics to detect or neutralize STX and related PSPs.

Results

Establishment of a Suite of Assays to Probe RsSxph Toxin-Binding Properties. To investigate the molecular details of the high-affinity RsSxph:STX interaction, we developed three assays to assess the effects of STX binding site mutations. A key criterion was to create assays that could be performed in parallel on many RsSxph mutants using minimal amounts of purified protein and toxin. To this end, we first tested whether we could detect STX binding using a TF assay (27, 28) in which STX binding would manifest as concentration-dependent change in $R_c$ (e.g., I782A and D785N) as well as those that caused complete $\sim$ 100-fold affinity changes further, we used ITC (10) to show no changes in the core STX binding site preorganization (10) showed no changes in the RsSxph:STX complex at 2.65-Å resolution by X-ray crystallography (SI Appendix, Fig. S3A and Table S1). Inspection of the STX binding pocket revealed clear electron density for the F-STX bis-guanidinium core as well as weaker density that we could assign to the fluorescein heterocycle (SI Appendix, Fig. S3B). Structural comparison with the RsSxph:STX complex (10) showed no changes in the core STX binding pose or STX binding pocket residues (RMSD$_{ca}$ = 0.279 Å) (SI Appendix, Fig. S3G). Together, these data demonstrate that both F-STX and STX bind to Sxph in the same manner and indicate that there are no substantial interactions with the fluorescein label.

FP measurement of the RsSxph alanine scan mutants uncovered binding affinity changes spanning a $\sim$ 13,000-fold range that correspond to free energy perturbations ($\Delta \Delta G$) of up to $\sim$ 5.60 kcal·mol$^{-1}$ (14). In almost all cases, diverse, encompassing enhanced affinity changes (Y558A $K_d$ = 1.2 nM $\pm$ 0.3) and large disruptions (E540A $K_d$ = 15.3 μM $\pm$ 4.1). As indicated by the TF data, each STX binding pocket residue contributes differently to STX recognition energetics. Comparison of the TF $\Delta T_m$ and FP $\Delta \Delta G$ values shows a strong correlation between the two measurements (Fig. 1A). This concordance between $\Delta T_m$ and $\Delta \Delta G$ indicates that the changes in unfolding free energies caused by protein mutation and changes in STX binding affinity do not incur large heat capacity or entropy changes relative to the wild-type protein (33, 34). Hence, $\Delta T_m$ values provide an accurate estimate of the STX binding affinity differences.

To investigate the STX affinity changes further, we used ITC (Fig. 1F and SI Appendix, Table S2), a label-free method that reports directly on ligand association energetics (35), to examine the interaction of STX with RsSxph and six mutants having varied effects on binding (E540D, Y558I, Y558A, F561A, P727A, and D794E) (Figs. 1F and 2A and SI Appendix, Fig. S5 A–C and Table S2). Experiments with RsSxph confirm the 1:1 stoichiometry and high affinity of the RsSxph:STX interaction ($K_d$ $\sim$ nanomolar) reported previously (8, 10, 14) and reveal a large, favorable binding enthalpy ($\Delta H$ $\sim$ -16.1 ± 0.2 kcal·mol$^{-1}$) in line with previous radioligand binding studies (14). In almost all mutants, binding affinity loss correlated with a reduction in enthalpy, consistent with a loss of interactions (SI Appendix, Table S2). The one exception to this trend is E540D for which STX association yielded a binding enthalpy ($\Delta H$ $\sim$ -16.3 ± 1.7 kcal·mol$^{-1}$) very similar to wild-type RsSxph that was offset by an approximately twofold unfavorable change in binding entropy. The ITC measurements were unable to measure the affinity enhancement for Y558A and Y558I accurately due to the fact that these mutants, as well as RsSxph, have $K_d$s at the detection limit of direct titration methods ($\sim$ 1 nM) (35). Nevertheless,
Fig. 1. Alanine scan of RsSxph binding. (A and B) Exemplar TF assay results for (A) RsSxph in the presence of the indicated concentrations of STX (Left) and TTX (Right) and (B) select RsSxph mutants in the presence of STX. STX and TTX concentrations are 0 nM (black), 19.5 nM (blue), 625 nM (cyan), 5,000 nM (orange), and 20,000 nM (red). Gray dashed lines indicate ΔTm. (C) F-STX diagram. STX and fluorescein (F) moieties are highlighted in blue and yellow, respectively. (D) Exemplar FP binding curves and Kds for RsSxph and the indicated mutants. (E) Comparison of RsSxph mutant ΔTm and ΔΔG values (line y = 3.49 – 0.7523x, R² = 0.886). (F) Exemplar isotherms for titration of 100 μM STX into 10 μM RsSxph, 100 μM STX into 10 μM RsSxph F561A, 100 μM STX into 10 μM RsSxph E540D, and 300 μM STX into 30 μM RsSxph D794E. Kd and ΔH values are indicated. (G) Comparison of ΔGITC for STX and ΔGFP for F-STX for RsSxph and mutants. Purple box highlights region of good correlation. Orange box indicates region outside of the ITC dynamic range. (line shows x = y). Colors in B, D, and E correspond to classifications in Table 1.
ΔG_{ITC} from mutants having STX K_{D} within the ITC dynamic range (K_{D} \sim 30 to 300 nM) showed an excellent agreement with ΔG_{FP} measurements made with F-STX (Fig. 1G). These data further validate the TP and FP assay trends and support the conclusion that RsSxph:F-STX binding interactions are very similar to the RsSxph:STX interactions. Together, these three assays (Fig. 1E and G) provide a robust and versatile suite of options for characterizing STX:Sxph interactions.

**Sxph STX binding code is focused on two sets of “hot spot” residues.** To understand the structural code underlying STX binding, we classified the effects of the alanine mutations into six groups based on ΔΔG values (Fig. 2A and Table 1) and mapped these onto the RsSxph structure (Fig. 2B). This analysis identified a binding “hot spot” comprising three residues that directly contact the STX bis-guanidinium core (Glu540, Phe784, and Asp794) (10) and an additional site near the carbamate (Pro727) where alanine mutations caused substantial STX binding losses (ΔΔG ≥ 1 kcal-mol^{-1}). Conversely, we also identified a site (Tyr558) where alanine caused a notable enhancement of STX binding (ΔΔG ≤ -1 kcal-mol^{-1}) (Fig. 2B and Table 1).

To examine the physicochemical nature of key residues critical for STX binding further, we made mutations at select positions guided by the alanine scan. Mutations at Glu540 and Asp794 (10), residues involved in charge pair interactions with the STX guanidinium rings, that neutralized the side chain while preserving shape and volume (Fig. 2A and Table 1) disrupted binding strongly, similar to their alanine counterparts (ΔΔG = 4.30 and 3.60 kcal-mol^{-1} for E540Q and D794N, respectively) (Fig. 2A, SI Appendix; Fig. S4, and Table 1). Altering side-chain length while preserving the negative charge at these sites also greatly diminished STX affinity but was notably less problematic at Glu540 (ΔΔG = 1.54 and 2.94 kcal-mol^{-1} for E540D and D794E, respectively). To probe contacts with Phe784, which makes a cation-π interaction (17) with the STX five-membered guanidinium ring (10), we tested changes that preserved this interface (F784Y), maintained side-chain volume and hydrophobicity (F784L), and that mimicked substitutions (F784C and F784S) found in the analogous residue in STX-resistant NaVs (NaV1.5, NaV1.8, and NaV1.9) (10, 17, 19–22) (Fig. 2A and Table 1). Preserving the cation-π interaction with F784Y caused a modest binding reduction (ΔΔG = 0.37 kcal-mol^{-1}), whereas F784L was disruptive (ΔΔG = 1.11 kcal-mol^{-1}) and F784C and F784S were even more destabilizing than F784A (ΔΔG = 3.15, 3.60, and 2.71 kcal-mol^{-1}, respectively).

We also examined two other positions that form part of the Sxph binding pocket near the five-membered STX guanidinium ring. Asp785 undergoes the most dramatic conformational change of any residue associated with STX binding, moving from an external-facing conformation to one that engages this STX element (10). Surprisingly, D785A and D785N mutations caused only relatively modest binding changes (Fig. 2A and Table 1) (ΔΔG = 0.57 and -0.30 kcal-mol^{-1} for D785A and D785N, respectively). Because of the proximity of the second-shell residue Glu787 to Asp785 and Asp794 (Fig. 2B), two residues that coordinate the five-membered STX guanidinium ring (10), we also asked whether adding additional negative charge to this part of the STX binding pocket would enhance toxin binding affinity. However, Q787E had essentially no effect on binding (ΔΔG = -0.09 kcal-mol^{-1}).

Two residues, Tyr558 and Ile782, stood out as sites where alanine substitutions enhanced STX affinity (Fig. 2A and B and Table 1). Tyr558 interacts with both the STX five-membered guanidinium ring and carbamate and moves away from the STX binding pocket upon toxin binding (10), whereas Ile782 is a...
second-shell site that buttresses Tyr558. Hence, we hypothesized that an affinity enhancement observed in the Tyr558 and Ile782 mutants resulted from the reduction of Tyr558–STX clashes. In accord with this idea, Y558F had little effect on STX binding ($\Delta G = -0.10 \text{ kcal-mol}^{-1}$), whereas shortening the side chain but preserving its hydrophobic character, Y558I, enhanced binding as much as Y558A ($\Delta G = -1.07$ and $-1.00 \text{ kcal-mol}^{-1}$, respectively). Conversely, increasing the side-chain volume at the buttressing position, I782F, a change expected to make it more difficult for Tyr558 to move out of the binding pocket, reduced STX binding affinity ($\Delta G = 0.46 \text{ kcal-mol}^{-1}$). Combining the two affinity enhancing mutants, Y558A/I782A, yielded only a marginal increase in affinity in comparison to Y558A ($\Delta G = -1.07$ and $-1.00 \text{ kcal-mol}^{-1}$, respectively) but was better than I782A alone ($\Delta G = -0.53 \text{ kcal-mol}^{-1}$). This nonadditivity in binding energetics (36) is in line with the physical interaction of the two sites and the direct contacts of Tyr558 with the toxin. Together, these data support the idea that the Tyr558 clash with STX is a key factor affecting STX affinity and suggest that it should be possible to engineer Sxph variants with enhanced binding properties by altering this site.

Taken together, these studies of the energetic map of the Rsxph STX binding pocket highlight the importance of two amino acid triads. One (Glu540, Phe784, and Asp794) engages the STX bis-guanidinium core of the toxin. The second (Tyr558, Phe561, and Pro727) forms the surface surrounding the STX carbamate in both structures has moved into a pocket formed by the mutation at Tyr558 (RMSD = 0.279 Å and 0.327 Å comparing apo- and STX-bound Rsxph-Y558A and Rsxph-Y558I, respectively) (Figs. 3A and 3B and Movies S1 and S2). In both, the largest conformational change is the rotation of Asp785 into the binding pocket upon ligand binding (RMSD = 0.209 Å and 0.308 Å comparing apo- and STX-bound Rsxph-Y558A and Rsxph-Y558I, respectively) (Fig. 3C). This structural change involves a repositioning of the carbamate carbon by 2 Å in the Rsxph-Y558I:STX complex relative to the Rsxph:STX complex. These findings are in line with the nearly equivalent toxin-binding affinities of Y558A and Y558I, as well as with the

### Table 1. Rsxph STX binding pocket mutant binding parameters

| Class                  | Construct              | $K_d$, nM | $\Delta G$, kcal-mol$^{-1}$ | $n$ |
|------------------------|------------------------|-----------|-----------------------------|-----|
| Enhanced binding       | I782A/Y558A            | 1.2 ± 0.2 | −1.07                       | 6   |
| $\Delta G < -1 \text{ kcal-mol}^{-1}$ | Y558I                 | 1.2 ± 0.2 | −1.07                       | 6   |
|                        | Y558A                  | 1.4 ± 0.3 | −1.00                       | 6   |
| Mild enhancement       | I782A                  | 3.0 ± 0.8 | −0.53                       | 4   |
| $-1 \leq \Delta G \leq 0 \text{ kcal-mol}^{-1}$ | D785N                 | 4.4 ± 0.6 | −0.30                       | 4   |
|                        | K789A                  | 5.1 ± 1.7 | −0.22                       | 4   |
|                        | T563A                  | 5.3 ± 0.5 | −0.20                       | 6   |
|                        | Y558F                  | 6.3 ± 2.3 | −0.10                       | 4   |
|                        | Q787E                  | 6.4 ± 1.6 | −0.09                       | 4   |
|                        | Rsxph                  | 7.4 ± 2.6 | 0                           | 10  |
| Mild disruption        | Y795A                  | 8.4 ± 2.1 | 0.08                        | 4   |
| $0 \geq \Delta G \geq 1 \text{ kcal-mol}^{-1}$ | Q787A                 | 11.3 ± 1.1| 0.25                        | 4   |
|                        | F784Y                  | 13.8 ± 1.0| 0.37                        | 4   |
|                        | I782F                  | 16.1 ± 4.1| 0.46                        | 4   |
|                        | F561A                  | 16.8 ± 6.0| 0.48                        | 4   |
|                        | D785A                  | 19.5 ± 2.5| 0.57                        | 4   |
| Disruption             | F784L                  | 48.0 ± 6.8| 1.11                        | 4   |
| $1 \geq \Delta G \geq 2 \text{ kcal-mol}^{-1}$ | P727A                 | 56.9 ± 12.1| 1.21                       | 4   |
|                        | E540D                  | 99.9 ± 25.1| 1.54                       | 4   |
| Strong disruption      | F784A                  | 725.1 ± 108.7| 2.71                      | 4   |
| $2 \geq \Delta G \geq 3 \text{ kcal-mol}^{-1}$ | D794E                 | 1,074.1 ± 69.3| 2.94                      | 4   |
| Very strong disruption | F784C                  | 1,510.5 ± 346.1| 3.15                      | 4   |
| $\Delta G \geq 3$      | D794N                  | 3,228 ± 397  | 3.60                      | 4   |
|                        | F784S                  | 3,240 ± 508  | 3.60                      | 4   |
|                        | E540Q                  | 10,640 ± 1,325 | 4.30                    | 4   |
|                        | D794A                  | 13,172 ± 6,871 | 4.43                     | 4   |
|                        | E540A                  | 15,294 ± 4,134 | 4.52                     | 4   |

$n$, number of observations. $\Delta G = RT \ln (K_d, \text{mutant}/K_d, \text{wild})$, $T = 298 \text{ K}$. Errors for measurements are SD.

Structures of enhanced-affinity Rsxph mutants. To investigate the structural underpinnings of the affinity enhancement caused by mutations at the Tyr558 site, we determined crystal structures of Rsxph-Y558A and Rsxph-Y558I (2.60 Å and 2.70 Å resolution, respectively) and as cocrystallized STX complexes (2.60 Å and 2.15 Å, respectively) (SI Appendix, Fig. S6 A–D and Table S1). Comparison of the apo- and STX-bound structures reveals little movement in the STX binding pocket upon ligand binding (RMSD = 0.209 Å and 0.308 Å comparing apo- and STX-bound Rsxph-Y558A and Rsxph-Y558I, respectively) (Fig. 3A and B and Movies S1 and S2). In both, the largest conformational change is the rotation of Asp785 into the binding pocket to interact with the five-membered guanidinium ring of STX, as seen for Rsxph (Fig. 3C) (10). By contrast, unlike in Rsxph, there is minimal movement of residue 558 and its supporting loop, indicating that both Y558A and Y558I eliminate the clash incurred by the Tyr558 side chain. Comparison with the Rsxph:STX complex also shows that the STX carbamate in both structures has moved into a pocket formed by the mutation at Tyr558 (RMSD = 0.279 Å and 0.327 Å comparing Rsxph:STX with Rsxph-Y558A:STX and Rsxph-Y558I:STX, respectively) (Fig. 3C). This structural change involves a repositioning of the carbamate carbon by 2 Å in the Rsxph-Y558I:STX complex relative to the Rsxph:STX complex. These findings are in line with the nearly equivalent toxin-binding affinities of Y558A and Y558I, as well as with the...
idea that changes at the Tyr558 buttressing residue, Ile782, relieve the steric clash with STX. They also demonstrate that one strategy for increasing STX affinity is to engineer a highly organized binding pocket that requires minimal conformational changes to bind STX.

**Sxph STX binding-affinity changes alter NaV rescue from STX block.** RcSxph acts as “a toxin sponge” that can reverse STX inhibition of NaV5.2 (9). To test the extent to which this property is linked to the intrinsic affinity of RcSxph for STX, we evaluated how STX-affinity-altering mutations affected RcSxph rescue of channels blocked by STX. As shown previously, titration of different RcSxph:STX ratios against Phylllobates terribilis NaV1.4 (PnNaV1.4), a NaV having a concentration that inhibits response by 50% for STX (IC50) of 12.6 nM (9), completely reverses the effects of STX at ratios of 2:1 RcSxph:STX or greater (Fig. 4 A and F). Incorporation of mutations that affect STX affinity altered the ability of RcSxph to rescue NaV5.2 and followed the binding assay trends. Mutants that increased STX affinity, Y558I and I782A, improved the ability of RcSxph to rescue PnNaV1.4 (Effective Rescue Ratio50 [ERR50] = 0.81 ± 0.01, 0.87 ± 0.02, and 1.07 ± 0.02 for Y558I, I782A, and RcSxph, respectively), whereas mutations that compromised STX binding reduced (P727A, ERR50 >4) or eliminated (E540A) the ability of RcSxph to reverse the STX inhibition (Fig. 4 B–F). This strong correlation indicates that the “toxin sponge” property of Sxph (9) depends on the capacity of Sxph to sequester STX and adds further support to the idea that Sxph has a role in toxin resistance mechanisms (8, 9).

**Expansion of the Sxph family.** STX binding activity has been reported in the plasma, hemolymph, and tissues of diverse arthropods, amphibians, fish, and reptiles (11, 13), suggesting that many organisms harbor Sxph-like proteins. Besides RcSxph, similar Sxphs have been identified in only two other frogs, the High Himalaya frog N. parkeri (10) and the little devil poison frog *O. sylvatica* (24). As a number of poison frogs exhibit resistance to STX poisoning (9), we asked whether the STX binding site “recognition code” could enable identification of Sxph homologs in other amphibians. To this end, we determined the sequences of 10 new Sxphs (Fig. 5 A and B and SI Appendix, Figs. S7 and S8). These include six Sxphs in two poison dart frog families (family Dendrobatidae: dyeing poison dart frog, *Dendrobates tinctorius*, little devil poison frog, *O. sylvatica*; mimic poison frog, *R. imitator*; golden dart frog, *P. terribilis*; phantasmas poison frog, *Epipedobates tricolor*; and brilliant-thighed poison frog, *Allobates femoralis* and family Mantellidae: golden mantella, *M. aurantiaca*) and three Sxphs in toads (Caucasion toad, *Bufo bufo*; Asiatic toad, *Bufo gargarizans*; and South American cane toad, *Rhinella marina*). The identification of the OStxh sequence confirms its prior identification by mass spectrometry (24) and the discovery of RmSxph agrees with prior reports of Sxph-like STX binding activity in the cane toad (*R. marina*) (13, 23).

Sequence comparisons ([SI Appendix, Figs. S7 and S8](https://doi.org/10.1073/pnas.2210114119)) show that all of the new Sxphs share the transferrin fold found in RcSxph comprising N and C lobes each having two subdomains (N1, N2 and C1, C2, respectively) (10, 12) and the signature “EFD” motif (10) or a close variant in the core of the C-lobe STX binding site (Fig. 5A). Similar to RcSxph, the new Sxphs also have amino acid differences relative to transferrin that should eliminate Fe3+ binding (10, 12, 37), as well as a number of protease inhibitor thryoglobulin domains (Thy1) inserted between the N1 and N2 N-lobe subdomains (10, 38) (Fig. 5A and [SI Appendix, Figs. S7–S9](https://doi.org/10.1073/pnas.2210114119)). These Thy1 domain insertions range from two in RcSxph, *NpSxph*, and *MaSxph* to three in the denderobatid poison frog and cane toad Sxphs to 16 and 15 in toad *BbSxph* and *BgSxph*, respectively (Fig. 5A and [SI Appendix, Figs. S7–S9](https://doi.org/10.1073/pnas.2210114119)).

We used the STX recognition code defined by our studies as a template for investigating cross-species variation in the residues that contribute to STX binding (Fig. 5B). This analysis shows a conservation of residues that interact with the STX bis-guanidinium core (Glu540, Phe784, Asp785, Asp794, and Tyr795) and carbamate (Phe561). Surprisingly, five of the Sxphs (*D. tinctorius*, *R. imitator*, *A. femorialis*, *B. bufo*, and *B. gargarizans*) have an aspartate instead of a glutamate at the Glu540 position in RcSxph that contributes the most binding energy (Fig. 2A). The equivalent change in RcSxph, E540D, reduced STX affinity by ~100-fold (Table 1) and uniquely alters enthalpy and entropy binding parameters compared to other affinity-lowering mutations ([SI Appendix, Table S2](https://doi.org/10.1073/pnas.2210114119)). Additionally, we identified variations at two sites for which mutations increase RcSxph STX binding, Tyr558 and Ile782 (Figs. 2A and 4C and E and Table 1). *NpSxph* and *MaSxph* have an Ile at the Tyr558 site, whereas eight of the new Sxphs have hydrophobic substitutions at the Ile782 position (Fig. 5B). The striking conservation of the Sxph scaffold and STX binding site indicate that this class of “toxin sponge” proteins is widespread among diverse anurans, while the amino acid variations in key positions (Glu540, Tyr558, and Ile782) raise the possibility that the different Sxph homologs have varied STX binding, Tyr558 and Ile782 properties. To explore the STX binding properties of this new set of Sxphs and to begin to understand whether changes in the binding site composition affect toxin affinity, we expressed and purified four representative variants. These included two Sxphs having STX binding-site sequences similar to RcSxph (*NpSxph* and *MaSxph*) and two Sxphs bearing more diverse amino acid differences (*RbSxph* and *OStxh*), including one displaying the E540D substitution (*RbSxph*). This set also represents Sxphs
Fig. 4. Rsxph mutants have differential effects on PtNaV1.4 rescue from STX block. (A–E) Exemplar two-electrode voltage-clamp recordings of PtNaV1.4 expressed in Xenopus oocytes in the presence of 100 nM STX and indicated [Sxph]:[STX] ratios for (A) Rsxph, (B) Rsxph E540A, (C) Rsxph Y558I, (D) Rsxph P727A, and (E) Rsxph I782A. (Inset) The stimulation protocol. (F) [Sxph]:[STX] dose-response curves for Rsxph (black open circles), Rsxph E540A (purple inverted triangles), Rsxph Y558I (orange triangles), Rsxph P727A (gold diamonds), and Rsxph I782A (blue squares) in the presence of 100 nM STX. Lines show fit to the Hill equation.
having either two Thy1 domains similar to \( R_{c} \)Sxph (\( N_{p} \)Sxph and \( M_{a} \)Sxph) or three Thy1 domains (\( O_{s} \)Sxph and \( R_{i} \)Sxph) (Fig. 5A). TF experiments showed STX-dependent \( \Delta T_{m} \) for all four Sxphs. By contrast, equivalent concentrations of TTX had no effect (Fig. 5C), indicating that, similar to \( R_{c} \)Sxph (Fig. 1A) (8, 9), all four Sxphs bind STX but not TTX. Unlike the other Sxphs, the \( R_{i} \)Sxph melting curve showed two thermal transitions; however, only the first transition was sensitive to STX concentration (Fig. 5C). FP binding assays showed that all four Sxphs bound F-STX and revealed affinities stronger than \( R_{c} \)Sxph (Fig. 5D and Table 2). The enhanced affinity of \( N_{p} \)Sxph and \( M_{a} \)Sxph for STX relative to \( R_{i} \)Sxph is consistent with the presence of the Y558I variant (Fig. 5B). Importantly, the observation that \( R_{i} \)Sxph has a higher affinity for STX than \( R_{c} \)Sxph despite the presence of the E540D difference suggests that the other sequence variations in the \( R_{i} \)Sxph STX binding pocket compensate for this Glu→Asp change at Glu540.

**Fig. 5.** Sxph family member properties. (A) Comparison of the human transferrin (TF) Fe\(^{3+}\) ligand positions (UniProtKB: P02787), \( R_{c} \)Sxph STX binding motif residues (10), and number of Thy1 domains for Sxphs from \( R. \)castesbeiana (PDB ID: 6O0D) (10), \( N. \)parkeri (NCBI: XP_018410833.1) (10), \( M. \)aurantiaca, \( D. \)tinctiorius, \( O. \)sylvatica, \( R. \)imitator, \( P. \)terribilis, \( E. \)tricolor, \( A. \)femoralis, \( R. \)marina, \( B. \)bufo (NCBI:XM_040427746.1), and \( B. \)garagarizans (NCBI:XP_044148290.1). TF Fe\(^{3+}\) (orange) and carbonate (blue) ligands are indicated. Blue highlights indicate residue conservation. (B) Comparison of STX binding pocket for the indicated Sxphs. Numbers denote \( R_{c} \)Sxph positions. Colors indicate the alanine scan classes as in Fig. 3B. Conserved residues are highlighted. Asterix indicates second-shell sites. (C) Exemplar TF curves for \( N_{p} \)Sxph, \( R_{i} \)Sxph, \( O_{s} \)Sxph, and \( M_{a} \)Sxph in the presence of the indicated concentrations of STX (purple box) or TTX (green box). \( \Delta T_{m} \) values are indicated. (D) Exemplar FP binding curves and \( K_{d} \)s for \( N_{p} \)Sxph (green), \( R_{i} \)Sxph (blue), \( O_{s} \)Sxph (orange), and \( M_{a} \)Sxph (purple). (E) Exemplar \( N_{p} \)Sxph I559Y TF curves in the presence of the indicated concentrations of STX (purple box) or TTX (green box) and FP binding (green). \( \Delta T_{m} \) and \( K_{d} \) values are indicated. Error bars are SEM.
Because NpSxph has a higher affinity for STX than RcSxph (Figs. 1D and 5D and Table 2) and has an isoelectric point at the Tyr558 site (Fig. 5B), we asked whether the NpSxph I559Y mutant that converts the NpSxph binding site to match RcSxph would lower STX affinity. TF measurements showed that NpSxph I559Y had a ~1°C smaller ΔTm than NpSxph (ΔTm = 3.2°C ± 0.3 versus 2.5°C ± 0.2 for NpSxph and NpSxph I559Y, respectively), indicative of a decreased binding affinity (Fig. 5 C and E). This result was validated by FP (ΔΔG = −1.56 kcal mol⁻¹), yielding a result of similar magnitude to the RcSxph Y558I differences (Fig. 5E and Tables 1 and 2). ITC confirmed the high affinity of the interaction (SI Appendix, Fig. S5 D, E and F), but could not yield an explicit Ki given its low nanomolar value (SI Appendix, Fig. S5F).

Nevertheless, these experiments validate the 1:1 stoichiometry of the NpSxph:STX interaction (SI Appendix, Table S2) and show that the I559Y change reduced the binding enthalpy, consistent with perturbation of NpSxph:STX interactions (ΔH = −18.7 ± 0.2 vs. −16.8 ± 0.2 kcal mol⁻¹, NpSxph and NpSxph I559Y, respectively) (SI Appendix, Table S2). Taken together, these experiments establish the conserved nature of the STX binding pocket among diverse Sxph homologs and show that the STX recognition code derived from RcSxph studies (Fig. 5B) can identify key changes that influence toxin binding.

**Structures of apo- and STX-bound NpSxph reveal a preorganized STX binding site.** To compare STX binding modes among Sxph family members, we crystallized and determined the structure of NpSxph, alone and cocystalized with STX. NpSxph and STX:NpSxph crystals diffracted X-rays to resolutions of 2.2 Å and 2.0 Å, respectively, and were solved by molecular replacement (Fig. 6A and SI Appendix, Fig. S10 A and B). As expected from the similarity to RcSxph, NpSxph is built on a transferrin fold (Fig. 6A) and has the same 21 disulfides found in RcSxph, as well as an additional 22nd disulfide in the Type 1A thyroglobulin domain of NpSxph Thy1-2. However, structural comparison of NpSxph and RcSxph reveals a number of unexpected large-scale domain rearrangements.

The NpSxph N lobe is displaced along the plane of the molecule by ~30° and rotated around the central axis by a similar amount (SI Appendix, Fig. S10C). NpSxph N lobe and C lobe lack Fe³⁺ binding sites (Fig. 5A), and despite the N-lobe displacement relative to RcSxph adopt closed and open conformations, respectively, as in RcSxph (10) (SI Appendix, Fig. S10 D and E) (RMSDCα = 1.160 Å and 1.373 Å for NpSxph and RcSxph N and C lobes, respectively). Surprisingly, the two NpSxph Thy1 domains are in different positions than in RcSxph and appear to move as a unit by ~90° with respect to the central transferrin scaffold (SI Appendix, Fig. S10F and Movie 5) and a translation of ~30 Å of Thy1-2 (SI Appendix, Fig. S10G). Thy1-1 is displaced from a site over the N lobe in RSCxph to one in which it interacts with the NpSxph C-lobe C2 subdomain and Thy1-2 moves from between the N and C lobes in RcSxph where it interacts with the C1 subdomain, to a position in NpSxph where it interacts with both N-lobe subdomains. Consequently, the interaction between the C-lobe β-strand β7C1 and Thy1-2 β5 observed in RcSxph is absent in NpSxph. Despite these domain-scale differences, Thy1-1 and Thy1-2 are structurally similar to each other (RMSDCα = 1.056 Å) and to their RcSxph counterparts (SI Appendix, Fig. S10H) (RMSDCα = 1.107 Å and 0.837, respectively). Further, none of these large-scale changes impact the STX binding site, which is found on the C1 domain as in RcSxph (Fig. 5A).

Comparison of the apo- and STX-bound NpSxph structures shows that there are essentially no STX binding site conformational changes upon STX engagement, apart from the movement of Asp786 to interact with the STX five-membered guanidinium ring (Fig. 6B and Movie S4). This conformational change is shared with RcSxph (10) and appears to be a common element of Sxph binding to STX. The movements of Tyr558 and its loop away from the STX binding site observed in RcSxph (10) are largely absent in NpSxph for the Tyr558 equivalent position, Ile559, and its supporting loop. Hence, the NpSxph STX binding site is better-organized to accommodate STX (Fig. 6B), similar to RcSxph Y558I (Fig. 3B). We also noted an electron density in the apo-NpSxph STX binding site that we assigned as a PEG-400 molecule from the crystallization solution (SI Appendix, Fig. S10A). This density occupies a site different from STX and is not present in the STX-bound complex (SI Appendix, Fig. S10B). Its presence suggests that other molecules may be able to bind the STX binding pocket. Similar to RcSxph, the NpSxph STX binding site is very electronegative (SI Appendix, Fig. S11), consistent with the strong conservation of the E540 and D794 positions (Fig. 5 A and B) that are the most energetically important and coordinate the positively charged STX bis-guanidinium core (Fig. 2 A and Table 1).

We also determined the structure of an NpSxph:F-STX complex at 2.2-Å resolution (SI Appendix, Table S1). This structure shows no density for the fluorescein moiety and has an identical STX pose to the NpSxph:STX complex (SI Appendix, Fig. S12). These data provide further evidence that fluorescein does not interact with Sxph (cf. SI Appendix, Fig. S3) even though it is tethered to the STX binding pocket and support the idea that the FP assay faithfully reports on Sxph:STX interactions. Comparison of the NpSxph and RcSxph STX poses shows essentially identical interactions with the tricyclic bis-guanidinium core and reveals that the carbamate is able to occupy the pocket opened by the Y1→I variant (Fig. 6C), as observed in RcSxph Y558I (Fig. 3B). This change, together with the more rigid nature of the NpSxph STX binding pocket, likely contributes to the higher affinity of NpSxph for STX relative to RcSxph (Table 1). Taken together, the various structures of Sxph:STX complexes show how subtle changes, particularly at the Tyr558 position, can influence STX binding and underscore that knowledge of the STX binding code can be used to tune the STX binding properties of different Sxphs.

**Discussion**

Our biochemical and structural characterization of a set of RcSxph mutants and Sxphs from diverse anurans reveals a conserved STX recognition code centered around six amino acid residues comprising two triads. One triad engages the STX...
bis-guanidinium core using carboxylate groups that coordinate each ring ($R_e$Sxph Glu540 and Asp794) and an aromatic residue that makes a cation–π interaction ($R_e$Sxph Phe784) with the STX concave face. This recognition motif is shared with NaVs, the primary target of STX in PSP (18, 39, 40) (Fig. 2C and D), and showcases a remarkably convergent STX recognition strategy for the tricyclic, bis-guandinium STX core. The second amino acid triad ($R_e$Sxph residues Tyr558, Phe561, and Pro727) largely interacts with the carbamate moiety and contains a site, Tyr558, and its supporting residue, Ile782, where amino acid changes, including those found in some anuran Sxphs (Fig. 5), enhance STX binding. Structural studies of $R_e$Sxph mutants and the High Himalaya frog $N_p$Sxph show that STX-affinity-enhancing substitutions in this area of the binding pocket act by reducing the degree of conformational change associated with STX binding (Figs. 3 and 6C and D). These findings reveal one strategy for creating high-affinity STX binding sites. Importantly, enhancing the affinity of Sxph for STX through changes at either residue increases the capacity of $R_e$Sxph to rescue $N_a$Sx from STX block (Fig. 4), demonstrating that an understanding of the STX recognition code enables rational modification of Sxph binding properties. Thus, exploiting the information in the STX recognition code defined here should enable design of Sxphs as STX sensors or agents for treating STX poisoning.

Although STX binding activity has been reported in a variety of diverse invertebrates (13) and vertebrates (13, 23), only two types of STX binding proteins have been identified and validated, frog Sxphs (8, 9) and the pufferfish STX and TTX binding proteins (41, 42). Our discovery of a set of 10 new Sxphs that bind STX with high affinity (Fig. 5 and SI Appendix, Figs. S7 and S8) that share a conserved STX binding site represents a substantial expansion of the Sxph family and reveals natural variation in the residues that are important for STX binding (Fig. 5B). Most notably, E540D, a change that reduces STX binding in $R_e$Sxph by $\sim 14$-fold, occurs in five of the newly identified Sxphs. Nevertheless, functional studies show that $R_e$Sxph, which bears an Asp at this site, binds STX more strongly than $R_e$Sxph (Table 2). Hence, the natural variations at other STX binding pocket residues must provide compensatory interactions to maintain a high STX binding affinity. Understanding how such variations impact STX engagement or influence the capacity of these proteins to discriminate among STX congeners (13) remain important unanswered questions. The striking abundance of Sxphs in diverse amphibians, representing lineages separated by $\sim 140$ My (25) and that are not known to carry STX, raises intriguing questions...
regarding the selective pressures that have caused these disparate amphibians to maintain this STX binding protein and its capacity to sequester this lethal toxin.

Besides the conserved STX binding site, all of the amphibian Sxphs possess a set of Thy1 domains similar to those in Rsxph that have been shown to act as protease inhibitors (38). Comparison of anuran Sxphs shows that these domains are a common feature of the Sxph family and occur in strikingly varied numbers, comprising two or three in most Sxphs but having a remarkable expansion to 15 to 16 in some toad Sxphs (Fig. S5 A and SI Appendix, Fig. S7–S9). Structural comparisons between Rsxph and NpSxph, representing the class that has two Thy1 domains (Fig. 5 A), show that these domains can adopt different positions with respect to the shared, modified transferrin core (SI Appendix, Fig. S10 F). Whether the Sxph Thy1 domains and their varied numbers are important for Sxph-mediated toxin resistance mechanisms (8, 9) or serve some other function remains unknown. Our de

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Materials and Methods

R. catesbeiana Sxph (Rsxph), N. parkeri Sxph (NpSxph), M. australica Sxph (MaSxph), O. sytycica Sxph (OsSxph), and R. imitator Sxph (Risxph), and mutants were expressed and purified using a previously described Rsxph baculovirus expression system (10). TF assays for STX and TTX binding were developed as outlined in ref. 27. FP assays were performed as described (50). Structure determination was done following previously described methods for Rsxph (10). Two-electrode voltage clamp experiments evaluating Rsxph rescue of NaVs from STX block were done as previously described (9). Details on cloning, expression, purification, binding assays, electrophysiology, structure determination, and F-STX synthesis can be found in SI Appendix.

Data, Materials, and Software Availability. Sequences of ApxSxph (OP265195), DxSxph (OP265194), EtSxph (OP265196), MaSxph (OP265197), OsSxph (OP311630), PsSxph (OP267560), RiSxph (OP265193), and RmSxph (OP267561) have been deposited and are available from the National Center for Biotechnology Information GenBank. Coordinates and structure factors and for Rsxph Y558A (BD65) (51), Rsxph Y558A-STX (BD65) (52), Rsxph Y558I (BD60) (53), Rsxph Y558I-STX (BD65) (54), Rsxph-F-STX (BD60) (55), NpSxph (BD66) (56), NpSxph:F-STX (BD65) (57), and NpSxph:F-STX (BD60) (58) have been deposited in the RCSB Protein Data Bank.

Acknowledgments. We thank Z. Wong and T.-J. Yen for technical help and K. Brej and J. Gross for comments on the manuscript. This work was supported by grants Department of Defense (DoD) HDTRA-1-19-1-0040 and HDTRA-1-21-1-10011 and University of California, San Francisco Program for Breakthrough Biomedical Research, which is partially funded by the Sandler Foundation, to D.L.M., NSF-1822025 to L.A.O, NIH-NIGMS R01-GM117263-05 to J.D.B., an American Heart Association postdoctoral fellowship to F.A.A., an NSF GRFP (DGE-1656518) and HHMI Gilliam Fellowship (GT13330) to A.A.-B., and a DoD National Defense Science and Engineering Graduate (NDSEG) Fellowship to H.S.H. H.S.H. is a Center for Molecular Analysis and Design Fellow at Stanford University.

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