X-ray structure of acid-sensing ion channel 1–snake toxin complex reveals open state of a Na^+-selective channel

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Supplemental Information Inventory

Figure S1. Heteromeric snake toxin, MitTx, activates Δ13, Related to Figure 1.

(A) Representative current traces of Δ13 upon application of low pH, and 600 nM α-MitTx and 300 nM β-MitTx at pH 7.4. (B) Activation of the Δ13 construct without (black trace) or with MitTx (red). These records are derived from the same cell which was held at pH 7.4 and then stepped in a perfusion solution at pH 5.5 (black trace). The cell was then returned to pH 7.4 in the presence of 300 nM MitTx and then into a perfusion solution at pH 5.5 which also contained MitTx at 300 nM. Fitting the records from 90% to 10% of the current amplitude to single exponentials yield time constants for decay in the absence of MitTx (pH 5.5) of 279 ms ± 21 ms (correlation of 0.987) and in the presence of MitTx (pH 5.5 + MitTx) of 466 ms ± 54 ms (correlation of 0.995), thus showing that in the presence of MitTx the rate of current decay slows. The presence of MitTx increases the extent of approximate 'steady state' current. Analysis of the ratios of the amplitude of current after 1 s of pH 5.5 application to peak current in the absence (black) and the presence (red) of MitTx shows that at 1 s, the current amplitude is ~11% (± 4%) of peak amplitude in the presence of MitTx whereas it is only ~1.3% (±1.0%) in the absence of MitTx. These statistics were derived from 5 independent cells or, in the case of the recordings, the records are representative examples from 5 independent cells.

Figure S2. Conserved sites of action in MitTx, Related to Figure 2.

(A) Interface between the α and β subunits, showing the multitude of hydrogen bonds and also the amino terminal PCA 1 residue. The first five residues of the α subunit form a 'clamp' around the aromatic side chain of Tyr 72 of the β subunit's β-wing, with the N-terminal pyroglutamate of the α subunit forming two hydrogen bonds with the backbone

amino group of Ala 74 and the carbonyl oxygen of Tyr 72 of the β subunit. Arg 3 of α augments subunit interactions, with its guanidinium group forming hydrogen bonds with carbonyl oxygens of Lys 10 and Cys 11 at the C-terminus of the α1 helix of the β subunit, with carbonyl oxygens of Cys 58 and His 59, and also with the hydroxyl group of Tyr 8 of the α subunit. Cation-π interactions between the guanidinium group of α Arg 3 and Tyr 8 and Phe 26 provide additional contacts within the α-ß interface. (B) A Na^+ ion fortifies intersubunit interactions and is coordinated by the backbone carbonyl oxygens of Ser 29 and Phe 68 of the α and ß subunits, respectively, and by a cation- interaction with Phe 68 of the ß subunit. Dashed lines indicate interaction with distances of 2.6-3.5 Å, unless specified. (C,D) Comparison of MitTx subunits to peptides adopting similar folds reveal conserved sites of action. The structures are shown in cyan for Kunitz proteins (C) or dark blue for PLA2s (D). Interfaces are shown in a separate color (dark green, dark blue, or cyan) depending on the binding partner, and residues in the interface are highlighted by displaying side chains. Interfaces are defined as any residue within 4 Å of the binding partner in structures (PDB codes are 3OTJ for BPTI, and 1BUN for β-bungarotoxin), or based on mutagenesis studies for δ-DTX (Imredy and MacKinnon, 2000), or based on deuterium exchange experiments for *Naja naja* PLA2 (Burke et al., 2008) (PDB code 1A3D). The PLA2 active site equivalents are in red. (E, F) The β subunit exploits its amino terminus and β-wing 'loop' to snugly adhere to the α4 and α5 helices of the thumb domain. Asn 1 and Asn 3, together with Gln 2 of β-MitTx, form a web of hydrogen bonds with the side chains of Asn 321 and Asn 323 on the α4 helix of the thumb. Positively charged residues, Arg 6 (E) and Lys 60 (F) of the β subunit, augment toxin-channel interactions by forming ionic interactions with Glu 320 on the α4 helix and with

Glu 339 and Glu 343 on the α5 helix, respectively. (G) Phe 65 packs directly alongside the α5 helix, bringing the polypeptide of the β subunit sufficiently close to the thumb so as to allow a direct hydrogen bond between the main chain nitrogen of Phe 65 and the carbonyl oxygen of Glu 343, reinforced by additional hydrogen bonds between Asn 321 (Δ13) and Gln 4 (β) and a salt bridge between Glu 320 (Δ13) and Arg 6 (β).

Figure S3. Superposition of the desensitized state and the Δ13-MitTx bound states demonstrate conserved intrasubunit interactions near the narrow ion pathway, Related to Figure 4.

Views of the desensitized state (PDB code: 3HGC, gray) and the MitTx-bound state transmembrane domains (red) as 'slabs' along the 3-fold axis of symmetry (A-D). (A) and (B) show how, within a subunit, TM1 and TM2 interactions remain intact via hydrophobic interactions preserving the conformation that define the narrow pathway. (C) and (D) show changes of interaction between TM1 and TM2 in the extracellular and intracellular regions, respectively. (E) Normalized amiloride concentration-response of Δ13 (IC50 of 15.2 μM) and Δ13_Q437A (IC50 of 14.5 μM) expressed in Chinese hamster ovary (CHO) cells. Perturbation at the 437 site demonstrates preserved conformation of the pore domain based on wild-type like effects of amiloride on the mutant. (F) Representative current traces of Δ13_Q437A expressed in CHO cells upon application of solution at pH 5.5 (left) and MItTx at pH 7.4 (right). MitTx-mediated macroscopic currents of Δ13_Q437A show about a three-fold increase of amplitude compared to parent contruct as shown in Figure S1.

Figure S4. Cation binding sites in the Δ13-MitTx complex reveal key regions in channel modulation and ion pathway, Related to Figure 7.

(A) Cs^+ ion sites in the Δ13-MitTx complex and the corresponding anomalous difference electron density map calculated from diffraction data measured from the Cs^+-soaked crystals. Each Δ13 subunit is in a different color and the MitTx α and β subunits are in cyan and dark blue, respectively. The map is contoured at 3.5 σ. (B) In the extracellular domain, within the central vestibule, we find two Cs^+ sites positioned on the threefold axis and coordinated by main chain carbonyl oxygens, near Leu 375 (13 σ) and near Lys 373 (7 σ), recalling the $Gd3^+$ binding site found within the central vestibule of the apo state of the P2X4 receptor. While the central vestibule provides a favorable site for cation binding, the role of these sites in channel conduction and gating awaits additional experiments. Boxed region in (A), Cs^+ sites (purple spheres) at the 'top' of the central vestibule are coordinated by main chain carbonyl oxygens from Leu 375 and Lys 373 and separated by a water molecule (red sphere). Difference anomalous map is contoured at 4.0 σ. (C) Overall view of Δ13-MitTx structure bound with amiloride molecules. The MitTx molecules are omitted for clarity. The Δ13 molecule is in both surface and ribbon representation. The ribbon representation is color coded like in figure 1 of the main text. Amiloride molecules are in sticks representation. (D) Close-up view of the amiloride binding site near the acidic pocket of Δ13 forming hydrogen bond and cation-π interactions. Dashed lines indicate bond distances of 2.5-3.5 Å unless specified. (E) In anomalous difference electron density maps derived from crystals soaked in Cs^+, there is also a substantial peak adjacent to the aromatic ring of Tyr 68. Here the map is contoured at 3.5 σ, also only immediately around the putative Cs^+ ion. (F) In Δ13-MitTx crystals

soaked in Cs^+ containing solutions, there is also a prominent peak in $2F_o$-F_c electron density maps. This map is contoured at 2.0 σ, only around the putative Cs^+ ion. (G) In the Δ13-MitTx-bound state, there is a prominent peak in a $2F_o$-F_c electron density map derived from 'native' crystals that we suggest is a bound Na^+ ion. Here the map is contoured at 0.9 σ around the putative Na^+ ion. (H) In the desensitized state (PDB code: 3HGC), the guanidinium group of Arg 65 forms a cation-π interaction with the aromatic ring of Tyr 68.

Table S1 Data collection and refinement statistics, Related to Figure 1.

	Δ13-MitTx	**Δ13-MitTx (Amiloride)**	**Δ13-MitTx (Cs^+)**
Data collection			
Beamline	ALS 5.0.2	ALS 5.0.2	ALS 5.0.2
Space group	*R*3	*R*3	*R*3
Cell dimensions			
a, b, c (Å)	151.9, 151.9, 123.8	151.6, 151.6, 123.2	151.5, 151.5, 123.8
α, β, γ (°)	90, 90, 120	90, 90, 120	90, 90, 120
Wavelength (Å)	1.000	1.000	1.378
Resolution (Å)*	28.0-1.97 (2.04-1.97)	29.25- 1.97 (2.04- 1.97)	50.00- 2.65 (2.70- 2.65)
R_{merge}*	0.050 (0.354)	0.060 (0.440)	0.093 (0.654)
$I/\sigma I$*	14.1 (1.6)	14.1 (1.15)	12.41 (2.25)
Completeness (%)*	93.87 (57.7)	95.3 (62.0)	99.8 (97.7)
Redundancy*	3.9 (1.5)	4.4 (1.5)	2.9 (2.8)
Refinement			
Resolution (Å)	28.0-2.07	29.25-2.27	50.0-2.65
No. of unique reflections	70881	71494	31004
R_{work}/ R_{free}	0.206/0.246	0.206/0.233	0.216/0.255
No. atoms			
Total	5011	4924	4739
Macromolecules	4637	4648	4630
Ligands (Na^+, Cs^+, Cl^-, Amiloride, PEG)	51	96	31
water	323	180	88
Average *B*-factors ($Å^2$)			
Total	52.1	58.1	49.4
Macromolecules	52.1	57.9	49.5
Ligands (Na^+, Cs^+, Cl^-, Amiloride, PEG)	70.0	79.7	64.6
water	49.5	53.20	37.5
R.m.s deviations			
Bond lengths (Å)	0.008	0.009	0.009
Bond angles (º)	1.09	1.28	1.05
Ramachandran			
Most favored (%)	96.0	96.0	95.0
Allowed (%)	4.0	4.0	5.0
Disallowed (%)	0	0	0

*Highest resolution shell is shown in parenthesis.

Table S2. Summary of organic cation reversal potentials, Related to Figure 6.

Cation (# of cells)	E_{rev} (mV)
Sodium ion (4)	32.4 ± 2.1
Hydrazine (6)	10.6 ± 1.3
Hydroxylamine (5)	17.9 ± 2.9
Methylamine (6)	impermeant

Extended Experimental Procedures

Refolding and purification of recombinant MitTxα and MiTxβ

Refolding of the MitTx subunits was carried out by dilution of the solubilized inclusion bodies 10-fold into a buffer composed of 50 mM Tris pH 8.5, 5 mM EDTA, 6 M GuCl, and 10 mM β-mercaptoethanol and clarification of the resulting solution by centrifugation at 125,000 x g. The supernatant was dialyzed overnight at 4°C against 20 mM Tris pH8.5, 1 mM EDTA, 200 mM NaCl, and 0.5 M arginine, followed by an additional overnight dialysis against 20 mM Tris pH 8, 200 mM NaCl, 1 mM EDTA and centrifugation at 125,000 x g to remove precipitate. Analysis of the resulting, refolded toxin subunits by fluorescence-detection size-exclusion chromatography (FSEC), using either tryptophan (MitTxβ) or tyrosine fluorescence (MitTxα), together with analysis by SDS-PAGE, demonstrated that the refolded subunits were >95% pure under native and denaturing conditions. Using 1.5 g of washed inclusion bodies, 116 mg and 9.2 mg of soluble fusion protein can be obtained for Trx-MitTxα and Trx-MitTxβ, respectively.

The Trx-tag was removed from both subunits by enterokinase digestion at RT for 2h using ~25 units of enterokinase for each 1 mg of Trx-fusion. Further purification of the MitTxα subunit was achieved by dilution of the digested sample with 20 mM MES pH 6, 200 mM NaCl, centrifugation to remove precipitate, concentration and finally application to a gel filtration column equilibrated in 20 mM MES pH 6, 200 mM NaCl. Monodisperse MitTxα fractions were pooled and treated with glutaminyl cyclase to catalyze N-terminal pyroglutamate formation. Purification of MitTxβ was accomplished by enterokinase digestion as described above, subsequent dialysis against 20 mM MES

pH 6, 10 mM NaCl, and chromatographic separation from the Trx tag and other impurities by cation exchange chromatography.

Crystallization and cryoprotection

Crystallization experiments that yielded crystals diffracting to 2.1 Å and 2.3 Å for the Δ13-MitTx and Δ13-MitTx (amiloride) complexes were in the presence of recombinantly expressed MitTx. Structures determined from both native and recombinant MitTx demonstrated that both samples were identical in structure. The Δ13-MitTx complex and amiloride-bound structures were derived from crystals grown in PEG 400 and cryoprotected using the reservoir solution supplemented with 35% PEG 400. To successfully introduce amiloride, crystals were initially soaked in reservoir solution supplemented with 17.5 mM amiloride and 5% DMSO overnight. Prior to flash cooling in liquid nitrogen, amiloride-soaked crystals were transferred in cryosolution containing 35 mM amiloride. For Cs^+ anomalous diffraction experiments, crystals grown in reservoir solution containing PEG 4000 were soaked in reservoir solution containing equimolar concentration of cesium acetate (pH 5.5) and supplemented with 500 mM CsCl prior to cryocooling in solution supplemented with 20-25% glycerol (v/v, final concentration) and 1 mM DDM.

Structure validation and analysis

Structure validation was performed using MolProbity (Davis et al., 2007). Pore surface and dimension were determined using the software HOLE (Smart et al., 1996) and APBS for calculation of electrostatic potential (Baker et al., 2001). AREAIMOL of the CCP4 package was used to calculate buried surface area (*CCP4* Project, 1994). Figures were prepared using the program PyMOL (DeLano, 2002).

Supplemental References

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