The Structure of YnaI Implies Structural and Mechanistic Conservation in the MscS Family of Mechanosensitive Channels

Highlights

- First structure of a large member of the MscS family of mechanosensitive channels
- The density map indicates extended sensor paddles of tilted transmembrane helices
- Lipids access the core of YnaI and fill cavities which are similar to gaps in MscS
- These cavities and the tilted helices could lay the basis for tension sensing

Authors

Bettina Böttcher, Vojtech Prazak, Akiko Rasmussen, Susan S. Black, Tim Rasmussen

Correspondence

t.rasmussen@abdn.ac.uk (T.R.), bettina.boettcher@ed.ac.uk (B.B.)

In Brief

Mechanosensitive channels of the MscS family are found in all kingdoms of life and protect bacteria against osmotic shock. Böttcher et al. provide the first structural insights into a larger family member, YnaI. Despite YnaI having additional transmembrane helices, important features are conserved, suggesting a common mechanism.
The Structure of Ynal Implies Structural and Mechanistic Conservation in the MscS Family of Mechanosensitive Channels

Bettina Böttcher, Vojtech Prazak, Akiko Rasmussen, Susan S. Black, and Tim Rasmussen

INTRODUCTION

Bacterial cells are directly exposed to the environment, and therefore must be able to adapt to sudden and drastic changes in external solute composition. Sudden decreases of osmolyte concentrations in the environment will cause a hypo-osmotic shock. Water is drawn into the cell with its higher solute concentrations, and consequently the internal pressure will increase to a minimum. At tensions close to the rupture of the membrane, MscL is activated and forms the largest pore, whereas at lower tensions MscS is triggered, which has much smaller conductance.

SUMMARY

Mechanosensitive channels protect bacteria against lysis caused by a sudden drop in osmolarity in their surroundings. Besides the channel of large conductance (MscL) and small conductance (MscS), Escherichia coli has five additional paralogs of MscS that are functional and widespread in the bacterial kingdom. Here, we present the structure of Ynal by cryo-electron microscopy to a resolution of 13 Å. While the cytosolic vestibule is structurally similar to that in MscS, additional density is seen in the transmembrane (TM) region consistent with the presence of two additional TM helices predicted for Ynal. The location of this density suggests that the extra TM helices are tilted, which could induce local membrane curvature extending the tension-sensing paddles seen in MscS. Off-center lipid-accessible cavities are seen that resemble gaps between the sensor paddles in MscS. The conservation of the tapered shape and the cavities in Ynal suggest a mechanism similar to that of MscS.

Multiple MS channel genes are often found within a single species. Escherichia coli, for example, has seven MS channels: MscL, MscS, and five further homologs of MscS (Edwards et al., 2012; Li et al., 2002, 2007; Schumann et al., 2010). It is likely that this functional redundancy is required for a graded emergency response mechanism that minimizes the unspecific release of solutes and keeps the disturbance of homeostasis to a minimum. At tensions close to the rupture of the membrane, MscL is activated and forms the largest pore, whereas at lower tensions MscS is triggered, which has much smaller conductance.

Crystal structures of MscS (Bass et al., 2002; Lai et al., 2013; Pliotas et al., 2012; Wang et al., 2008; Zhang et al., 2012) show that the channel is formed by seven subunits. Each of the subunits contributes three transmembrane (TM) helices, TM1–3. TM1 and TM2 form a flexible paddle that is likely to be involved in tension sensing. TM3 is a long kinked helix, which consists of the TM pore-forming part, TM3a, followed by TM3b that links the membrane part with a large cytosolic domain. This domain forms an enclosed vestibule at the entrance of the pore.

Sequence alignments show that all homologs of MscS in E. coli have an N-terminal extension while the cytosolic domains have a similar size except for YbdG, which has a small insertion within the cytosolic domain (Schumann et al., 2010). Analysis of the sequences also indicates that these N-terminal extensions form TM helices in addition to the three found in MscS. Ynal and YbdG are predicted to contain two additional TM helices, whereas MscK, YjeP, and YbiO are predicted to have eight additional helices (Krogh et al., 2001). It is not fully understood whether these additional TM helices isolate the sensor paddle from direct contact to the lipids, provide access routes for lipids to the sensor paddle (Booth et al., 2011) or even extend the paddle.

Although it is generally agreed that the paddles must be important for tension sensing and rotationally rearrange giving rise to different conformations of MscS (Wang et al., 2008), it is not clear how they are actually sensing the tension in the membrane. Recently, we demonstrated that lipids can intercalate between the paddles in MscS, which may be important for the sensing mechanism (C. Pliotas et al., personal communication). In addition, it was proposed that gating could be driven by a change of local membrane curvature due to the tilted paddles (Phillips et al., 2009). However, it is unclear whether these mechanisms are transferable to the larger homologs.

Here, we present a first glimpse at the structure of one of the larger homologs, Ynal, which suggests that the additional N-terminal helices may induce membrane curvature and may move and act as an extended sensor paddle. Furthermore, we...
obtained evidence that the core of the membrane domain is fully accessible to lipids, and saw lipid-filled pockets comparable with the pockets seen between the sensor paddles in MscS. We found that the tryptophan W184 at the portals of the cytosolic domain is required for function.

RESULTS

Cryo-Electron Microscopy of YnaI

Electron micrographs showed that YnaI aggregated at one site and had very little overlap to neighboring particles in the smaller aggregates (Figure S1, arrows). We selected a total 34,700 of YnaI channels, of which 18,985 particle images grouped into well-defined classes in 2D classification. This subset was used in further analysis. The 2D class averages showed predominantly side views with two distinct moieties of different sizes (Figure 1). The smaller moiety had a distinct central cavity, which resembled the cytosolic domain of MscS and contained the major contact sites to other particles in the aggregates (Figure 1A, white arrowheads).

Next, we calculated a 3D map using a previously determined map of negatively stained particles as starting reference (see Supplemental Information; Figure S1). To identify those particles that were least disturbed by the aggregation, we performed a 3D classification of the aligned vitrified particles into three classes. All three 3D classes showed a similar shape of YnaI with extensions of different sizes at the top and at the side of the cytosolic domain (Figure 1B, arrows). These extensions were attributed to neighboring particles in the aggregates. On the inside (Figure 1B), all three classes had a similar distribution of cavities, with the cavity of the cytoplasmic vestibule clearly resolved and distinct cavities in the center and at the side of the membrane part. For further refinement, we used the particle images of class 3 (7,202 particles) that had the least additional density from aggregation (Figure 1B, black arrows). The refined map of class 3 (Figure 2; accession number EMDB: 3035) had a resolution of 12.6 Å.

Structure of the Cytosolic Domain

The structure of YnaI shows on one side a large vestibule, which had an overall cross-correlation of 0.7 to the cytosolic domain of MscS (see Figure S4 for local cross-correlation). This domain provides in MscS stability (Rasmussen et al., 2007; Schumann et al., 2004) and selectivity (Maksaev and Haswell, 2012, 2013; Zhang et al., 2012), and is conserved between different channels of the MscS family. For structural comparison, we fitted the structure of MscS in the non-conducting state (PDB: 2OAU; Bass et al., 2002) into the electron microscopy (EM) map of YnaI (Figure 2). The fit showed excellent agreement of the cytosolic vestibule, but did not resolve the portals and the openings in the β-barrel structure of the vestibule. Furthermore, the EM map of YnaI had unaccounted density beyond the C-terminal β barrel of MscS (Figure 2A, arrow). This unaccounted density is most likely built by the seven C-terminal residues of MscS that are not resolved in the crystal structure plus the C-terminal purification tag of YnaI and density from the neighboring particles in the YnaI aggregates.
Structure of the Membrane Domain

The membrane domain in YnaI was funnel shaped. The cytosolic-facing site was narrower and had the same diameter (8 nm) as that of MscS. The diameter at the periplasm was 12.5 nm, compared with 5.5 nm in MscS. The total length of the membrane domain perpendicular to the plane of membrane was 7.5 nm, which was much larger than the 4.5 nm in MscS (Figure 2A). This difference in size was probably due to the two additional TM helices of YnaI and the tightly bound detergent micelle (Figure 2A, green semi-circles of 5 nm diameter indicate the extent of a typical dodecyl maltoside micelle) that are absent in MscS. The membrane part, which formed a distinct pore at the inside of fragmented density at the periplasmic side, which we think is accounted for by the detergent micelle.

The EM map of YnaI revealed a hollow cylinder in the center of the membrane part, which formed a distinct pore at the inside that was obstructed at both the cytosolic (Figure 3, red arrow) and periplasmic side (Figure 3, blue arrow). In MscS the central pore is formed by the seven tightly packed TM3 helices, which fit well into the hollow cylinder in their closed conformation (PDB: 2OAU; Bass et al., 2002), but are not accommodated in their open conformation (PDB: 2VV5; Wang et al., 2008), suggesting that YnaI was in a closed conformation. In the closed state, the pore of MscS is sealed by the hydrophobic residues L105 and L109 (corresponding L154 and M158 in YnaI), which aligned well with the obstruction of the pore at the cytosolic side of YnaI.

Sequence alignment of MscS and YnaI showed that YnaI has a 49-amino-acid long extension at the N terminus. Furthermore, structures of MscS lack the first 25 N-terminal amino acids. This N-terminal region of MscS does not align with the sequence of YnaI, and predictions of its structure have been made on the basis of spectroscopic data and simulations (Anishkin et al., 2008; Vásquez et al., 2008). Based on secondary structure prediction by JPRED, YnaI had two more N-terminal helices (residues 3–31 and 41–67; Figure 4A). These predicted helices largely overlapped with predictions of TM helices between 10–32 and 39–61 using TMHMM (Krogh et al., 2001).

To illustrate the possible contribution of the N-terminal helices to the structure of YnaI, we have placed a homology model of YnaI together with the predicted N-terminal helices in the EM map (Figures 4B and 4C). Since we cannot resolve the helices in our EM map, we have assumed that the additional helices were similarly staggered and tilted as the helices are in the structure of MscS, and that the ends of the N-terminal helices were close to the surface of the EM map of YnaI. Our illustration accounts for most of the observed EM density apart from a belt of fragmented density at the periplasmic side, which we think is accounted for by the detergent micelle.

The Core TM Helices Are Accessible to Lipids

The off-center cavities in the membrane domain of YnaI (Figure 3; green arrow) resembled crevices seen in the crystal structures of MscS between the sensor paddles and TM3. Recently, we proved lipid penetration into these crevices in MscS and hypothesized that this could also be important for the sensing of membrane tension (C. Pliotas et al., personal communication). In YnaI, these cavities are shielded by 14 more helices than in MscS. To test whether this had any impact on the accessibility to lipids, we used collisional quenching of tryptophan fluorescence by bromine atoms attached to fatty acid chains of the lipids (Kasha, 1952). Fluorescence quenching occurs if the tryptophan is in van der Waals contact with the bromine atoms in the lipids, and thus reports lipid contact for a particular location on a membrane protein (East and Lee, 1982). We constructed a mutant of YnaI where the five tryptophan residues were replaced by phenylalanine (Δ5W), allowing introduction of new tryptophan residues as probes. Based on sequence alignment of YnaI to MscS, we chose three residues that were likely to line the cavities: G152W, F168W, and the native tryptophan W201 (Δ4W). These positions were homologous to A103W (TM3a), A119W (TM3b), and I150W in E. coli MscS, respectively (Figures 5A and 5D). We showed that G152W and F168W were highly accessible to lipids (Figure 5C) as were the corresponding residues in MscS (C. Pliotas et al., personal communication). YnaI W201 had limited accessibility for lipids, similarly to the corresponding MscS I150W. Emission spectra of MscS and YnaI samples solubilized in n-dodecyl-β-D-maltopyranoside (DDM) showed peak positions that correlated well with the lipid accessibility (Figure 5B). The fatty acid tails of the phospholipids are predominantly located where the tryptophan probes indicate a low-polarity environment. There is good agreement of fluorescence
embedded cavities that are also seen in the crystal structure of MscS. The red balls and sticks. The green arrow indicates the off-center membrane-inner ring of the closed channel (magenta) is accounted for by the EM density. The positions L105 and L109 of the hydrophobic seal of MscS are indicated by the glycine-rich sequence GGIGG (Figure 5D) and fitted open channel of MscS (PDB: 2VV5;Wang et al., 2008; blue) are shown. The left slice is parallel to the symmetry axis; the right slice is perpendicular to the symmetry axis at the position indicated on the right panel. The inner ring of helices, which forms the central pore of the open channel (blue) in MscS, is not accommodated by the EM density, whereas the inner ring of the closed channel (magenta) is accounted for by the EM density. The positions L105 and L109 of the hydrophobic seal of MscS are indicated by balls and sticks. The green arrow indicates the off-center membrane-embedded cavities that are also seen in the crystal structure of MscS. The red arrow highlights the cytotoxic constriction, and the blue arrow the periplasmatic constriction of the pore.

properties between YnaI and MscS for these three residues, suggesting conservation of the lipid-filled cavities next to the pore-forming helices in these two proteins as indicated by the structural similarity of the EM map of YnaI. However, YnaI ∆5W L154W did not follow this trend. L154W was highly quenched, indicating greater lipid accessibility than its MscS counterpart, the pore-facing L105W (C. Pliotas et al., personal communication). Moving just two residues along the helix, however, restores the behavioral correlation, as G152W fluorescence data agree well with the MscS counterpart A103W. A possible explanation for the anomaly of the L154W data may be provided by the glycine-rich sequence GGIGG (Figure 5D) adjacent to L154W in YnaI. This sequence could introduce high flexibility and enable accessibility to lipids, e.g. by partial unwinding of the helix in YnaI.

Position of the First TM Relative to the Lipid Bilayer
Analysis of the sequence of YnaI (Krogh et al., 2001) and hydrophobicity plots suggested that YnaI had two additional TM helices N-terminal to the three conserved TM helices found in MscS. The fluorescence of one turn of the predicted first TM helix from V14W to F17W was investigated. All mutants showed high lipid accessibility, with the highest for V14W and the lowest for I16W (Figure 6A), confirming their location in the lipid bilayer. Emission peak positions for I15W and I16W were more red shifted than for V14W and F17W (Figure 6B), characteristic of a more hydrophilic environment. If a simple helical model is made (using PyMol v1.7.1), it can be seen that on both adjacent turns hydrophilic residues, N12 and S19, face in the same direction as I15W and I16W (Figure 6D). Therefore, it seemed likely that a helical structure was present and that this face of the helix was in contact with another TM helix, which is often promoted by formation of hydrogen bonds (Adamian and Liang, 2002). On this face of the helix, C20 and S27 may form additional hydrogen bonds with their side chains to neighboring helices. Lipids brominated at different positions along the fatty acid chain allow depth-dependent quenching experiments, which inform on the relative position of the tryptophan probe within the bilayer (Bolen and Holloway, 1990). Only F17W showed depth dependence in the quenching (Figure S5). The distribution analysis (Ladokhin, 1997, 2014) suggested that F17W was 7 ± 1 Å away from the membrane center (Figure 6C; dispersion σ = 3.4 ± 0.7 Å; area parameter S = 12 ± 2), which agreed well with the predicted extend of the first TM helix from residues 10 to 32 (Figure 6D; Krogh et al., 2001).

Tryptophan W184 Is Required for Function
Wild-type (WT) YnaI provides less protection in osmotic downshock assays than MscS, requiring induction when expressed from the pTrc plasmid (Edwards et al., 2012). When assayed for function, the tryptophan mutants used for the fluorescence experiments in this study failed to protect cells against hypo-osmotic shock (Figure S6) while the corresponding MscS mutants retained functionality (C. Pliotas et al., personal communication). The similarity of spectroscopic properties between YnaI and MscS (Figure 5B) indicated that structural features are similarly independent of functionality. However, we sought to obtain evidence that structural conclusions drawn from our fluorescence experiments hold true for the active and native form of YnaI. Structures obtained from negatively stained particles of the mutants ∆5W G152W and F168W were similar to those of WT, indicating that these mutations do not cause a gross change in structure (Figure S7).

Several mutants of the native tryptophan residues were tested for functionality to better understand the cause of the loss of function. The double mutant W29F/W201F (Δ2W) is functional (Figure S6). These are the two tryptophan residues predicted to be located in the membrane domain. Δ2W shows no quenching by brominated lipids (Figure 7), consistent with the prediction that the three remaining tryptophan residues are located close together at the portals in the cytosolic domain away from the membrane. The related mutant W29F (Δ1W) is active and reports the quenching of tryptophan W201 by brominated lipids. The small quenching seen is consistent with that seen for W201 in the tryptophan-free background reported above (Δ4W) but with the constant, non-quenchable contributions from the three cytosolic tryptophan residues. Similarly, quenching of W201 is seen in the mutant Δ3W (Figure 7; see below).

The mutant W29F/W184Y (Δ2Wb) is inactive while W29F/W299Y/W302Y (Δ3W) is active, which shows that W184 is required for function and cannot be substituted even by the conservative mutation to tyrosine (Figure S6). The role of tryptophan residues in YnaI and MscS are clearly different because the two residues of MscS in the cytosolic domain are located at the subunit interface and are important for complex stability (Rasmussen et al., 2007). Phenylalanine mutations can retain stability of the heptameric MscS complex, and function is not influenced (Rasmussen et al., 2007). On the other hand, the three tryptophan...
residues in the cytosolic domain of YnaI are not predicted to be at the subunit interface. All mutations we tested form stable heptameric complexes, but the function is lost by mutation of W184. The single tryptophan in MscS close to the membrane interface, W16, is functionally sensitive to mutation while this is not the case for the only tryptophan in YnaI on a TM helix, W29, as far as we can tell from our downshock assay. The lack of tryptophan residues at the membrane-water interfaces in MS channels is striking and has been proposed to be important for their function (Booth et al., 2007, 2011). Usually tryptophan residues serve as “anchors” to localize membrane proteins in the membrane (Killian and von Heijne, 2000).

In summary, we see similar quenching for W201 in two active mutants, although with constant fluorescence contributions from the cytosolic tryptophan residues overlaid, compared with W201 in the tryptophan-free background (Figure 7). Furthermore, the gross structure of two other mutants was similar to that of WT (Figure S7). Therefore, it seems reasonable to assume that the subunit interface is also filled with lipids in the native YnaI. Interestingly, we saw a functional contribution of W184 at the portals of the cytosolic domain. MscS homologs often seem to have a tryptophan at this position while in the case of E. coli MscS itself, tyrosine Y135 is aligned (Figure 5D). W184 is far away from the membrane regions investigated by fluorescence in this study.

**DISCUSSION**

We have reconstructed the 3D structure of YnaI at intermediate resolution. The map confirms that YnaI has a seven-fold symmetric organization similar to that of the related MscS, with an almost identical organization of the C-terminal cytosolic domain and a significantly larger membrane domain, which was consistent with the N-terminal extension of YnaI and the presence of a detergent micelle.

The size of the central pore in the EM map of YnaI agreed very well with the diameter of the pore formed by TM3a helices in closed structures of MscS. Furthermore, the seal region toward the cytosolic site of the pore has the highest sequence homology between YnaI and MscS, and the seal residues L154 and M158 are of similar size to L105 and L109 in MscS.

Electrophysiological characterization of YnaI showed very different properties of a conducting open state between YnaI and MscS (Edwards et al., 2012). YnaI opens less frequently and appears to require more pressure for activation, and the conductance is much smaller (Edwards et al., 2012). There may be several reasons for this. One could be the second constriction of the pore toward the periplasm in YnaI (Figure 3, blue arrow), which could stabilize the closed state and might restrict the pore size in the open state. For example, the large side chain of L142 could cause this second constriction. Another reason could be an extended sensor paddle (four TM instead of two in MscS), which forms a larger interaction interface with the lipids and so might require larger tension for changing the lipid content and, thus, for driving conformational switching. The off-center cavities in the EM map of YnaI agree in their position with cavities seen between TM3 and the sensor paddle in the crystal structures of MscS, suggesting that the inner core of the sensor paddle is structurally preserved despite the two additional TM helices in YnaI. Our fluorescence experiments on tryptophan mutants confirmed that three residues that are predicted to be exposed to the cavity in YnaI are similarly accessible to lipids as the corresponding residues in MscS.

Several MscS crystal structures from different organisms produced by different laboratories agree on structural features of the membrane domain (Bass et al., 2002; Lai et al., 2013; Wang et al., 2008; Zhang et al., 2012), which seem also be conserved in YnaI seen in our EM study. However, molecular dynamics (MD) simulations were performed by two independent groups who proposed that the gaps and tilted paddles are an artifact of crystallography and are not present in the active MscS in the membrane (Anishkin et al., 2008; Vásquez et al., 2008). One of these studies constrained its simulation on experimental continuous-wave electron spin resonance data, however, without distance measurements (Vásquez et al., 2008). Because of these contradicting views of the membrane domain...
structure, a follow-up study was performed in which distances of MscS reconstituted into membranes were measured by pulsed electron-electron double resonance (PELDOR) (Ward et al., 2014). These data agreed with the crystal structures and not with the MD simulations. Our recent fluorescence quenching data on MscS (C. Pliotas et al., personal communication) and YnaI in this study provide an independent experimental approach to the PELDOR experiments, showing that the gaps between the paddles are present and filled with lipids when these MS channels are located in the membrane. Thus, considering the currently available evidence, we believe that structural features suggested by the crystal structures are more likely to represent the active form of MscS and, indeed, homology of YnaI.

We noticed that the thickness of the membrane domain of YnaI perpendicular to the plane of the membrane was significantly larger than the typical width of a membrane and also as observed for MscS. We can exclude that this is caused by an extended N-terminal domain in the periplasm, as the predicted first TM helix showed strong lipid interaction and the depth-dependent fluorescence quenching agreed with the proposed location of this TM relative to the membrane. The highly charged loops between TM1 and TM2 and between TM3 and TM4 also give a good estimation for the length of the TM helices, which is similar to those in MscS. It therefore seemed unlikely that the membrane domain of the channel of YnaI had an extraordinarily large hydrophobic extent perpendicular to the plane of membrane that could lead to an atypical stretching of the membrane in the vicinity of the channels (Figure 8A). The likely position of the detergent micelle at the bottom of the channel suggested that the hydrophobic domain of the membrane-embedded part was further away from the cytosolic domain as in MscS (Figure 8B). This is probably caused by the outer ring of additional N-terminal helices in YnaI being vertically displaced toward the periplasm. A good indication for such a displacement is the fact that the diameter of the membrane part at the cytosol is the same in MscS and YnaI, which would be too small to accommodate the additional 14 N-terminal helices in YnaI at the same level close to the cytosol. However, toward the periplasm the diameter of YnaI increased, in contrast to MscS, providing plenty of space for an additional ring of helices, in agreement with a displacement toward the periplasm.

The preserved off-center cavities of YnaI suggested that the TM helices were most likely tilted similarly to those in MscS. Such tilted helices would induce local tension in the membrane that could increase the local curvature of the membrane (Figure 8C). This is probably caused by the outer ring of additional N-terminal helices in YnaI being vertically displaced toward the periplasm. A good indication for such a displacement is the fact that the diameter of the membrane part at the cytosol is the same in MscS and YnaI, which would be too small to accommodate the additional 14 N-terminal helices in YnaI at the same level close to the cytosol. However, toward the periplasm the diameter of YnaI increased, in contrast to MscS, providing plenty of space for an additional ring of helices, in agreement with a displacement toward the periplasm.

The preserved off-center cavities of YnaI suggested that the TM helices were most likely tilted similarly to those in MscS. Such tilted helices would induce local tension in the membrane that could increase the local curvature of the membrane (Figure 8C), which is in agreement with simulations and models of MscS (C. Pliotas et al., personal communication) (Phillips et al., 2009; Sotomayor and Schulten, 2004).

In summary, we illustrate an unusual overall shape of the membrane domain of YnaI which is best explained with four similarly tilted TM helices that probably form an extended sensor paddle compared with that of MscS. The sensor paddle fully maintains lipid accessibility to the core of the channel. Similar to the proposal for MscS, changes in the curvature of the membrane could represent the tension-sensing mechanisms. The proposed extension of the sensor paddle would provide a larger lipid interaction interface, and thus would require larger tensions for changing the lipid content and thus for triggering the opening of the YnaI channel, as observed in patch-clamp experiments (Edwards et al., 2012). The presence of seven distinct “knobs” in this part of the EM map also supports the presence of seven
defined sensor paddles, in contrast to an isolating ring of 14 evenly distributed TMs.

**EXPERIMENTAL PROCEDURES**

**Purification of YnaI**

Purification of YnaI was performed as described in detail previously (Edwards et al., 2012). In brief, YnaI was expressed from a pTrc99A vector in E. coli strain MJF641 missing all seven genes of the known MS channels (Edwards et al., 2012). Expression was induced for 4 hr at 30°C with 0.8 mM isopropyl β-D-1-thiogalactopyranoside. Cell pellets were stored at −80°C until further use.

Cells were lysed by a single passage through a French press, and membranes were collected by ultracentrifugation at 100,000 g for 1 hr at 4°C. Membranes were then solubilized in 1% DDM (Glycon) for 1 hr at 4°C and were loaded onto a prepacked Ni-nitrilotriacetic agarose column (Sigma) using a C-terminal His6-tag on YnaI. After washing and elution, the peak fraction was further purified on a size-exclusion Superose 6 10/300 column (GE Healthcare). The MscS mutant YFF I150W was purified following a protocol very similar to that for YnaI and was described earlier (Rasmussen et al., 2010). A size-exclusion Superdex200 10/300 column (GE Healthcare) was used in this case.

**Sample Preparation for EM**

Quantifoil Grids (1.2/1.3) were glow discharged for 1 min in a Quorumtech mini sputter coater (Quorumtech SC7620) at 35 mA and used within 1 hr. Grids were collected by ultracentrifugation at 100,000 x g for 1 hr at 4°C. Membranes were then solubilized in 1% DDM (Glycon) for 1 hr at 4°C and were loaded onto a prepacked Ni-nitrilotriacetic agarose column (Sigma) using a C-terminal His6-tag on YnaI. After washing and elution, the peak fraction was further purified on a size-exclusion Superose 6 10/300 column (GE Healthcare). The MscS mutant YFF I150W was purified following a protocol very similar to that for YnaI and was described earlier (Rasmussen et al., 2010). A size-exclusion Superdex200 10/300 column (GE Healthcare) was used in this case.

**Figure 6. Fluorescence of YnaI V14W to F17W on the Proposed First TM Helix**

(A) Lipid contacts were determined by reconstitution into brominated and non-brominated lipids. Fractional quenching (F0 – F)/F0 is shown as mean with SD.

(B) Emission peak positions of detergent-solubilized samples.

(C) Distribution analysis (blue line) of depth-dependent fluorescence quenching with three different brominated lipids for mutant F17W (black circles). Mean values with SD are shown (n = 5).

(D) Helical model (produced with PyMol) of the TM helix (residues 10–30) including the residues used for the fluorescence experiments. Hydrophilic residues are shown in orange. The prediction of this TM helix is in agreement with the results shown in (C) considering the positioning of F17W. See also Figures S5, S6; Table S1.

**Figure 7. Quenching of W201 in Different Mutants and Properties of Tryptophan Mutants**

Quenching of BrPC for the mutants Δ4W, Δ1W, Δ2W, Δ3W, and Δ4Wb are shown in the first row. For comparison of the quenching of W201 the constant, non-quenchable contributions of the cytosolic tryptophan residues were eliminated by subtracting spectra of Δ2W and Δ4Wb from Δ1W and Δ3W, respectively (second row). Fluorescence intensities in both rows are each normalized for protein concentration. The table on the bottom gives an overview of the used mutants, showing that W184 is required for function. The two tryptophan residues located in the membrane region have an orange background. The quenching by BrPC is given without and with correction for the fluorescence contributions of the cytosolic tryptophan residues. See also Figures S6 and S7.
mounted in a plunge freezer with an environmental chamber (Bellare et al., 2007). The chamber was humidified with two water-soaked sponges and mounted in a plunge freezer with an environmental chamber (Bellare et al., 2007). The direction of the forces is indicated by arrows. (A) The large size of the membrane part could cause a local increase of the width of the membrane to bury the membrane part completely. (B) However, the likely position of the detergent micelle suggests that the hydrophobic part is located further away from the cytosolic domain at the bottom of the channel. (C) The tilt of the helices could induce a local curvature of the membrane as indicated.

Electron Microscopy

EM was done with an FEI-F20 electron microscope with field emission gun and a TVIPS F416 4k x 4k CMOS camera. The microscope was operated at 200 kV, spot size 5, with a C2 condenser aperture and an objective aperture of 100 μm diameter. For data acquisition of negatively stained samples at room temperature, a standard room temperature holder was used. Vitri-fied samples were transferred with a Gatan 626 cryo-holder with a matching Gatan transfer station. Image acquisition was done semi-automatically with EM tools (TVIPS; see Supplemental Experimental Procedures for more details).

Image Processing

Particles were manually selected with e2boxer of the EMAN 2 package (Tang et al., 2007) using the auto-center option and windowed with a box size of 256 x 256 pixels. For each micrograph the defocus was determined with ctffind3 (Mindell and Grigorieff, 2003). Further image processing was done with Relion (Scheres, 2012) (for more details see Supplemental Information).

Fitting of Atomic Models to EM Maps

Fitting of the models of MscS (PDB: 2OAU [Bass et al., 2002] and 2V6S [Wang et al., 2008]) was done with the “fit into map” option of Chimera (Pettersen et al., 2004). The homology model of YnaI was generated using the automated mode of the Swiss-Model server (Kief er et al., 2009). The homology model of YnaI was placed into the EM map using the match maker option of Chimera and aligning the homology model to one of the chains of the fitted MscS channel. The models of the individual helices (poly-Ala-chain of appropriate length) were generated with the “built” option of the structure-editing tool of Chimera. The helices were manually placed in the EM map.

Fluorescence Experiments

The five native tryptophan residues in YnaI were replaced by phenylalanine, and new tryptophan residues were introduced by site-directed mutagenesis (Stratagen e). Furthermore, several intermediate mutants were obtained whereby some of the tryptophan residues were replaced by phenylalanine or tyrosine. A mutant with only one of the native tryptophan residues remaining, W201 (Δ4W), was also used. The mutant forms were purified as described for WT YnaI, and resulted in homogeneous heptameric YnaI complexes. Then YnaI was reconstituted into lipid bilayers in the same way as described earlier (Carney et al., 2008). In short, DDM-solubilized YnaI was mixed with cholate-solubilized lipids at a ratio of 1:100 (mol/mol) and incubated at room temperature for 15 min. Then YnaI was reconstituted into bilayers by diluting into a buffer containing 40 mM HEPES (pH 7.2), 100 mM KCl, and 1 mM EGTA. After 5 min of incubation at 20°C, fluorescence spectra were recorded using an FLS920 spectrometer (Edinburgh Instruments) with an excitation wavelength of 295 nm and emission from 300 to 420 nm. Spectra were compared for reconstitution into 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; Avanti Polar Lipids) or 1,2-di-(9,10-dibromo)stearoyl-sn-glycero-3-phosphocholine (BrPC). The fractional quenching was calculated by (F0 − F)/F0, where F0 is the fluorescence intensity at 340 nm for samples reconstituted into DOPC and F for reconstitution into BrPC. Emission spectra were also recorded for DDM-solubilized YnaI samples. For depth-dependent quenching experiments, YnaI was reconstituted into 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine as non-brominated reference sample and into brominated lipids, 1-palmitoyl-2-(6,7-dibromo)stearoyl-sn-glycero-3-phosphocholine or similar lipids with bromination at 9,10- or the 11,12-positions (all obtained from Avanti Polar Lipids). Data were analyzed according to Ladokhin (1997) with distribution analysis using Gaussian functions and the program Origin 8.0 for fitting.

The quenching of the W201 fluorescence in the mutants W29F (Δ1W) and W29F/W201F (Δ3W) were determined by following procedure. The mutant forms were reconstituted in DOPC and BrPC as described above, and the fluorescence was measured. The intensity was normalized by the final protein concentration, ignoring the inner filter effect because of low absorption.

ACCESSION NUMBERS

The accession number for the EM map reported in this paper is EMDB: 3035.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2015.06.023.

AUTHOR CONTRIBUTIONS

B.B. and T.R. designed the study and analyzed data; all authors conducted experiments and prepared the manuscript.

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Supplemental Information

The Structure of YnaI Implies Structural and Mechanistic Conservation in the MscS Family of Mechanosensitive Channels

Bettina Böttcher, Vojtech Prazak, Akiko Rasmussen, Susan S. Black, and Tim Rasmussen
Figure S3
Figure S5
| name  | mutation                        | comment       |
|-------|---------------------------------|---------------|
| Δ1W   | W29F                            |               |
| Δ2W   | W29F/W201F                       |               |
| Δ2Wb  | W29F/W184Y                       |               |
| Δ3W   | W29F/W299Y/W302Y                 |               |
| Δ3Wb  | W29F/W184Y/W201F                 |               |
| Δ4W   | W29F/W184F/W299F/W302F           |               |
| Δ4Wb  | W29F/W201F/W299Y/W302Y           | Not determined|
| Δ4Wc  | W29F/W184Y/W299Y/W302Y           |               |
| Δ5W   | W29F/W184F/W201F/W299F/W302F     |               |

Figure S6
Figure S7

|     | WT       | G152W    | F168W    |
|-----|----------|----------|----------|
| a   | 74 Å     | 75 Å     | 76 Å     |
| b   | 108 Å    | 108 Å    | 106 Å    |
| c   | 58 Å     | 57 Å     | 59 Å     |
| α   | 62°      | 60°      | 66°      |
Supplemental Figures

**Figure S1:** Electron micrograph of vitrified YnaI (related to Figure 1).

The particles are dark against a brighter background. Some individual particles in the aggregates are marked by arrows.

**Figure S2:** Negatively stained YnaI and its image analysis (related to experimental procedures).

A A micrograph of negatively stained YnaI (50 μg/ml) is shown. Particles are evenly distributed and show little aggregation.

B The symmetry of the particles was tested by extracting top-views and generating 10 copies each with random in-plane rotation. These particles were subjected to multivariate statistical analysis. The panel shows the most significant Eigen images obtained by multivariate statistical analysis. The second and third Eigen images have a seven-fold symmetric pattern with opposite phase. This is indicative of seven-fold symmetry in the particle population (Dube et al., 1993).

C Results of the image analysis of stained YnaI. The top row shows selected surface representations of the channel, the middle row shows projections of the map in the same direction as the surface views and the lower panel shows matching averages from supervised classification. The length of the scale bar equals 10 nm.

D Class averages obtained by multivariate statistical analysis. They agree well with the averages from supervised classification in C.

**Figure S3:** Fourier-Shell Correlation curve (related to experimental procedures).

The Fourier-Shell Correlation curve (blue) of two independently calculated half-maps of vitrified YnaI after post processing (the half maps were masked and the contribution of the mask was corrected by phase randomization). The Fourier-Shell-correlation curve is shown up to Nyquist frequency. The correlation drops to 0.143 (dotted line) at 1/12.6 Å⁻¹.

**Figure S4:** Local Cross-Correlation between the EM-map of YnaI and the structure of MscS (2OAU) (related to Figure 2).

A and B The structure of MscS (2OAU) truncated at 13 Å resolution (2OAU, blue) and fitted into the EM-map of YnaI.

In A the correlation between the cytosolic domain (133-286) of MscS and the vestibule of YnaI was maximized and in B the correlation between the membrane domain (1-132) of MscS and the vestibule of YnaI was maximized.
C-and D EM-Map of YnaI coloured with the local correlation to the map of MscS in the orientations given in the panels above.

The local correlation was highest when the vestibule of YnaI superimposed with the cytosolic domain of MscS. The overall correlation between the vestibule of YnaI and the cytosolic domain of MscS (133-286; orientation shown in A) was 0.72 compared to 0.15 between the vestibule and the membrane domain of MscS (1-133; orientation shown in B) supporting the chosen assignment of the cytosolic domain to the vestibule of YnaI.

**Figure S5: Fluorescence quenching of mutants V14W to F17W dependent on the position of the bromine on the fatty acid chain (related to Figure 6).**

Fractional quenching for the YnaI mutants V14W to F17W are shown reconstituted into brominated lipids with the bromines at different positions. Mean values with standard deviation are shown (V14W to I16W: n = 3; F17W: n = 5).

**Figure S6: Functional assay of YnaI tryptophan mutants (related to Figures 5, 6 and 7).**

Hypo-osmotic downshock assay. YnaI constructs were transformed in MJF641, grown in LB medium + 0.3 M NaCl and then diluted into a LB only medium. Before the shock expression of YnaI was induced with 0.3 mM IPTG during the main culture (~2 h). Serial dilutions were made and plated. Shown survivals are number of counted colonies relative to control samples which were diluted in LB + 0.3 M NaCl. Mean values with standard deviation are shown (n=3). An overview of the naming of the YnaI mutants is given in the table below.

**Figure S7: Image analysis of the negatively stained mutants G152W and F168W of YnaI (related to Figure 7).**

A Typical micrographs of stained G152W (left) and F168W (right). The particles are bright against a darker background and show a homogeneous spread.

B Selected class averages (six out of 20) of G152W (left) and of F168W (right). The class averages were determined by multivariate statistical analysis using Imagic. The length of the scale bar indicates 10 nm.

C Surface presentation of the 3D-map of G152W (left) and the same view with the front half removed (right).

D Shows the same views of G152W (grey) as shown in C but with the surface representation of the low-pass filtered map of the vitrified YnaI WT (magenta) superimposed.

E Surface presentation of the 3D-map of F168W (left) and the same view with the front half removed (right).
F Shows the same views of F168W (grey) as shown in D but with the surface representation of the low-pass filtered map of the vitrified YnaI WT (magenta) superimposed.

G Surface presentation of the low pass filtered map of vitrified YnaI WT (magenta) in the same orientations as in C-F.

H Table with key dimensions and angles of the membrane parts of the WT-particle and the mutants. The positions where the measurements were taken are indicated in the low pass filtered map of the vitrified WT-YnaI in G.
Supplemental Tables

Table S1: Fluorescence spectroscopic properties of YnaI mutants (related to Figures 5 and 6).

Fractional quenching for reconstituted YnaI samples and the position of the emission peak of solubilised samples are compared with MscS. Mean values with standard deviation are given (number of repeats in brackets).

| YnaI-mutant | Fractional quenching | Peak position λ_{max} (nm) | homologue MscS-mutant* | Fractional quenching | Peak position λ_{max} (nm) |
|-------------|----------------------|-----------------------------|------------------------|----------------------|-----------------------------|
| Δ4W         | 0.43±0.05 (7)        | 322.7±0.1 (3)               | I16W                   | 0.57±0.04 (4)        | 326.0±0.2 (4)               |
| G152W       | 0.82±0.04 (3)        | 309.7±0.1 (3)               | A103W                  | 0.75±0.08 (17)       | 310.7±0.4 (5)               |
| F168W       | 0.81±0.01 (3)        | 314.3±0.4 (3)               | A119W                  | 0.88±0.04 (17)       | 313.6±0.2 (4)               |
| L154W       | 0.83±0.02 (9)        | 320.8±0.1 (3)               | L105W                  | 0.32±0.04 (4)        | 327.7±0.6 (7)               |
| V14W        | 0.92±0.01 (8)        | 317.4±0.1 (6)               |                        |                      |                             |
| I15W        | 0.89±0.04 (4)        | 320.5±0.1 (4)               |                        |                      |                             |
| I16W        | 0.76±0.09 (4)        | 320.7±0.1 (4)               |                        |                      |                             |
| F17W        | 0.81±0.09 (8)        | 313.1±1.1 (5)               |                        |                      |                             |

*Pliotas; manuscript in preparation

Supplemental experimental procedures

Sample Preparation (Negative stain)

Negative stain for WT YnaI was done using the sandwich method: Freshly cleaved mica was coated with a thin layer of carbon and used within a couple of weeks after coating. For staining, 4 µl of the undiluted sample (50 µg/ml) was pipetted between carbon and mica of an approximately 2x2 mm large piece of coated mica. Then, the carbon was floated on 1 ml of 2% uranyl acetate in a porcelain spot plate. A grid was placed on top of the floating carbon and picked up with a small piece of paper. Then the paper with the grid was turned around that the grid with the sample was pointing upwards and was placed at the bottom of a second well with 1 ml 2% Uranyl-acetate solution. A second piece of carbon was floated on top of the Uranyl-acetate solution. Then the floating carbon was picked up with the grid, sandwiching the sample and the stain between two layers of carbon. Excess liquid was removed with filter paper and the grid was dried.

Negative stain of the YnaI mutants G152W and F168W was done on carbon-coated grids without sandwich. In brief: Carbon coated (17 nm thick) copper grids were glow discharged for 1 min and used within 1 hour. 3 µl of sample (6 µg/ml for G152W and 20 µg/ml for F168W) were applied to the glow discharged grids. The sample was incubated for ca. 1 min.
Then the grids were washed 3 times with water and 4 times with 2% Uranyl Acetate. In the last wash step the Uranyl Acetate was incubated for 3-5 min before removal. Excess liquid was removed with Whatman filter paper No 50. Care was taken that the grids did not dry between the wash-steps.

**Electron Microscopy: semi-automatic data acquisition**

Semi-automatic data acquisition was done with the single particle application software EM-tools (TVIPS). Micrographs of vitrified samples were recorded at a primary magnification of 62000 (pixel size 1.73 Å/pixel, calibrated with catalase) and with an approximate dose of 17-22 electrons/Å². Data acquisition was done with the spot scan option of EM-tools, which electronically shifts the image for positioning the spots to take several exposures for one stage position. The spot scan pattern was triangular with a distance of 900 nm between the three spots. The approximate beam diameter for each spot was 780 nm. For each hole three none-overlapping spots were recorded. A total of 4191 spots from 1397 different holes were recorded in 8 sessions. The measured defocus range was between 1300 nm and 5800 nm (determined with ctffind3, (Mindell and Grigorieff, 2003)).

For recording data of negatively stained YnaI the procedure was similar as for vitrified particles with the following modifications: The spot scan pattern was hexagonal (7 spots with a distance of 1.4 μm). We acquired 1316 micrographs from 188 different positions in a single session for WT-YnaI. The calculated defocus range of the spots was between 400 and 1600 nm.

Micrographs of the negatively stained mutants G152W and F168W were recorded on a conventional 8kx8k TVIPS CMOS-camera at a primary magnification of 50,000. 2 by 2 pixels were binned giving a calibrated pixel size of 3.06 Å. Otherwise the data acquisition scheme was the same as for negatively stained WT-YnaI with a distance between spots of 1.8 μm.

**Image Processing**

The selected particle images were pre-processed with Relion to normalize the grey value distribution and reboxed to a box size of 160x160 pixels. Particle images were aligned and classified in 2D. All particle images were excluded, which could not be aligned with a rotational accuracy better than 10° in this step.

3D-refinement was started with the final 3D-map of the negatively stained YnaI (see below) as starting reference. To avoid that the two independent halves in the refinement were of opposite hand at the end of the refinement, the resolution to which the refinement in the first iteration was limited was 35 Å.

After having established the parameters, 3D autorefine was started with the current best map and the current best information on the particle orientations. The resolution was limited to 35Å in the first iteration. Particles were masked with a spherical mask with zeros (diameter 190 Å). Processing assumed C7 symmetry based on the Eigen image analysis of the negatively stained particles (see below). Alignment was followed by 3D classification into 3
classes. The class, which showed least distortion by neighbouring particles was refined with the auto-refine option. The resolution was assessed by Fourier-shell correlation of independently processed halves (gold standard, (Scheres and Chen, 2012) and using a cut-off for resolution of 0.143 (Rosenthal and Henderson, 2003)).

**Image analysis of negatively stained data**

**WT-YnaI**

For negatively stained data of WT YnaI, the particle images were processed with IMAGIC (van Heel et al., 1996). The defocus of the particles was determined for class averages of masked and band-pass filtered power spectra of individual particles. These class averages showed the typical Thon-ring pattern (Thon, 1966) for different defocus values. The defocus for each of the class averages was determined with the integrated ctf-find option in IMAGIC. Then the defocus parameters were used for inverting the phase information in every other transfer band (phase-flipping). The phase flipped particle images were binned by a factor of 2 (3.5 Å/pixel) and the box size was reduced to 64x64 pixels in order to speed up processing. Particle images were low-pass filtered with a Gaussian cut-off of 22 Å and normalized in their grey value distribution. Without prior alignment, the filtered and normalized particle images were classified using multivariate statistical analysis. The resulting class averages were centred and rotationally aligned in respect to each other followed by realigning the members of a class to their realigned average. This was followed by a few rounds of classification using multivariate statistical analysis followed by multi-reference alignment. The references for the multi-reference alignment were a few selected, well defined class averages from the previous classification. After class averages did not improve further, we selected the most elongated view, centred it and oriented its long axis upright. This view was assigned with Euler angles for an equatorial view and a three-dimensional reconstruction was calculated from this view using exactly weighted back-projection and imposing C7-symmetry. Projections from the resulting 3D-map were used as anchor projections for assigning Euler angles to individual class averages using sinogram-correlation. From selected class averages a new 3D-map was calculated. Class averages were selected based on whether they were similar to projections of the current 3D-map in the direction of the assigned Euler angles. Assignment of Euler angles with sinogram-correlation was followed by calculation of 3D-maps and of new anchor projections. The process was iterated until no further improvement of the 3D-map occurred.

This was followed by iterative projection matching (five iterations): In short, we aligned the particle images to projections of the current best map and assigned the same Euler angles to the particles as the reference projection had to which they showed the highest cross-correlation. Then, for each reference orientation we calculated averages from the 20 images with the highest cross correlation. This gave averages of similar quality evenly distributed across the asymmetric unit with no preferential views. The averages were back-projected into 3D-maps using the exact weighting scheme. The resulting 3D-maps appeared isotropic in resolution.
We also performed several tests to test the plausibility of the results:

1) **Different start-up strategy**: We started 3D-reconstruction with assigning Euler angles to selected class averages using sinogram correlation between class averages and assuming C7-symmetry. This was followed by refinement as described above. The resulting 3D-map was similar to the 3D-map obtained with the approach above. It could be reproduced from different sub-sets of the data and with different class averages in the start-up.

2) **Symmetry check 1**: We used the same approach as described in 1 but assumed C6 or C8 symmetry. In both cases the projections of the resulting 3D maps did not resemble the initial class averages, suggesting that the particles had neither C6 nor C8 symmetry.

3) **Symmetry check 2**: We selected particle images from the final alignment of the projection matching, which were assigned with beta angles between 0 and 10 degree (top-views). This accounted for 436 particles (ca. 2% of the total data). These top views should have approximate seven-fold rotational symmetry, if the particles have C7-symmetry. The top-views were extracted. For each particle 10 randomly rotated copies were generated and included in the test data. Then without further alignment, Eigen images were calculated using multivariate statistical analysis. The predominant Eigen images were checked for rotational symmetry.

To exclude a bias from the previous alignment to projections of a seven-fold symmetric volume, the randomly rotated particles were aligned several times to the sixfold averaged total sum of the test data (rotate and shift). There was no obvious enhancement of the 6-fold symmetry. Afterwards particles were again, randomly rotated and classes and Eigen images were calculated. This gave back the seven-fold symmetry. However, most of the Eigen images now represented a shift rather than a variation in rotation.

4) **Reference Bias towards MscS**: We started with a low pass filtered map of the related MscS channel (2OAU) to generate reference projections for projection matching or for sinogram correlation as described above. However, refinements did not stabilize and projections of the final map did not match with the initial class averages. This suggested that the structure of YnaI and MscS differed too much to justify this approach.

**Structure of YnaI reconstructed from negatively stained particles.**

Electron micrographs of negatively stained YnaI illustrate a homogeneous distribution of particles with few aggregates before concentration (Figure S2A). Particles were selected semi automatically and class averages were calculated by multivariate statistical analysis. The class averages composed mainly of elongated views and very few spherical ones.

To establish the rotational symmetry of the particles we identified top views, centred them and generated ten randomly rotated copies for each top-view. This was followed by multivariate statistical analysis. The second and third Eigen image of this analysis showed two sevenfold-symmetric rings (Figure S2B), which suggested that the channels had a
sevenfold symmetry. This observation was consistent with the MALS-analysis that showed that YnaI was a heptamer similar to MscS (Edwards et al., 2012).

Next, we calculated a 3D-map by weighted back-projection and assuming C7-symmetry. Projections of the 3D-map of the negatively stained data together with re-projections and matching classes from the supervised classification demonstrated a good consistency of the data (Figure S2C). The projections of the map were also similar to class averages from unsupervised classification (Figure S2D).

The image reconstruction of the negatively stained particles that is shown in Figure S2C was used as starting reference for determining the orientations of the vitrified particle images.

**Functional assays for YnaI**

The function of YnaI tryptophan mutants were assessed as described for YnaI WT in the literature (Edwards et al., 2012) in osmotic downshock assays (Booth et al., 2007). In short, YnaI constructs were transformed into strain MJF 641 where all 7 genes for MS channels are knocked out (Edwards et al., 2012) and overnight cultures were grown in LB medium supplemented with 25 µg/ml ampicillin at 37°C. Next day a pre-culture was grown in the same medium and then diluted 10 times into a LB medium containing additional 0.3 M NaCl and 0.3 mM IPTG. The culture was grown to an OD₆₅₀nm of 0.3 and then diluted 10 times into LB medium (shock) or LB + 0.3 M NaCl (control). After 10 min incubation at 37°C serial dilutions were made and plated on LB or LB + 0.3 M NaCl plates, respectively. Next day colonies were counted and the survival was calculated as ratio number of colonies on shock to control plates. Mean values with standard deviation (n=3) are shown in Figure S6.

**Structural Integrity of Tryptophan Mutants G152W and F168W**

The exchange of single residues against Tryptophan in the membrane part of YnaI led to a reduced protection of bacteria against hypo-osmotic shock. This suggested that the mutant channels had reduced activity. To exclude that this reduced activity was caused by a loss of the structural integrity or by major structural rearrangements in the membrane part, we investigated the structure of two of the mutants (YnaI Δ5W G152W and F168W) by electron microscopy of negatively stained particles followed by image analysis. Micrographs of the stained mutants showed homogeneous particle populations (Figure S7A).

For further analysis, we selected some 3000 particles images of G152W semi-automatically (e2boxer, swarm) and calculated class averages of the selected particles with Relion. Two representative class averages were used as templates (low pass filtered to 1/40 Å) for subsequent automatic particle picking (Relion) from the remaining micrographs. The template based picking yielded 93138 particles for F168W and 27471 particle images for G152W. Particle selection and extraction was followed by 2D classification, in which particles were excluded that did not group into consistent classes. All further analysis was done with the remaining particles (60246 for F168W and 22974 for G152W).
Next, we calculated 2D class averages with Imagic (Figure S7B) using multivariate statistical analysis. The class averages showed bi-partite projections with a tapered, larger membrane part and a smaller vestibule, similar to that observed previously for WT-YnaI. To test whether the three-dimensional shape was also maintained, we calculated independent 3D-reconstructions for both mutants. To avoid a reference bias towards the WT-particle, we used an *ab-initio* approach in which Euler angles were determined by angular reconstitution in IMAGIC. In brief: We assumed C7-symmetry and calculated the relative euler-angles of the class averages using sinogram correlation. Then the 3D-map of each mutant was determined by weighted back-projection of the class averages. This was followed by 2 rounds of refinement, which consisted of 1) multi-reference alignment of the particle images to 10 projections of the current 3D-map; 2) the classification of the aligned particle images using multivariate statistical analysis; 3) the assignment of Euler-angles of the class averages by sinogram correlation against 30 anchor projections of the current 3D-map and 4) the determination of the refined 3D-map by weighted back-projection of the class averages. The refined 3D-maps of both mutants were used as starting models in iterative alignment and image reconstruction with Relion.

The 3D-maps of the negatively stained mutants looked very similar to each other (Figure S7C and D) and showed a clear separation of the vestibule and the membrane part. Comparison of the mutants to the low pass filtered map of the vitrified WT-YnaI (Figure S7E and F) showed an increase in the diameter of the vestibule, which was probably caused by the flattening of this hollow structure during drying rather than structural changes in the mutants. This is further supported by our earlier observation on vitrified and stained YnaI WT that showed similar changes in the diameter of the vestibule.

The membrane part is much more compact than the vestibule. Therefore, drying will have a much smaller effect on the dimensions of the membrane part than on the vestibule. For comparison of YnaI WT and the two mutants, we measured the cross sections of the membrane part close to the inner and outer surface of the membrane as well as the membrane spanning width and the angle between the plane of the membrane and outer surface of the membrane part (Figure S7G and H). The measurements (Figure S7H) show no significant differences in the dimensions and the angles between YnaI WT and G152W and F168W. Thus, despite the limited resolution, we can exclude that the mutants have undergone major rearrangements in their helical packing. In particular it is unlikely that there is a major change in the tilt of the helices or their staggered packing, which would have impacted on the dimensions and/or the slope of the outer surface of the membrane part. The general structural preservation of the membrane part in the mutants suggests that they have maintained their general architecture and thus their gross lipid environment despite their reduced activity.
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