

# Crystal structure and functional mechanism of a human antimicrobial membrane channel

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Multicellular organisms fight bacterial and fungal infections by producing peptide-derived broad-spectrum antibiotics. These host-defense peptides compromise the integrity of microbial cell membranes and thus evade pathways by which bacteria develop rapid antibiotic resistance. Although more than 1,700 host-defense peptides have been identified, the structural and mechanistic basis of their action remains speculative. This impedes the desired rational development of these agents into next-generation antibiotics. We present the X-ray crystal structure as well as solid-state NMR spectroscopy, electrophysiology, and MD simulations of human dermcidin in membranes that reveal the antibiotic mechanism of this major human antimicrobial, found to suppress *Staphylococcus aureus* growth on the epidermal surface. Dermcidin forms an architecture of high-conductance transmembrane channels, composed of zinc-connected trimers of antiparallel helix pairs. Molecular dynamics simulations elucidate the unusual membrane permeation pathway for ions and show adjustment of the pore to various membranes. Our study unravels the comprehensive mechanism for the membrane-disruptive action of this mammalian host-defense peptide at atomistic level. The results may form a foundation for the structure-based design of peptide antibiotics.

crystallography | electrophysiology | ion conduction | molecular dynamics

Host-defense peptides actively control a wide range of microbes across most tissues of the animal and plant kingdoms, which signifies their importance during the evolution of multicellular organisms (1–3). In comparison with traditional small-molecule antibiotics, host-defense or antimicrobial peptides (AMPs) are often considered to have a distinctly superior property, as they target the microbial Achilles heel, i.e., the unique but essential features of all microbial cellular membranes (albeit at somewhat lower efficacy) (1–5). Thus, microbes have not been able to develop efficient resistance mechanisms against AMPs within the time frame of their parallel evolution (3, 5).

In recent years, a steep global rise in infections by multi-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) has been recorded (6, 7). These often affect the patients' skin and epithelial injuries, and are particularly hard to treat with conventional small-molecule antibiotics (8). The development of high-efficiency antibiotic agents, less prone to evoking resistance, is thus essential (4–7). However, the rational design of AMPs requires a detailed understanding of their structural and mechanistic determinants of antimicrobial action, which has not been achieved to date (4, 9, 10). The lack of molecular-based understanding has been named as the main obstacle hampering progress in this field (11).

The human epithelium exposes a large external surface for the growth of microbes (12). Among the major AMPs detected on human skin is the negatively charged peptide dermcidin (DCD; refs. 13–15), which is constitutively produced in sweat glands as

a precursor protein, further processed and finally secreted into human sweat (refs. 13 and 16; Fig. S1 A–C). DCD is active against a broad spectrum of bacteria including MRSA and rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* at concentrations of  $\sim 1 \mu\text{g/mL}$  (16). Its antimicrobial activity is particularly robust against changes in pH and ionic strength (13, 16). When isolated from sweat or after recombinant expression, DCD forms an equilibrium mixture of oligomers of varying size, both in solution and in membrane mimetics (16, 17). Human sweat is enriched in divalent ions, among which  $\text{Zn}^{2+}$  is of particular importance and has previously been demonstrated to be essential for AMP action on some microbes (18, 19).

AMPs are classified according to their overall charge, secondary structure, and more specifically the presence of certain amino acid combinations such as cysteines or prolines (1, 9). Many AMPs carry an excess of positive charges to interact favorably with the negatively charged surface of bacterial membranes (1–3). Although a number of models for the membrane-disrupting action of AMPs have been proposed, detailed and compelling structural and mechanistic evidence for any of these models involving mammalian (or human) AMPs has so far been elusive (4, 9, 10). To elucidate the antibiotic mechanism of DCD and reveal the underlying structural determinants, including the level of oligomerization, we crystallized the 48-residue DCD peptide (Fig. S1A), determined its channel-forming structure, and looked at membrane interaction with solid-state (ss) NMR spectroscopy. We then conducted electrophysiology experiments in which we characterized the activity of DCD in membranes under various conditions. The experimental results are in excellent agreement with data we obtained from extended computational electrophysiology and molecular dynamics (MD) simulations of the channel assembly, which further reveal the ion-transfer mechanism of DCD in atomic detail.

## Results

**Structural Architecture of the Hexameric AMP Channel.** The structure of assembled DCD solved at 2.5-Å resolution exhibits a channel architecture comprising a hexameric bundle formed by

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) (PDB ID code 2YMK).

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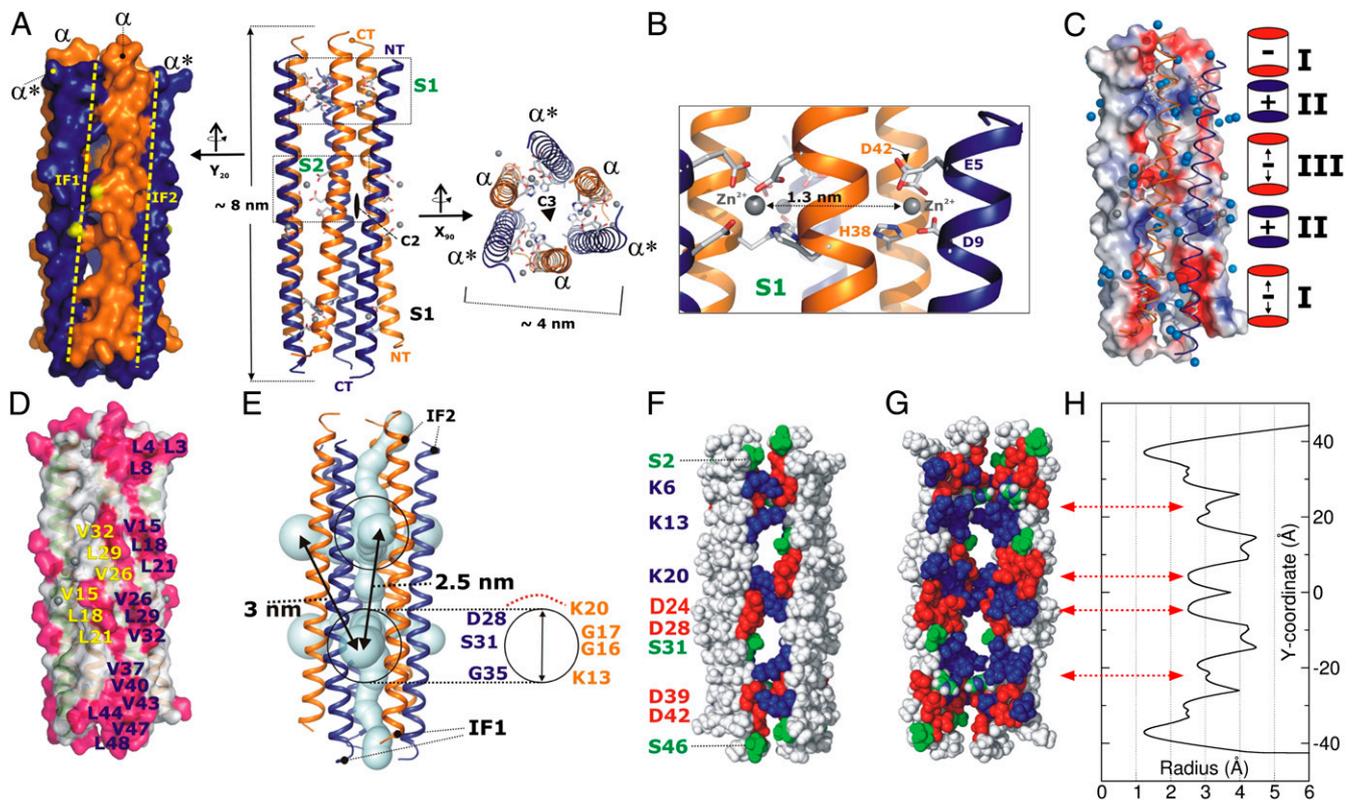
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elongated  $\alpha$ -helices, which adopts overall dimensions of  $\sim 8 \times 4$  nm (Fig. 1A). Channel formation involves trimerization of antiparallel peptide dimers resulting in a firmly enclosed channel structure. Each monomer presents two distinct interfaces to neighboring subunits (Fig. 1A). The extended interface, displaying a contact surface of  $930 \text{ \AA}^2$ , is mainly formed by salt bridges, and the second interface covers  $520 \text{ \AA}^2$  (IF1) and is primarily stabilized by  $\text{Zn}^{2+}$  ions that intercalate between the two helices (Fig. 1A and Fig. S1D). The zinc ions in DCD are coordinated by *N*- and *C*-terminal residues of dimerizing peptides (residues involved: Glu5, Glu9, His38', and Asp42') (Fig. 1A and B). They are attached to the inner wall of the channel and substantially change the overall charge of the assembly (from  $-12e$  to neutral). In addition, they modify the local charge distribution especially at the entrance of the channel.

DCD shows a unique distribution of charges, reflecting peptide oligomers that occur both in soluble and membrane-bound forms. The entire hexameric channel comprises 96 ionizable residues, which are all oriented toward the channel interior (Fig. 1C, F, and G). This enormous charge density, which is not completely shielded to the exterior, is likely to contribute to the relatively high aqueous solubility of DCD. The inner space of the peptide channel has an apparent separation into five radially symmetric charge girdles I/II/III/II/I (Fig. 1C). Residues facing

the acyl chain region of the membrane are exclusively hydrophobic (Ala, Val, Leu) (Fig. 1D and Fig. S1A). However, no aromatic residues are found that are commonly considered to be important for the lateral adjustment of proteins in membranes (20, 21). The channel diameter is not homogeneous and varies along the *y* axis with two rather narrow entry sites, followed by a widened interior with windowlike eyelets in the IF1 interface (Fig. 1A, E, and H). The six lateral openings have a diameter of  $\sim 1$  nm and are surrounded by small amino acids as well as positively charged residues that may have an influence on the selection of ion entry. The distance between two adjacent eyelets is 2.5 (same plane) and 3 nm (opposite plane), respectively, roughly corresponding to the width of the membrane hydrophobic core.

**Interaction of DCD with Lipid Bilayers.** To investigate the interaction of DCD with bilayers, we conducted ss-NMR spectroscopy experiments. The structural preferences of DCD were investigated after labeling of the peptide with  $^{15}\text{N}$  at the Gly22 position and  $^2\text{H}_3$ -Ala at position 25, its reconstitution into oriented lipid bilayers and investigation by proton-decoupled  $^{15}\text{N}$  ss-NMR spectroscopy. DCD labeled with  $^{15}\text{N}$  at Gly22 exhibits a  $^{15}\text{N}$  chemical shift of  $(62 \pm 2)$  ppm in oriented 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleoyl-



**Fig. 1.** Crystal structure and surface characteristics of the human dermcidin channel. (A) X-ray structure of the hexameric DCD channel shown in cartoon representation from the side and top (*Middle and Right*), and as surface representation (*Left*). The different orientations of the individual peptides relative to the membrane normal are marked in orange and dark blue, and termini are marked (NT, N terminus; CT, C terminus). Arrows combined with tilt angle and axes give the relative orientation. Residues involved in Zn binding are shown in stick representation and Zn ions are marked in gray. The symmetry axis of the channel is marked with C2 (for the side view) and C3 (for the top view). Two interfaces of different surface area are formed after trimerization and named IF1 and IF2. (B) Close-up into the Zn-binding site S1. Four residues (Glu5 and Asp11 from one peptide and Asp41 and His38 from the second) form each Zn-binding site. The distance between the Zn ions is marked by arrows. (C) Electrostatic surface representation of the channel with two monomers marked in ribbon representation. The channel comprises five alternating patches of elongated negative (red) and ring-like positive (blue) charge. (D) Side view of DCD (hydrophobic residues in magenta). (E) Ribbon model of DCD. The pore diameter is represented by spheres. The lateral entry points are marked with circles. (F) Hydrophilic residues on the trimeric interface (negatively charged residues in red, positively charged residues in blue, polar residues in green). Nonpolar residues are shown in white. (G) The hydrophilic channel interior. For clarity, the front dimer is omitted; colors as in F. (H) Channel radius along the pore axis.

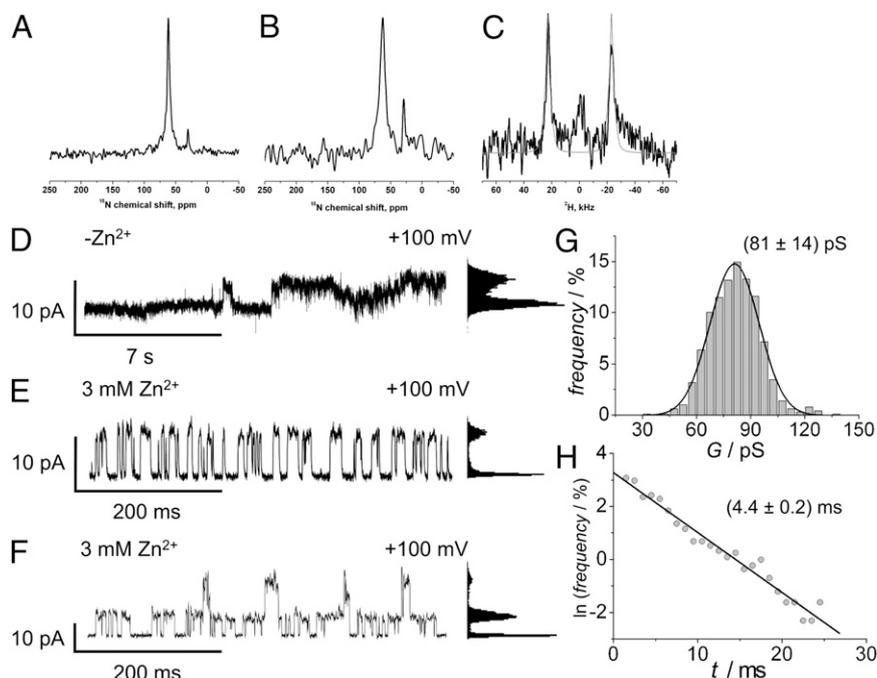
*sn*-glycero-3-phospho(1'-*rac*-glycerol) (POPE/POPG, 3:1) membranes both in the absence or presence of a 10-fold excess of  $Zn^{2+}$  (Fig. 2 *A* and *B*). This indicates an in-planar alignment of the major population of the labeled peptide domain (22), in agreement with previous oriented circular dichroism data (16). The chemical shifts are within experimental error identical in POPE/POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and DPhPC/cholesterol 9:1 membranes and also independent of zinc ions ( $ZnCl_2$  salt) and the sample preparation protocol. The  $^2H$  ss-NMR spectrum of the  $^2H_3$ -Ala25 labeled site exhibits a  $^2H$  quadrupolar splitting of  $(45 \pm 3)$  kHz (Fig. 2*C*) and further restricts the tilt and pitch angle (23). A detailed topological analysis is shown by the red and black traces in Fig. S2 for the  $^{15}N$  chemical shift and the  $^2H$  quadrupolar splitting, respectively (see ref. 23). Both NMR parameters agree when their corresponding traces intersect leading to a set of tilt/pitch angular pairs in the proximity of  $(90^\circ/90^\circ)$ , which would represent an ideal alignment of the amphipathic helix with the membrane interface (Fig. 2*B*). In agreement, the  $^2H$  spectra of the fatty acyl chains show pronounced membrane disordering by the presence of DCD (Fig. S3).

**Functional Properties of the Channel Highlight the Importance of Zinc as Cofactor.** The solid-state NMR data represent probably the lowest energy state after extensive equilibration of the major population of the peptide and, in particular, in the absence of a membrane potential. In contrast, electrophysiological recordings monitor a population that may occur as a minor, but functionally relevant, species under the application of a transmembrane voltage. To obtain functional evidence for the activity of DCD channels in the membrane, we collected conductivity data in planar lipid bilayers. First, membranes were prepared in 1 M NaCl, 5 mM Hepes, pH 7.1 in the absence of  $Zn^{2+}$ . After the addition of DCD

in micromolar concentrations, two out of the six membranes showed weak activity of the peptide (Fig. 2*D*). Altogether, only 20 current steps were observed, from which a mean conductance of  $G = (31 \pm 8)$  pS was evaluated.

By contrast, in the presence of  $Zn^{2+}$ , the addition of DCD at concentrations of 850 nM or higher resulted in current fluctuations for every membrane preparation, which eventually led to rupture of the membrane (Fig. 2*E* and *F*). These concentrations are similar to DCD concentrations used to efficiently remove bacteria in antimicrobial assays. Evaluation of 1,009 events revealed a mean conductance of  $G = (81 \pm 14)$  pS (Fig. 2*G*) and the mean open lifetime was determined to be  $\tau = (4.4 \pm 0.2)$  ms (Fig. 2*H*). This shows that, in the presence of zinc ions, specific and individual channels with a defined conductance are formed in the membrane bilayer. Neither the membrane with DCD alone nor the addition of only  $Zn^{2+}$  gave rise to the occurrence of individual channels in the electrophysiology experiments. However, we found that the polarity of the electric field had a significant impact on the efficiency of DCD- $Zn^{2+}$  insertion.

To further investigate the importance of the specific  $Zn^{2+}$  interactions and their impact on the active membrane-bound form of DCD, we mutated His38, one of the main interaction partners of DCD with  $Zn^{2+}$ , to alanine and studied this mutant through electrophysiology. The single mutation was sufficient to abolish channel formation in membranes (Fig. S4). This finding is most compatible with the notion that the assembled structure, which is characterized by specific  $Zn^{2+}$ -conferred peptide-peptide interactions, is the most prominent membrane-disruptive form. It is important to note that the current fluctuations seen for DCD- $Zn^{2+}$  in electrophysiology are representative of a small number of channels efficiently conducting ions across membranes,

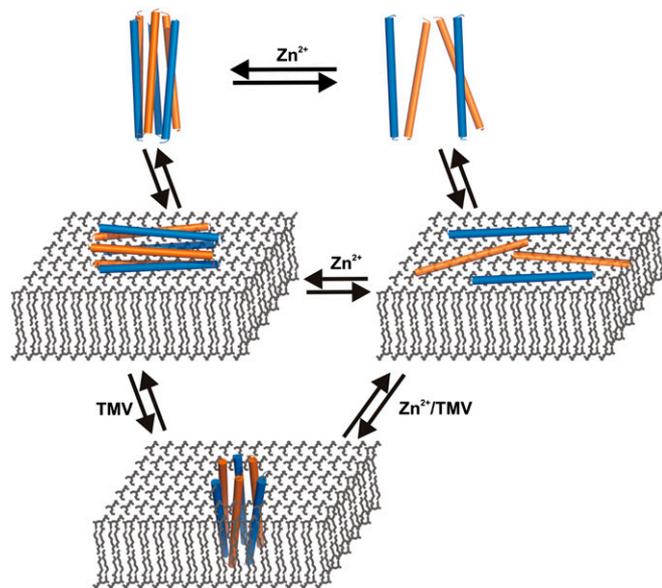


**Fig. 2.** Electrophysiology and ss-NMR of DCD in the presence and absence of zinc ions. (*A* and *B*) Proton-decoupled  $^{15}N$  and (*C*)  $^2H$  ss-NMR spectra of 2 mol % [ $^{15}N$ -Gly22, $^2H_3$ -Ala25]-DCD in oriented POPE/POPG without  $ZnCl_2$  (*A*) and with fivefold molar excess of  $ZnCl_2$  relative to the peptide concentration (*B* and *C*).  $^{15}NH_4Cl$  (40.0 ppm) and  $^2H_2O$  (0 Hz) were used as external references and the spectra processed with an exponential line broadening of 50 (*A* and *B*) and 500 Hz (*C*), respectively. The gray line in *C* shows a simulated spectrum arising from a  $3^\circ$  Gaussian distribution for the peptide alignment and a spectral line width of 2 kHz. (*D*) Current trace and point amplitude histogram representing DCD activity in 1 M NaCl, 5 mM Hepes, pH 7.1 recorded at a holding potential of +100 mV. Protein activity was only rarely observed under these conditions. (*E* and *F*) Current traces and point amplitude histograms of DCD-1L in 1 M NaCl, 3 mM  $ZnCl_2$ , 5 mM Hepes, pH 7.1 recorded at a holding potential of +100 mV; (*E*) 3.3  $\mu M$  DCD-1L was added resulting in one defined conductance state. In (*F*) 7.9  $\mu M$  DCD-1L was used and two distinct conductance levels each of about 80 pS were monitored, suggesting the insertion of two channels. (*G*) An event histogram of the conductance levels ( $n = 1,009$ ) shows a mean conductance of  $G = (81 \pm 14)$  pS. (*H*) Open lifetime analysis with  $\tau = (4.4 \pm 0.2)$  ms.



Here, we present a comprehensive functional mechanism of a human antimicrobial peptide in its active form, including its high-resolution structure, single-channel functional measurements, and atomistic simulations of permeation. In the absence of transmembrane voltage (TMV), in-plane alignments have been observed for DCD and other helical antimicrobial peptides such as magainins or cecropins (Fig. 2 A–C; reviewed in ref. 35). However, important differences exist between the latter peptides and DCD, already when their size and charge are compared. Of note is that when TMV is applied, the channel recordings reveal mostly membrane lysis in the case of magainins or cecropins, and only rarely are the stepwise conduction increases clearly observed with DCD. The electrophysiological recordings of lysis or stochastic membrane deformations, however, are erratic, highly variable, and characterized by considerable fluctuations. Therefore, it is likely that the tilted hexamer of DCD provides an example for a hitherto undescribed mechanism for the membrane interactions of antimicrobial peptides (for reviews of other mechanisms, see ref. 36). The ensemble of data from the herein and previously presented studies (16) suggest that DCD occurs in a number of states that are interconnected by sensitive equilibria as illustrated in Fig. 4, including oligomers and monomers in solution, as well as monomers or small oligomers lying parallel to the membrane surface (Fig. 2 A–C). Furthermore, transmembrane potentials and the presence of  $Zn^{2+}$ , by neutralizing the anionic charges of the DCD polypeptides, favor the formation of oligomeric structures spanning the membrane (Fig. 2 D–H). These channels display a conductance of about 80 to 110 pS.

Although the suggested mechanisms of AMP action have so far remained largely speculative (4, 9, 10), our study on DCD provides a structurally detailed and experimentally validated mechanism for membrane perturbation by an AMP. It is striking that none of the currently debated models explicitly predict this channel. Our X-ray, electrophysiology, and simulation data show a barrel-stave-like channel (10), which is held together by divalent



**Fig. 4.** Model of DCD interactions with membranes. Peptide monomers of DCD are in equilibrium with the hexameric channel form, which is stabilized by the presence of zinc. The channel and DCD monomers can interact with membrane surfaces through lateral association as shown by our NMR studies. The channel can be translocated into the membrane upon application of a TMV, as shown in planar lipid membrane conductance measurements, and forms a representative of the barrel-stave model. Once the channel is integrated into a membrane, channel conductance can be recorded by planar lipid membrane experiments and estimated by molecular dynamics studies.

cations and displays a high permeability for water and ions, as ions can enter and exit through side eyelets of a tilted pore. DCD thereby forms a site of severe membrane disruption. As recently noted, a single ion channel with a conductance near 100 pS can dissipate the bacterial transmembrane potential on a time scale of as little as  $10^{-4}$  s (37). The DCD channel characterized here thus represents highly efficient channels, and even a few of which are capable of rapidly abrogating the bacterial transmembrane potential that is essential for cell survival. Its structure and functional mechanism may form a foundation for the rational design of antibacterial, peptide-derived drugs that are less liable to evoke resistance.

## Material and Methods

**Crystallization and Structure Determination.** The DCD peptide sequence (Fig. S1) was synthesized by the company Peptide2.0 ([www.peptide2.com](http://www.peptide2.com)) to yield a product of >97% purity. For crystallization, the peptide was solved in 10 mM Hepes at a concentration of 50 mg/mL (~10 mM final concentration). Crystallization drops were prepared by mixing 400 nL of the peptide solution with the same amount of reservoir, and drops were incubated by vapor diffusion (sitting drops) under a variety of different conditions (800 conditions from Qiagen screens). Two conditions, both of which contained Zn-acetate, yielded crystals suitable for X-ray diffraction analysis. Crystals for X-ray diffraction were taken from a drop containing 0.2 M Zn(ac)<sub>2</sub>, 0.1 M Na-cacodylate, 18% (wt/vol) PEG8000 at pH 6.5. The channel has been crystallized in the absence of lipophilic molecules such as detergents or lipids. Crystals were taken from the mother liquor and flash-frozen in liquid nitrogen without adding cryoprotectant. Data were collected at the Swiss Light Source, beamline PXII at 100 K and 20 eV above the theoretical Zn-edge (9.658 keV). Three hundred sixty 1° images were collected at 2% beam intensity to generate a highly redundant dataset for single anomalous dispersion data processing. Diffraction data were processed with XDS/XSCALE (38). The structure was solved with the Phenix program package (39) with Phaser (38) for heavy atom detection and Resolve (40) for initial solvent flattening. Solvent flattening was repeated by Pirate of the CCP4 suite (41) and a model 85% complete was built with Buccaneer (42). The final model was obtained after several rounds of manual rebuilding and refinement with Coot (43), REFMAC (44), and PHENIX (39). For more details see *SI Materials and Methods* and Table S1.

**Solid-State NMR Spectroscopy.** Samples were prepared by dissolving 4 mg of peptide and ~30 mg of lipid in TFE/water 50/50 vol/vol, dried in air and under high vacuum, applied onto 20 ultra-thin cover glasses, and equilibrated at 93% relative humidity, as described previously (45). Where appropriate, ZnCl<sub>2</sub> was added in five- to ten-fold molar excess over the peptide. As an alternate, peptide and ZnCl<sub>2</sub> were added to preformed vesicles, and the resulting mixture was applied onto the glass plates (45). The ss-NMR spectra were recorded on a Bruker Avance wide-bore NMR spectrometer operating at 9.4 T with flat-coil probes as described previously (46). Orientational restraints were calculated from the solid-state NMR spectra as described in (46) by using the chemical shift tensor (44, 65, 211) ppm, a maximum quadrupolar splitting of 74 kHz for the alanine <sup>2</sup>H<sub>3</sub>C group and DCD coordinates from the crystal structure. For further details of ss-NMR methods and data analysis see *SI Materials and Methods*.

**Electrophysiology.** Giant unilamellar vesicles (GUVs) composed of DPhPC/cholesterol (9:1) were prepared by the electroformation method with a 3-V peak-to-peak AC voltage at a frequency of 5 Hz for 2 h at 20 °C in the presence of 1 M sorbitol (47–49). A freestanding membrane was prepared by spreading a GUV in 1 M NaCl, 5 mM Hepes, pH 7.1 on an aperture in a borosilicate chip (Port-a-Patch; Nanion Technologies) by applying 15–40-mbar negative pressure resulting in a solvent-free membrane with a resistance in the GΩ range. After successful membrane formation, varying amounts of DCD stock solution (100 μM) were added to 50-μL buffer solution, while applying a DC potential of +100 mV. Current traces were recorded at a sampling rate of 50 kHz using an Axopatch 200B amplifier (Axon Instruments). They were filtered with a low-pass four-pole Bessel filter of 1 kHz and digitized by an A/D converter (Digidata 1322; Axon Instruments). Data evaluation was performed with the pClamp 9 software package (Axon Instruments). For additional details see *SI Materials and Methods*.

**Molecular Dynamics and Computational Electrophysiology Simulations.** All MD simulations were performed with the GROMACS package, version 4.5 (50) in combination with the CHARMM36 force field (51). Unless otherwise stated, the simulation temperature was 310 K. The protein, lipids, and water/ions

were coupled separately to a temperature bath with the v-rescale method with a time constant of 0.1 ps (52). Short-range electrostatics were calculated with a cutoff of 1.3 nm. Long-range electrostatics were treated with the particle-mesh Ewald method (53). Short-range Van der Waals (VdW) interactions were calculated explicitly up to a distance of 0.8 nm, beyond which a switch function was used to smoothly switch off the VdW interactions to reach 0 at 1.2 nm. All bonds were constrained using the LINCS method (54). The time step was 2 fs for all-atom MD simulations and 4 fs for simulations with the virtual site model for hydrogen atoms (55), respectively. To study whether the oligomer is ion conductive, we used the computational electrophysiology method (26). For additional details see *SI Materials and Methods*.

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