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Functional truncated membrane pores

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Membrane proteins are generally divided into two classes. Integral proteins span the lipid bilayer, and peripheral proteins are located at the membrane surface. Here, we provide evidence for membrane proteins of a third class that stabilize lipid pores, most probably as toroidal structures. We examined mutants of the staphylococcal α-hemolysin pore so severely truncated that the protein cannot span a bilayer. Nonetheless, the doughnut-like structures elicited well-defined transmembrane ionic currents by inducing pore formation in the underlying lipid. The formation of lipid pores, produced here by a structurally defined protein, is supported by the lipid and voltage dependences of pore formation, and by molecular dynamics simulations. We discuss the role of stabilized lipid pores in amyloid disease, the action of antimicrobial peptides, and the assembly of the membrane-attack complexes of the immune system.

alpha-hemolysin | beta-barrel | lipid reorganization | nanopore

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ells, and compartments within cells, are bounded by membranes, which are based on lipid bilayers. The overall width of a lipid bilayer from lipid head group to lipid head group is around 40 Å, with a hydrocarbon core that is about 30 Å across (1) (SI Appendix, Table S1). Proteins associated with membranes are usually divided into two classes. Integral proteins span the lipid bilayer, and peripheral proteins are located at the membrane surface, bound in many cases to integral proteins (2). Here we provide evidence for a third class of membrane proteins that stabilize lipid pores. These pores are most likely toroids, i.e., holes in the bilayer with a roughly circular cross-section and lined by lipid head groups. By contrast, integral membrane proteins are generally believed to span bilayers by displacing lipid molecules laterally rather than by reorganizing the bilayer structure. Toroidal lipid pores have been proposed previously in several contexts, for example for antimicrobial peptides (3, 4), but without a firm structural basis for the peptide or protein component.

We have explored mutants of the heptameric staphylococcal α-hemolysin (αHL) pore (5) in which the transmembrane β-barrel is so severely truncated that it cannot span a lipid bilayer (Fig. L4). The barrel comprises 14 β-strands, two contributed by each of the seven subunits (5). Like the wild-type (WT) αHL pore, the truncated pores form conductive channels in lipid bilayers. The conductive pathway must traverse the center of the doughnut-like truncated structure and continue through the reorganized lipid bilayer. Further, the existence of these lipid pores is supported by the lipid and voltage dependences of pore formation, and by molecular dynamics simulations. Our observation of lipid pores induced by structurally defined proteins is of importance in several areas including amyloid disease (6, 7), the action of antimicrobial agents (8, 9), and the assembly of membrane-attack complexes of the immune system (10).

Results

Heptamertric α-Hemolysin Pores with Truncated β-Barrels. Truncated barrel mutants (TBM) were made from the αHL NN mutant (Fig. L4) (11) by pairwise removal of amino acids from both β-strands to yield barrels shortened by 2, 4, 6, 8, and 10 amino acids (Fig. 1 B and C). Although the full-length αHL NN monomer is stable in solution and assembles into heptamers only in the presence of rabbit erythrocyte membranes (rRBCm), the truncated αHL polypeptides assembled spontaneously during cell-free synthesis as judged by the formation of SDS-resistant oligomers (Fig. 1 D and E) that also resisted proteolysis (Fig. 1 F). The latter properties are characteristic of the heptameric αHL pore containing full-length subunits. Native gel electrophoresis showed that TBMΔ2, Δ4, Δ6, and Δ8 formed structures with mobilities similar to the αHL NN heptamer, and TBMΔ10 formed a mixture of oligomeric structures. TBMΔ2 and Δ4 showed only weak hemolytic activity toward rabbit erythrocytes, and TBMΔ6, Δ8, and Δ10 were inactive (SI Appendix, Fig. S1), suggesting that the spontaneously formed heptamers cannot penetrate erythrocyte membranes, which is also the case for WT αHL heptamers (as opposed to WT monomers) (12).

Truncated α-Hemolysin Pores Conduct Ions Across Lipid Bilayers. TBMΔ2, Δ4, Δ6, and Δ8 pores were assembled in the presence of rRBCm, purified by SDS–polyacrylamide gel electrophoresis and examined by planar lipid bilayer recording (Fig. 24 and SI Appendix, Fig. S2). TBMΔ2 and Δ4 exhibited increased unitary conductance values compared with the full-length αHL NN pore, which would be expected for shortened pores based on the behavior of an electrolyte in a cylinder (13). By contrast, the unitary conductance values of TBMΔ6 and Δ8 were reduced, which is suggestive of a constricted or elongated conductive pathway (13). TBMΔ4 and Δ6 showed excess current noise by comparison with αHL NN (Fig. 2A), indicating conformational flexibility of these pores (SI Appendix, Table S2).

Significance

Cells and compartments within them are bounded by membranes, the underlying structure of which is the lipid bilayer. Molecules that move into and out of cells or between compartments are transported through protein channels and pores, which according to conventional thinking completely span the intact bilayer. However, indirect evidence has suggested the existence of a separate class of membrane proteins that perturb the bilayer structure to create transmembrane conduits. We present direct evidence for such proteins, and suggest that they are protagonists in important physiological processes, including defense by the immune system against microorganisms and neuronal damage in Alzheimer’s disease.

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TBMAΔ8 was examined in more detail. When the lipid bilayer was formed from 100% 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), the TBMAΔ8 pore opened and closed in bursts at +200 mV (Fig. 2B). The mean closed time was 13 ± 4 s, and the mean open time was 1.3 ± 0.3 s (n = 3 experiments, total of 490 events). By contrast, when the lipid was DPhPC containing 10% 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), the TBMAΔ8 pore remained open almost continuously at +200 mV, with frequent spikes toward zero current (Fig. 2C). DLPC has shorter fatty acyl chains than DPhPC and the chains are un-branched (Fig. 2C and SI Appendix, Fig. S10). Therefore, this experiment suggests that the bilayer thins in the vicinity of the pore, possibly forming a toroidal lipid ring. The latter structure is favored by DLPC (Fig. 2C), which has a greater tendency than DPhPC to form micelles with curved surfaces (14, 15). The TBMAΔ8 pore also closed at low applied membrane potentials (SI Appendix, Fig. S3), supporting the idea of a lipid toroid, which is favored at high potentials (16–18).

**The Truncated Pores Bind Cyclodextrin Adapters.** Cyclodextrins (CD) bind within the β-barrel of the WT αHL pore (19–21), and binding is sensitive to small perturbations in the structure of the pore (21) or the cyclodextrin itself (22). To test the integrity of the barrel in TBM mutants, we measured the association and dissociation rate constants of β-cyclodextrin (βCD), heptakis-(6-deoxy-6-amino)-β-cyclodextrin (amβCD) and γ-cyclodextrin (γCD) (SI Appendix, Figs. S4–S7). The binding of γCD showed a clear decrease in affinity (increase in $K_D$) as the pores became shorter. This is reasonable, because the bulky γCD may bind at the lower cyclodextrin site near residue 139 (20), rather than at the upper site near the central constriction (Fig. 1A; ref. 21). The effects of truncation on βCD and amβCD binding were less marked in the TBMAΔ2 and TBMAΔ4 pores, whereas the affinity for TBMAΔ6 pore was significantly reduced. TBMAΔ8 and TBMAΔ10 do not bind the cyclodextrins. We display representative current traces showing the interaction of βCD, amβCD, and γCD with TBMAΔ6 (Fig. 2D). It is interesting to note that although TBMAΔ6 binds amβCD weakly...
lipid molecules were normal to the plane of the bilayer. The system was solvated and $K^+$ and $Cl^-$ ions were added to give 0.5 M KCl. Seven additional $Cl^-$ ions were added to neutralize the positive charge on the protein. Steepest descent energy minimization and 4 ns of preconditioning MD were run with a constant number of particles and at constant volume. At 0 ns after preconditioning, the protein sat in a thinned bilayer in which the $trans$ opening of the truncated $\beta$-barrel was not occluded by lipids (Fig. 3A, Left and SI Appendix, Fig. S8A). In the case of TBM\(\Delta6\), after 400 ns, the bilayer assumed a largely toroidal character with the lipid head groups lining the bottom part of the conductive pathway. The radial distribution of the DLPC molecules was determined at 0, 400, 800, and 1,600 ns (Fig. 3A and SI Appendix, Fig. S8). The mole fraction of DLPC remained at ~10% at distances more than 4 nm from the central axis of the protein for the entire simulation (Fig. 3). However, after 800 ns, the mole
fraction of DLPC in the vicinity of TBMΔ6 rose to 30% (Fig. 3A), i.e., DLPC became concentrated in the toroid. TBMΔ8 showed reduced affinity for the lipid bilayer, and was extruded onto the bilayer surface (SI Appendix, Fig. S8B), consistent with the frequent closures observed for TBMΔ8 at low applied potentials (Fig. 2B and SI Appendix, Fig. S3). However, we cannot distinguish between various mechanisms for the opening and closing of the TBM pores in electrical recordings. Gating could be caused by the truncated end of the pore collapsing and reopening, or by pores leaving the bilayer, as seen in the MD simulations of TBMΔ8, and reinserting into it.

**Short-Chain Fluorinated Amphiphiles Facilitate Pore Formation.** The interaction of TBMΔ8 with bilayers containing one fluorinated leaflet was examined by using droplet interface bilayers (DIBs) (24). In DIBs formed from two DPhPC leaflets, TBMΔ8 behaved in much the same way as it did in conventional planar lipid bilayers of the same composition, producing pores with short-lived openings that closed at applied potentials below +100 mV (Fig. 3B). In asymmetric bilayers (25) with one leaflet of DPhPC and the other comprising the short single-chain fluorinated amphiphile fluorinated fos-choline (F–FC; Fig. 3B), TBMΔ8 added from the DPhPC side of the bilayer formed long-lived pores, which stayed open at low applied potentials (±25 to ±100 mV); mean open times 2.2 ± 0.8 s (−25 mV), 6.4 ± 4.4 s (+25 mV); mean closed times 190 ± 40 ms (−25 mV), 46 ± 17 ms (+25 mV). TBMΔ8 inserted into the asymmetric bilayers from the DPhPC side far more readily than it inserted into symmetric DPhPC (i.e., at 25 times higher dilution). By contrast, TBMΔ8 inserted into the F–FC side of the asymmetric bilayer only when it was applied at 50 concentrations the required for the DPhPC side, and in this case the pores that formed were short lived. In accord with the idea of a lipid toroid, the presence of the wedge-shaped F–FC in the trans leaflet of the asymmetric bilayer facilitated pore opening and led to longer open times, by comparison with symmetrical bilayers with two DPhPC leaflets.

**Discussion**

Here, we have explored the functional properties of a series of truncation mutants of the heptameric staphylococcal αHL pore. Our results indicate that, except for the deleted residues, the truncated pores (TBMΔ2 to Δ8) have structures that are closely similar to that of the full-length heptameric αHL pore (5). The truncated pores are SDS and protease resistant indicating that they are correctly folded. They have mobilities in SDS and native gels that are similar to that of the full-length heptamer indicating that they too are heptamers. Additional evidence indicates that the internal structures of the truncated pores are similar to that of the full-length heptamer: TBMΔ2 to Δ6 bind cyclodextrins (in particular, the mutant TBMΔ6/M113F binds amyl-βCD very tightly) and the unitary conductance values of the pores are in the same range as that of the full-length pore.

TBMΔ6 and TBMΔ8, which are missing a remarkable 12 and 16 amino acids per subunit, form pores in planar lipid bilayers. We suggest that an open TBM protein ring sits on the bilayer surface and stabilizes a lipid pore, most likely in the form of a toroidal defect. In keeping with the latter, the conductance values of the pores that formed were short lived. In accord with the idea of a lipid toroid, the presence of the wedge-shaped F–FC in the trans leaflet of the asymmetric bilayer facilitated pore opening and led to longer open times, by comparison with symmetrical bilayers with two DPhPC leaflets.

**Fig. 4.** Lipid pores in assembly intermediates and functional pores. (A) A revised model for the assembly of pore-forming toxins and related membrane proteins. (i) A monomeric toxin, such as αHL (depicted), binds to the bilayer surface (ii). This step may be receptor mediated (46). (iii) The membrane-bound monomer assembles (47) into an oligomeric prepore (35, 36), which does not penetrate the lipid bilayer. In the case of αHL, the prepore (and pore) is a heptamer. In some cases, prepore formation may occur in the bulk aqueous phase (48), before binding to the bilayer surface. (iv) The prepore induces lipid pore formation as described in the present paper. (v) The transmembrane β-barrel is formed when polypeptide sequences stored in the central cavity of the prepore descend into the aperture provided by the lipid pore. (B, i) Annular structures formed by short pentapeptide sequences stored in the central cavity of the prepore descend into the aperture provided by the lipid pore. (B, ii) The phi29 connector (PDB ID code 1F0U) (43), (iii) The dodecameric SP1 ring (PDB ID code 1TR0) (44), (iv) Ring formed by the TatA protein from the twin-arginine transport system (PDB ID code 2L2S) (45). Negatively charged residues are colored in red and positively charged residues in blue.
ClyA (38) and FraC (39). In the case of αHL, the prepore resembles the truncated heptameric structures of the present work, except that the cavity within the cap domain contains the poly-peptide sequences that go on to form the membrane-spanning β-barrel. We now suggest an elaboration of the assembly model in which a transient lipid pore appears in the bilayer beneath the prepore structure. On the first opening of this pore, or perhaps during subsequent openings, the barrel-forming residues move into the aperture, and irreversible barrel formation occurs without the need to displace bilayer lipid (Fig. 4A).

Other pores might assemble through a similar pathway including the membrane-attack complexes and perforin pores of the immune system (10). However, other pore-forming proteins might stabilize lipid pores as the endpoint in their assembly, including the proteins involved in permeabilizing mitochondria during apoptosis (40, 41) and the membrane-active bacteriocins, such as colicin E1 (42). Peptide antimicrobial agents, such as magainins, have also been proposed to stabilize or line toroidal pores (3, 4, 8, 9). It is also possible that the annular amyloid peptide aggregates that have been suggested to mimic bacterial pore-forming toxins (6, 7) operate through a lipid-pore mechanism (Fig. 4B, i). Protein rings that permeabilize bilayers but do not have a hydrophobic surface may also function from on top of the bilayer, including the phi29 connector (43) (Fig. 4B, ii) and the dodecameric SPI (44) (Fig. 4B, iii). Rings too thin to span a bilayer, such as the TatA protein from the twin-arginine transport system (45), may act in a similar way (Fig. 4B, iv).

Materials and Methods

Protein Procedures. The αHL truncated barrel mutants were generated from the NNαHL gene (11) in a pIT7 vector by PCR mutagenesis and ligation-free in vivo recombination. The αHL polypeptides were obtained by in vitro transcription and translation; the supernatant was used after centrifugation at 25,000 rpm. Hemolytic assays were carried out by monitoring the decrease in light scattered at 595 nm with a microplate reader. Oligomeric structures formed by TBD and their sensitivity to SDS were examined with the NativePAGE gel system (Invitrogen, Ltd.). The products of limited proteolysis with proteinase K were analyzed with 12% SDS-polyacrylamide gels.

Electrical Recordings. Ionic current measurements were performed with a conventional planar bilayer apparatus, except for the study of fluorinated bilayers, which was carried out with droplet interface bilayers (24). Data were analyzed and prepared for presentation with pClamp (version 10.1, Molecular Devices). OriginPro 8 was used for further analysis.

MD Simulations. Coarse-grained molecular dynamics simulations were performed placing the TMβα16 and TMβα18 αHL pores in a simulation box of 18 x 18 x 18 nm dimensions, containing an equilibrated 90 mol% DPPC / 10 mol% DLPC bilayer. K⁺ and Cl⁻ ions were added to give a final concentration of 0.5 M KCl. The final system included ~60,000 beads. Simulation analysis was carried out with GROMACS software with periodic boundary conditions at a constant temperature and pressure and with a 20-fs time step. Full Methods and associated references are available in the SI Appendix.

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