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Ion-Channels: Goals for Function-Oriented Synthesis

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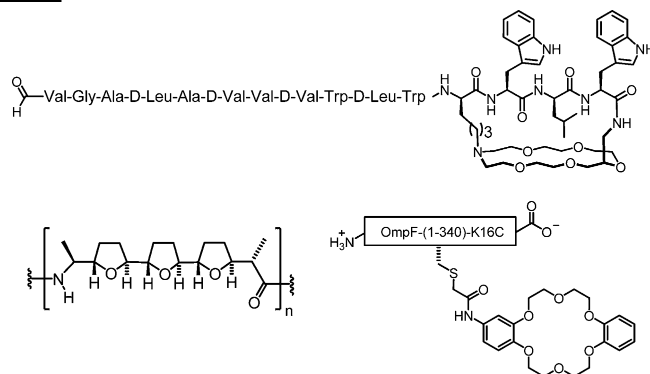

Ion channels provide a conductance pathway for the passive transport of ions across membranes. These functional molecules perform key tasks in biological systems such as neuronal signaling, muscular control, and sensing. Recently, function-oriented synthesis researchers began to focus on ion channels with the goal of modifying the function of existing ion channels (ion selectivity, gating) or creating new channels with novel functions. Both approaches, ion channel engineering and de novo design, have involved synthetic chemists, biochemists, structural biologists, and neurochemists.

Researchers characterize the function of ion channels by measuring their conductance in samples of biological membranes (patch clamp) or artificial membranes (planar lipid bilayers). At the single molecule level, these measurements require special attention to the purity of the sample, a challenge that synthetic chemists should be aware of. Ideally, researchers study the function of channels while also acquiring structural data (X-ray, NMR) to understand and predict how synthetic modifications alter channel function.

Long-term oriented researchers would like to apply synthetic ion channels to single molecule sensing and to implantat these synthetic systems in living organisms as tools or for the treatment of channelopathies. In this Account, we discuss our own work on synthetic ion channels and explain the shift of our research focus from a de novo design of oligo-THFs and oligo-THF-amino acids to ion channel engineering. We introduce details about two biological lead structures for ion channel engineering: the gramicidin $\beta^{6,3}$ helix as an example of a channel with a narrow ion conductance pathway and the outer membrane porins (OmpF, OmpG) with their open β-barrel structure. The increase and the reversal of ion selectivity of these systems and the hydrophobic match/mismatch of the channel with the phospholipid bilayer are of particular interest. For engineering ion channels, we need to supplement the single-point attachment of a synthetic modulator with the synthesis of a more challenging two-point attachment.

The successful function-oriented synthesis of ion channels will require interdisciplinary efforts that include new electrophysiology techniques, efficient synthesis (peptide/protein/organic), and good structural analysis.

Introduction

Function has developed into a core theme for Chemical Synthesis over the last two decades. The transfer of function from biology - its original field - into chemistry was initiated and pioneered by supramolecular chemistry.[1] Supramolecular Chemistry has made recognition and transport equally as important as catalysis and reactivity. Historically, the synthesis of enzyme inhibitors for medical purposes was another source for interdiciplinary research between biology and chemistry. Pharmaceutical/Medicinal chemistry was always focused on (medical) function. Today, chemical synthesis is oriented toward two main

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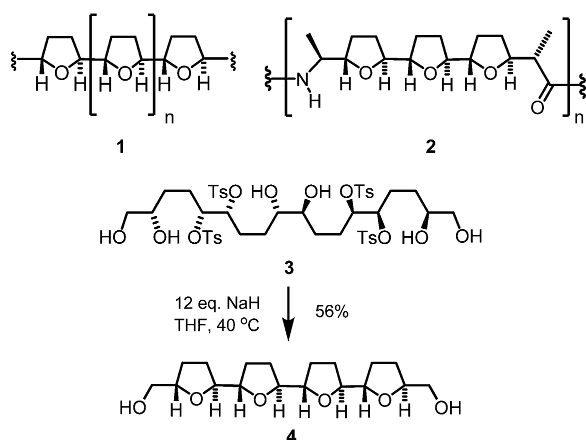

FIGURE 1. De novo design of transmembrane ion channels: structures of *trans* oligo-tetrahydrofurans **1** and ter-THF-peptides **2**; multiple Williamson reaction (**3** → **4**) for the stereoselective synthesis of oligo-tetrahydrofurans.

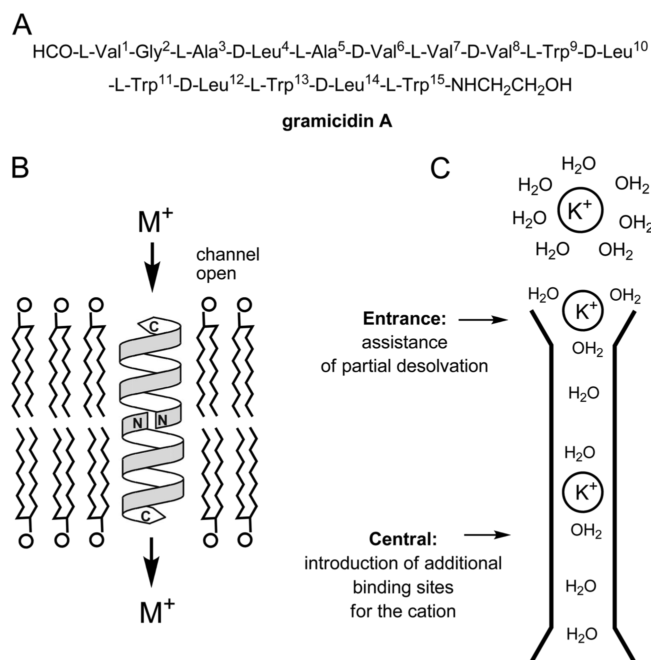

FIGURE 2. (A) Structure of the pentadecapeptide gramicidin A. (B) Schematic representation of the dimeric β-helical secondary structure of gramicidin a in a membrane environment. This structure corresponds to the ion-channel active conformation (open state). (C) Passage of a hydrated potassium ion through the narrow ion-conductance pathway of gramicidine A and points of operation for function-oriented synthesis: assistance of partial desolvation at the channel entrance and introduction of additional binding sites for the cation in the central part of the channel.

areas of functional significance: life sciences and material sciences.

This Account describes our own efforts in function-oriented synthesis in the area of transmembrane ion channels. Recent contributions from various other groups have been covered in excellent reviews.[2]

Biological ion channels have key functions in muscular control, neuronal signal transduction, and sensing.[3] They have evolutionarily developed as a response to compartment formation in multicellular organisms to allow a controlled passive transport of ions across the phospholipid bilayer of biological membranes. In addition to highly ion-selective channels with a narrow ion-conductance pathway, wider pores for the transport of ions and polar molecules are known. A distinction must been made between the passive transport of ion channels/pores and active transporters/ion pumps. The driving force for passive ion transport through channels and pores is an ion concentration gradient at the membrane and its associated transmembrane potential. Gating of biological ion channels occurs via recognition of molecular signals (ligand gating), change in the membrane potential (voltage gating), mechanical/conformational changes (mechano-sensitive channels), and (temporarily) covalent modification, for example, phosphorylation.

De Novo Design or Ion Channel Engineering? The de novo synthesis of a structure that can span the 2.5−3 nm hydrophobic part of a membrane is a challenging task. Two possible solutions are dimerization/oligomerization or a self-assembly approach. It is beyond the scope of this Account to discuss valuable other contributions along these lines, for example, of Kobuke and co-workers,[4] Gokel and co-workers,[5] Fyles et al.,[6] Voyer and co-workers,[7] and Matile et al.[8] Our contribution to the oligomerization route consisted of the synthesis of *trans* oligo-tetrahydrofurans **1** and *trans* ter-THF amino acids which were connected to oligo-THF-peptides **2** (Figure 1).[9,10]

Efficient stereoselective routes to oligo-2,5-tetrahydrofurans such as the multiple Williamson reaction **3** → **4** were developed.[11] These oligomeric compounds exhibited ion-transport properties; their structural analysis, however, was complicated, for example, by severe signal-overlay in the NMR spectra. A function-oriented synthesis without reliable correlation to structural data of the postulated channel structure (NMR, X-ray) is difficult. The lack of profound structural information for our channel-active oligomers moved us from de novo design into the area of engineering channel structures known from biological systems which can be analyzed by NMR and/or X-ray structural analysis. Two different types of channels were selected for function-oriented synthetic modifications. One with a narrow ion-conductance pathway, where partial ion desolvation was a prerequisite for transport. The other was a wider pore with a

robust pore scaffold suitable for attachment of chemical modulators. Gramicidin A was chosen for the first type of channel and porins served as prototype for wide-pore channels.

Narrow Ion-Conductance Pathway: Gramicidin A

Gramicidin A isolated from *Bacillus brevis* is a 15 amino acid peptide with formyl-*N*- and aminoethanol-*C*-termini displaying alternate L- and D-amino acids which favors the formation of β-helical secondary structures (Figure 2A).[12] In a membrane environment, gramicidin A forms a head-to-head dimer of two right-handed single-stranded β helices with 6.3 residues per turn (Figure 2B).[13] Gramicidin A was used as the lead structure for ion-channel engineering by several other groups extensively.[14]

The pore diameter of the gramicidin $\beta^{6,3}$ helix is as narrow as 400 pm.[12] A fully hydrated cation has a diameter larger than this pore, therefore transport of cations through the gramicidin channel requires partial desolvation. Gramicidin A exhibits a low Eisenman I selectivity for monovalent cations ($Cs^+ > K^+ > Na^+ > Li^+$). For alkali metal cations the hydration energy decreases from lithium to cesium (Li^+, −131 kcal/mol; Na^+, −105 kcal/mol; K^+, −85 kcal/mol; Cs^+, −71 kcal/mol).[3] Partial desolvation is energetically favored for Cs^+ over Na^+. This is the reason for the ion selectivity Cs^+/K^+ for gramicidin A of 2:1. The transport of divalent cations such as Mg^{2+} or Ca^{2+} is blocked for the gramicidin channel, which is a direct consequence of the much higher desolvation energies. (hydration energy: Mg^{2+}, 476 kcal/mol; Ca^{2+}, 397 kcal/mol). Figure 2C illustrates the partial desolvation necessary for a potassium cation during the transport through the narrow gramicidin channel. At the channel entrance part of the hydration shell is ripped off. In the channel, the cation is coordinated only by one top and one bottom water molecule. The remaining coordination sites for the cation in the channel interior are provided by the amide bonds (π-orbital, O-lone pair). Function directed synthesis can modulate this process at two positions. First, additional binding sites such as oxygen lone pairs could be added at the central part of the channel. Second, by assisting the partial desolvation of the ions at the channel entrance.

The δ-amino acid **5** is a dipeptide mimetic that combines a conformational biased cyclohexane core with an ether oxygen offering its lone pair as cation binding site (Figure 3A).[15,16] Having developed a stereoselective synthesis for compound **5** using an asymmetric azide opening of cyclohexene epoxide, it was possible to introduce two units of this cyclohexylether

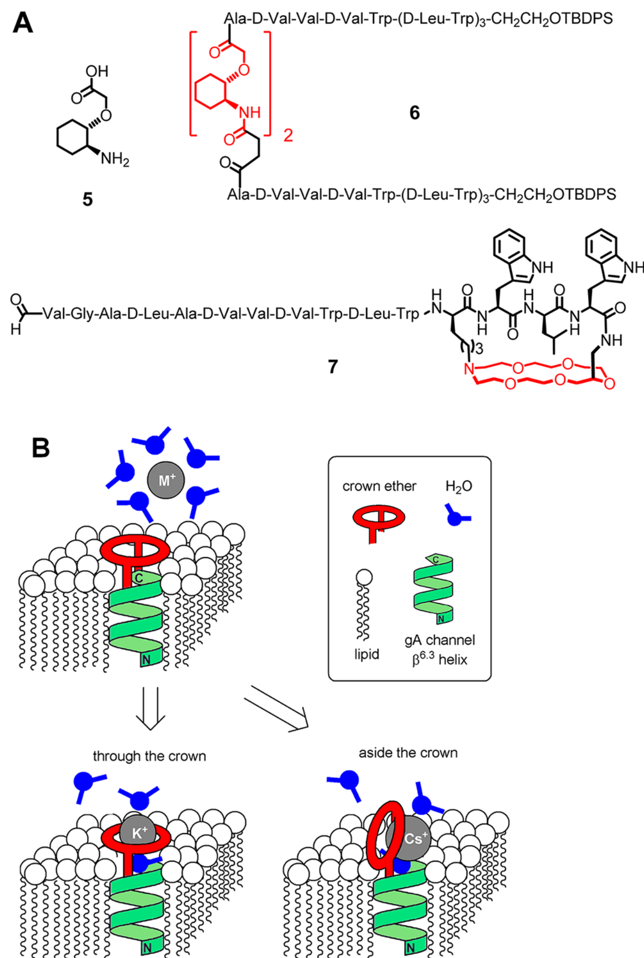

FIGURE 3. Ion-channel engineering of the gramicidin channel. (A) Structures of the cyclohexylether-gramicidin hybrid channel **6**, that displays increases Cs^+/K^+ ion selectivity and the crown-gramicidin hybrid **7** that exhibits reversed Cs^+/K^+ ion selectivity. (B) Schematic representation of the $K^+ > Cs^+$ selectivity observed for compound 7 due to optimal partial desolvation of potassium ions by the azacrown ether at the channel entrance; the larger cesium cation slides aside the crown into the channel.

δ-amino acid into the central region of the gramicidin channel by the synthesis of the gramicidin hybrid compound **6**. The functional analysis of ion channels was done by single-channel current measurement in a planar phospholipid bilayer (black lipid technique). Characteristic values of ion channels are the conductivity and the dwell time for a single channel event. This method is a single-molecule analytical technique, that requires particular attention to be paid to sample purity. In our hands, fragment-coupling in solution with chromatographic purification after each coupling step was superior to solid-phase peptide synthesis with respect to purity of the resulting gramicidin hybrid channels. Purity was checked after each coupling step not only by standard NMR-analysis but also by single-channel

current measurements. In the membrane environment, compound **6** exhibited well-defined channels with a remarkable selectivity among monovalent cations: Cs^+ and NH_4^+ were conducted well, but K^+-single-channels events met the detection limit and for Na^+ and Li^+, single channel events could not be resolved.[15] Thus, the introduction of ether oxygen atoms as additional binding in the central part of the narrow ion conductance channel of the gramicidin $\beta^{6,3}$ helix increased, for example, the Cs^+/K^+ selectivity from of 2:1 for wildtype gramicidin A to 10:1 for the gramicidin hybrid compound **6**. Asymmetric compounds such as **6** may adopt two different orientations of the $\beta^{6,3}$ helix within the membrane, with respect to the membrane normal, which explains the presence of two detectable conductance levels for most investigated cations.

Crown ethers are promising structural units for a function-oriented synthesis of ion channels, because of their known capability to bind solvated alkaline cations selectively. Their complexation/decomplexation rates in water are compatible with an ionic flux of 10^7 s^{-1} through the channel, which should not lead to a blockage of the ion transport. With the goal assisting the partial desolvation at the entrance of the gramicidin $\beta^{6,3}$ helix, we tried to position a crown ether at the entrance motif of gramicidin A.[17] A simple one-point attachment of a crown ether via a flexible linker to the C terminus has no significant conductance or ion selectivity effect probably due to the structural flexibility.[18] We therefore designed a two-point attachment to position the crown ether more rigidly before the channel entrance. The C terminus was chosen as first attachment point and the second attachment point was created by changing in the gramicidin sequence D-Leu(12) into D-Lys and to use the lysine side-chain amino group for the installation of an aza crown ether. Toward this end, the azacrown gramicidin hybrid **7** was synthesized (Figure 3A).[17] The single-channel measurements of **7** showed a reversal in the Cs^+/K^+ selectivity from of 2:1 to 1:2. The aza-[18]-crown-6-ether is optimal for partial desolvation of the potassium cation which results in an optimal transport for this cation (Figure 3B). The larger cesium cation is less able to be desolvated by the aza-[18]-crown-6-ether. and cannot be pulled through the crown, therefore probably sliding beside the crown into the channel lumen. The approach to modulate ion selectivity by facilitating desolvation is a promising concept. Modulating ion selectivity by varying the crown ether size it could be feasible to design hybrid gramicidin channels with different ion selective transport properties (e.g., Na^+/K^+). Compounds **6** and **7** are a proof of concept for function-directed synthesis of gramicidin ion-channels: it is possible to increase the wild-type ion selectivity with **6** and to reverse it with **7**.

Tetrahydrofuran- and tetrahydropyran-amino acids could also be incorporated into the gramicidin motif to generate hybrid channels which were functionally characterized by conductance measurements.[19,20] Gramicidin-hybrid channels were transferred into Chinese hamster ovary (CHO) cells and studied in vivo by patch clamp.[21] The Cs^+ ion selectivity of compound **6** seen in BLM measurements could also be observed in CHO cells. For the case of tetrahydrofuran-amino acids, the electrophysiological response of trabecular meshwork cells to tetrahydrofuran-gramicidin hybrid channels was investigated.[22]

Further functional features which were addressed with gramicidin hybrid channels were voltage gating and hydrophobic matching with the phospholipid bilayer. A gramicidin derivative with an ammonium group that is linked to the C-terminus via an ether linkage showed rectified current behavior (voltage gating).[23] The interplay of the membrane thickness and an integral membrane protein in terms of hydrophobic match and hydrophobic mismatch is an important issue in biological systems. Minigramicidin compounds with 11 instead of 15 amino acids were synthesized and their ion-channel function in membranes of various lipid-bilayer thickness were studied.[24] In 1,2-dioleylphosphatidylcholine [DOPC(18:1)], the hydrophobic mismatch was so strong that no stable channel-dimer was formed. In contrast, thinner membranes made of 1,2-dipalmitoleylphosphatidylcholine [DPPC(16:1)] or 1,2-dimyristoleylphosphatidylcholine [DMPC(14:1)] exhibited channel formation. The best hydrophobic match was found in DMPC(14:1)] resulting in long dwell times.

Engineering Wide-Pore Channels: OmpF and OmpG

The β-barrel architecture of porins with their wide pore offers a structural motif suitable for synthetic modifications.[25] In contrast to the oligomeric hemolysin, which has been used for ion-channel engineering by Bayley and co-workers,[26] porins have a conductance pathway built up by a single polypeptide chain, which makes synthetic modification in the pore interior more straightforward. This allows the incorporation of asymmetric, sterically demanding synthetic modulators by covalent attachment at one or more sites. The porins from bacterial outer membranes (outer membrane protein: Omp) form wide, water filled channels. OmpF (340 amino acids) is a well characterized porin from the outer membrane of *Escherichia coli* that can be refolded in

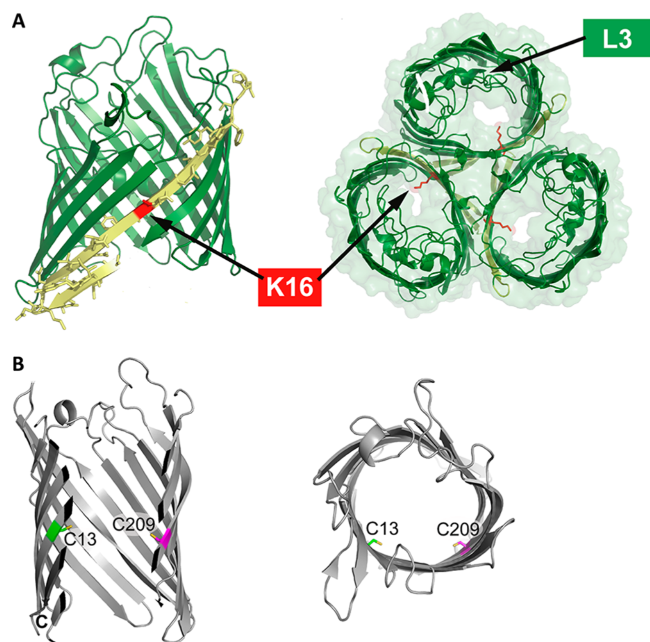

FIGURE 4. Structures of β-barrel porins used for ion-channel engineering. (A) Side view of one OmpF monomer and top-view of the OmpF trimer with highlighted point of operation (lysine 16) for function oriented synthesis. (B) Side view and top view of OmpG with possible positions for cystein mutations to introduce attachment points for synthetic modulators.

vitro to SDS (sodium dodecyl sulfate) resistant trimers[27] allowing chemical modification to be carried out in the unfolded state. As revealed by X-ray crystallography, the 16-stranded antiparallel β-barrel of each OmpF subunit forms a central, asymmetrical pore (Figure 4).[28] A loop region folds inside the pore and contributes to a unique channel constriction zone, the OmpF eyelet.

OmpF is only weakly cation-selective and shows distinct channel conductances and voltage-gating behavior when reconstituted into planar lipid bilayers. The lack of significant ion selectivity (Li, Na, K, Cs) results from the relatively wide pore diameter, which allows the passage of fully hydrated cations. The OmpF pore is maximally restricted at the eyelet to an elliptical cross section of 7 $\times$ 11 Å, thus providing a region that can tolerate a variety of synthetic modulators. We selected the N-terminal region (Ala1-Gly 26) for synthetic modification, because this segment is accessible via solid-phase peptide synthesis, which allows the introduction of artificial building blocks and the preparation of a thioester suitable for native chemical ligation and protein semisynthesis.[29] In particular, lysine-16 is located in the center of the OmpF eyelet constriction zone and was therefore chosen as modulator attachment point. For protein semisynthesis, an N-terminal fragment, where lysine 16 was altered to a propargyl-tyrosine ether, was synthesized by Fmoc solid phase synthesis. The C-terminal OmpF fragment missing the first 26 amino acids and harboring a Asn27Cys mutation was produced as inclusion bodies using a porin-deficient *E. coli* strain. The ligation between both fragments proceeded under denaturing conditions to produce an OpmF hybrid with a terminal alkyne in the side chain of position 16. The bioorthogonal triple bond could be used for further synthetic modifications. A Cu(I)-mediated cycloaddition with a dansyl azide led to the OmpF hybrid **8** bearing a dansyl moiety linked via a triazol to position 16 (Figure 5a).[29] A different route for the introduction of synthetic modulators used the OmpF-K16C mutant as starting point. A chemoselective S-alkylation with various substituted iodoacetamides led to various OmpF hybrids with modulators linked via a thioether bridge to position 16. One example is the OmpF hybrid **9** with a dibenzo-18-crown-6 a synthetic modulator (Figure 5a).[29] The synthesized OmpF hybrids **8** and **9** were refolded by insertion into mixed unilamellar vesicles comprising a 1:1 ratio of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/*n*-dodecyl-β-D-maltoside. For **9** the structural consequences of the synthetic modification could be studied by X-ray crystallography. The dibenzo crown transverses the constriction zone. This spanning conformation is stabilized by noncovalent interactions within the OmpF eyelet. The synthetic modification within the pore of the OmpF β-barrel for **8** and **9** resulted in significant reductions of the trimer conductances.[29] Although a crown ether was used as modulator, no ion-selective transport was observed for compound **9**. The modulator does not fit perfectly into the pore to span the OmpF eyelet completely leaving space for the unselective passage of fully hydrated cations. The single-point only attachment of the modulator to position 16 can cause structural mobility, which results in unselective ion transport too. These results call for a two-point attachment of the modulator within the pore in order to get improved function. Protein *trans*-splicing via intein-splicing was used as synthetic alternative to protein semisynthesis for fragment ligation to produce OmpF-hybrid channels. By that means a benzo-18-crown ether moiety was introduced into the OmpF pore via a triazol/thioether linker.[30]

The trimeric structure of OmpF can lead to a collective closure of all three monomers which complicates the interpretation of the conductance measurements. In order to simplify the functional analysis of the hybrid channels we switched our focus to monomeric porins. OmpG from *Escherichia coli* resembles OmpF in its architecture, but due to its monomeric nature it exhibits advantages for single-molecule detection. OmpG consists of 280 amino acids forming a stable β-barrel structure with 14 antiparallel β-sheets. The monomeric OmpG pore shows short turns and N- and

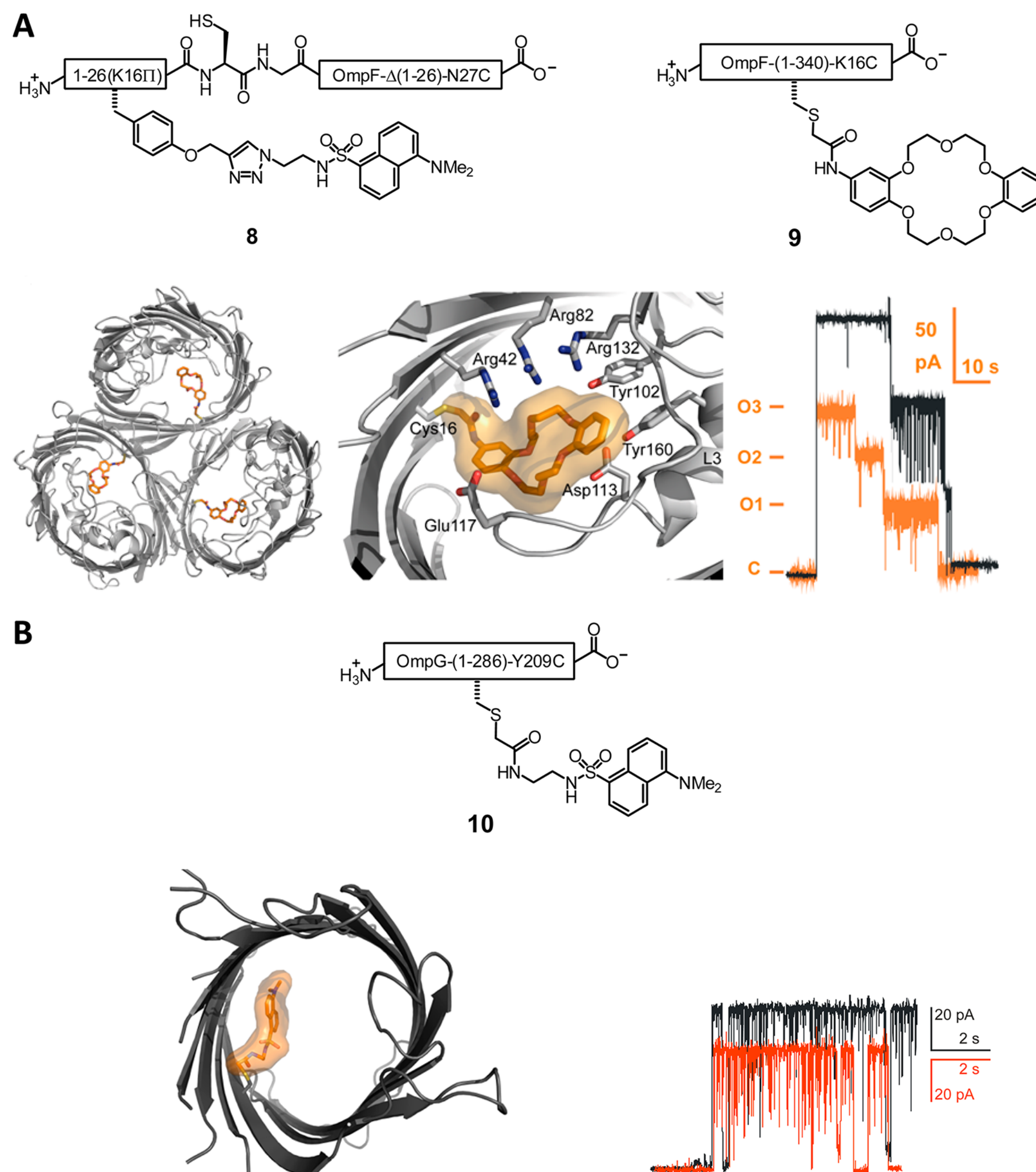

FIGURE 5. Ion-channel engineering of OmpF and OmpG. (A) OmpF hybrid channels with synthetic modulators attached via alkyne/azide cycloaddition (**8**) and via iodoacetamide S-alkylation (**9**); X-ray structure of the dibenzocrown-OmpF hybrid **9** [lower part left: top view of the trimer; center: expansion of the dibenzocrown region in the pore; right: single-channel conductance of wild type in black and hybrid channel **9** in orange]. (B) OmpG hybrid with a dansyl group attached via iodoacetamide S-alkylation (**10**); X-ray structure of hybrid porin **10**; and single-channel conductance of wild type OmpG in black and hybrid channel **10** in red.

C-termini on the inner, periplasmic side, but longer loops on the outer, extracellular side. Due to its large pore with a diameter of ~11 × 15 Å, this porin is rather unspecific and unlike OmpF it lacks a constriction zone narrowing the central region of the pore.[31] The L6 loop of OmpG exhibits structural flexibility and causes a fast gating behavior (flickering) in single-channel recordings. An OmpG pore suitable for biosensing was engineered by reducing the L6-loop mobility via introduction of a disulfide bond and by optimizing interstrand hydrogen bonding.[32] Bayley and co-workers used OmpG in planar lipid bilayers for cyclodextrine-dependent ADP detection.[33] We selected two positions in the OmpG pore, M13 and Y209, as attachment point for synthetic modulators.[34] The inward facing nature of these side chains implies functional consequences upon chemical modifications and therefore these residues were mutated to cysteines (Figure 5b). Using S-alkylation the OmpG/C209-dansyl hybrid **10** was synthesized. Single channel recordings showed a reduction of ion conductances for compound **10** by 28% compared to unmodified OmpG/C209. The dansyl

moiety of this modulator resulted in partial blockage of current though the OmpG channel with its gating characteristics mainly unaffected. The crystal structure of an OmpG-dansyl hybrid at 2.4 Å resolution corroborates this finding by showing that the modulator lines the inner wall of the OmpG pore.[34] In contrast to the structure of the OmpF hybrid **9**, the dansyl modulator in **10** exhibits more conformational mobility as indicated by the crystal disorder of the naphthyl substructure.

Our studies on OmpF- and OmpG-hybrid channels prove the β-barrel structure as suitable lead structure for ion-channel engineering.[35] Protein semisynthesis, *trans* splicing and S-alkylation are a reliable synthetic repertoire for various synthetic modifications. It should be possible to advance from a covalent single-point attachment to a two-point attachment of the modulator to reduce the mobility and to make functional consequences more predictable. Readily available crystallization and refolding protocols allow structural analysis of the hybrid channels at the atomic level and the necessary structure/function correlations. It is possible to expand this work on bacterial porins to mitochondrial β-barrel structures of the voltage-dependent anion channel (VDAC),[36] Fixation of the N-terminal segment of VDAC1 at the base or the midpoint of the pore forms channels with altered gating characteristics relative to the native protein.[37]

Concluding Remarks

Ion channels are important goals for function-oriented synthesis with potential applications in sensing and in vivo for biological/medical purposes. While de novo design is a valuable route for smaller structures, channels with dimensions of protein size are better approached by ion channel engineering. Future progress on synthetic ion channels will benefit from the collaboration of various disciplines such as electrophysiology, synthesis (peptide/protein/organic), and structural analysis.

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BIOGRAPHICAL INFORMATION

Ulrich Koert is Professor for Organic Chemistry at the Philipps university Marburg/Germany. He studied chemistry at Goethe university in Frankfurt and got his doctoral degree in 1988 with G. Quinkert. After a postdoc with J.-M. Lehn in Strasbourg (1988-90) he did habilitation in 1994 at Marburg. In 1994/1995 he was visiting associate professor at the university of Wisconsin at Madison. In 1996 he moved to München at the Ludwig-Maximilians university as an associate C3 professor for organic chemistry. From 1996 until 2001 Prof. Koert was professor for organic and bioorganic chemistry at the Humboldt University in Berlin. Since October 2001 he is professor at Marburg in Germany. His research interests include multistep synthesis of complex targets (natural products, molecular switches, synthetic ion channels) and the development of efficient synthetic methods.

Philipp Reiß is a research associate at the Philipps university Marburg. He studied chemistry at Marburg and received his Ph.D. with R.W Hoffmann in 2002 from the same university. Since 2002 he has a research associate position in Marburg and heads the ion-channel conductance analysis lab at the chemistry department and supports the studies on synthetic ion-channels in the Koert group. In addition, he is in charge of teaching organic chemistry for future teachers.

FOOTNOTES

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The authors declare no competing financial interest.

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