Supramolecular Chemistry

Single DNA Rotaxanes of a Transmembrane Pore Protein**

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Dedicated to Professor Julius Rebek, Jr. on the occasion of his 60th birthday

Recent advances in stochastic DNA sensing technologies,[1] especially those that exploit the transmembrane protein pore α-hemolysin (α-HL), have led to the realization that rapid single-molecule DNA sequencing may be feasible.[2] As part of an ongoing study to better ascertain the nucleobase recognition capacity of α-HL and its potential utility in DNA sequencing, we report herein methods for capturing single-stranded DNA–poly(ethylene glycol) (DNA-PEG) hybrid molecules inside the α-HL pore. The DNA–α-HL rotaxanes were characterized at the single-species level by electrophysiological techniques and display a stable and reversible two-state switching capacity that depends on the applied potential, the orientation, and the method of threading and capture used.

Staphyloccocus aureus α-HL forms large heptameric protein pores in lipid bilayers. Its crystal structure shows a mushroom-shaped assembly with a central channel approximately 10 nm long with a diameter of 1.5 nm at the narrowest point.[3] Macrocyclic adapters have been shown to lodge inside and modify the conductance characteristics of the α-HL pore. This approach has been used to devise stochastic sensors for a range of small organic molecules.[4] In addition, the pore structure of α-HL can accommodate PEG molecules, which can be used either to measure the pore size[5] or in α-HL-based biosensors for protein recognition.[6] Nucleic acids

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can also be detected during transit transport through the α-HL pore.[2,7] Homopolymers and block copolymers of DNA such as poly(A) and poly(C) can be distinguished by different reductions in the α-HL channel conductance caused by the presence of these polymers.[2b] These elegant studies provide the basis for the designs and studies reported herein.

Rotaxanes are among the most commonly used classes of functional supramolecular structures. These assemblies have promising applications in nanotechnology as molecular switches in which the thread unit contains at least two dynamic binding sites for the macrocyclic component, each defined by a distinct signal.[8] In the study described herein, we sought to form rotaxanes by threading single-stranded (ss) DNA–PEG copolymers of defined lengths through the α-HL transmembrane pore. The pore has a dual function in our design: its protein structure plays a role equivalent to that of the macrocyclic subunit of typical small-molecule rotaxanes, and perturbations of its native ion conductance provide the sensing signature for evaluating the functional characteristics of the rotaxanes.

The thread component of our rotaxane is an appropriately functionalized ss-DNA–PEG hybrid molecule designed to afford two different conductance states, depending on the portion of the strand (DNA or PEG) that is occupying the channel. DNA hybrid **1** was constructed as a continuous linear arrangement of the following four segments: The 3′-end consists of a 20-base region designed to bind to a specific oligonucleotide of complementary sequence. It is followed by a poly d(A) 40-mer. This segment is extended with 10 units of hexaethylene glycol phosphate and is capped with a biotinyl group at the 5′-terminus. This design includes DNA and PEG regions of the appropriate length for full threading of the α-HL pore by either of the two polymeric segments. The design also provides a mechanism to lock thread **1** at both ends after its insertion into the pore. Formation of a DNA duplex[2a] at the 3′-end of the polymer and binding of streptavidin to the 5′-biotin unit[6,7b,d] create structures at the ends that are wider than the α-HL entrances and thus can be used to lock-in the rotaxane complex (Figure 1a). Similarly, DNA hybrid **2** has a double-stranded 3′-DNA stem-loop structure[2c] designed to allow control of the threading orientation and rotaxane formation (Figure 1b). We exploited the anionic character of the ss-DNA–PEG chains to drive the threading process by applying a transmembrane potential. Once a rotaxane has been formed, the relative position of the thread inside the pore can be switched simply

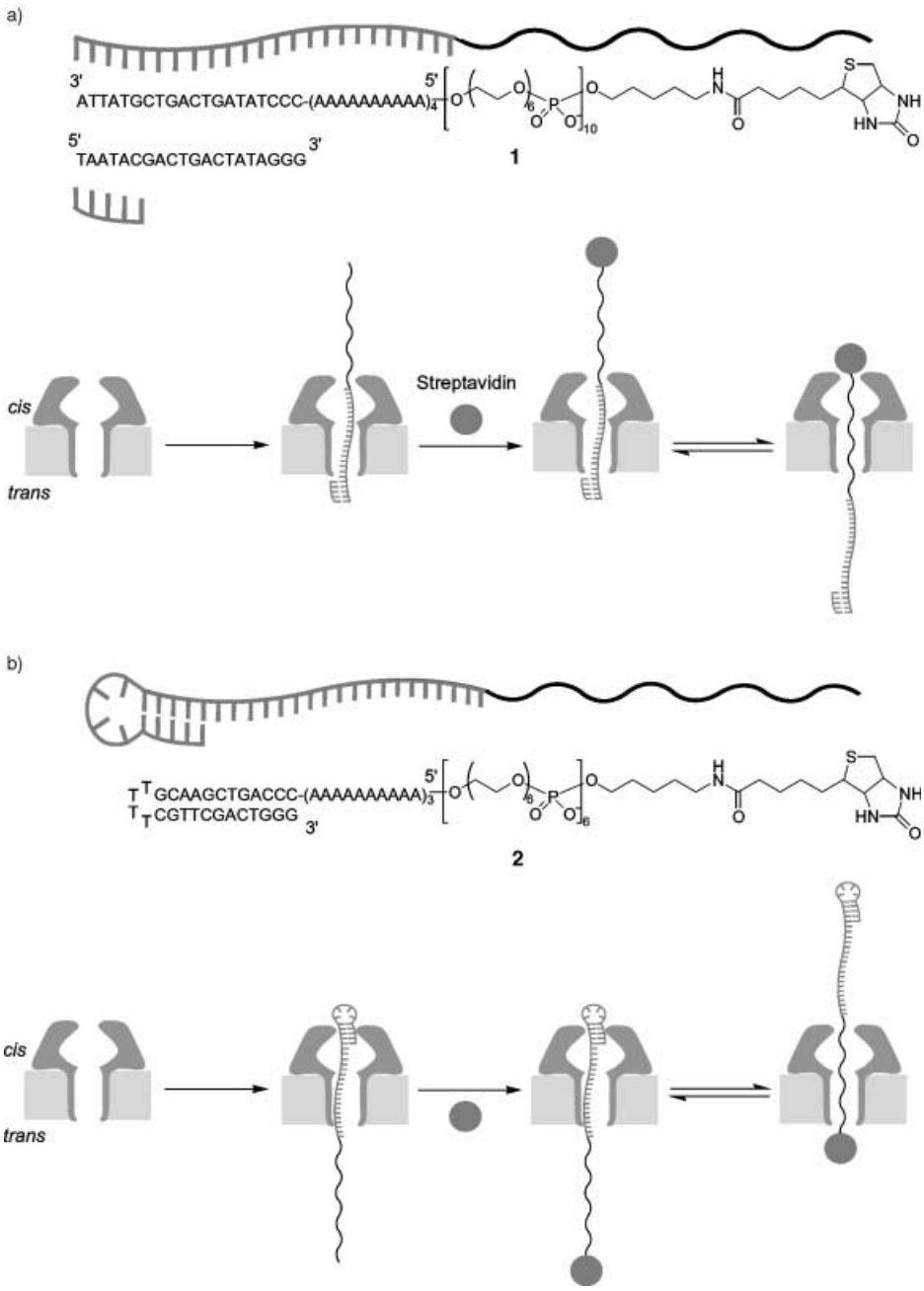

Figure 1. Schematic representation of the molecular components and processes employed for synthesis of transmembrane protein rotaxanes. a) System employed for rotaxane formation through capture by streptavidin on the *cis* side of the pore. b) Thread molecule with a DNA hairpin for rotaxane formation by capture with streptavidin on the *trans* side.

by adjusting the sign and magnitude of the applied transmembrane potential.

Initial experiments were performed with the uncapped ss-DNA–PEG thread **1**, which was added either to the *cis* or the *trans* side of the bilayer.[9] At positive transmembrane holding potentials (negative when **1** is added in the *trans* chamber), blocking events were detected at two distinct current levels, which suggests that the PEG and DNA segments can be differentiated (Figure 2a, b). The events can be grouped in two classes according to which level is detected first. We have established (see below) that the higher blocking step (Level 2) results from the DNA segment traversing the pore, while the lower blocking level (Level 1) corresponds to the passage of the PEG portion of the strand. Events that display the higher blocking level first are more numerous than those that begin with the lower level, which indicates that the favored orientation for strand threading is with the 3′-DNA tail first.

Topoisomeric pseudorotaxanes of α-HL, which differ only in the orientation of the threading polymer **1**, were used to assign the two observed conductance states unequivocally. The conductance levels attributed to PEG and DNA could be established by sterically blocking the 3′- or the 5′-terminus of **1** from entering the pore, either by DNA duplex formation at the 3′-tail or complexation of streptavidin to the 5′-biotin moiety. In experiments with the thread and streptavidin both present in the same chamber, the 5′-tail was blocked and thus only the DNA end was available to go through the α-HL pore. As expected, the current showed an initial high-level of block (passage of DNA), followed by a recovery of the channel conductance to a lower level which is assigned to the assembly with the PEG segment within the channel (Figure 2c, e). The conductance level was stable as long as the transmembrane voltage was applied, as we expect for a pseudorotaxane. This result indicates thatthe strength of the interaction between biotin and streptavidin as well as the steric bulk of the complex prohibit the strand from translocating through the channel pore to the other side of the bilayer.

Experiments performed under asymmetric salt conditions allowed the charge selectivity of the threaded channel to be determined.[10] Free α-HL displays a slight anion selectivity ($P_{K^+}/P_{Cl^-} = 0.6$ in bilayers that separate solutions containing 300 and 500 mM KCl on the *cis* and *trans* sides, respectively). When the pore is threaded from the *trans* side by the streptavidin–biotin strand complex, the P_{K^+}/P_{Cl^-} value is 2.0, which suggests that the negatively charged phosphate groups in the PEG segment of the strand change the charge density inside the pore and transform α-HL into a somewhat cation-selective pore (Figure 3a).

Hybridization of thread **1** to its complementary 20-mer DNA sequence prevents the 3′-DNA tail from entering the α-HL lumen; only the 5′-tail (PEG segment) is free to enter the pore. When **1** and the complementary short DNA were both added to the *trans* side of the pore (Figure 2f), the blocking events observed under negative transmembrane potentials remained stable over time and the high blocking level associated with the presence of the DNA segment of the thread inside the α-HL pore was seen. High charge selectivity was observed ($P_{K^+}/P_{Cl^-} > 100$) when the same experiment was

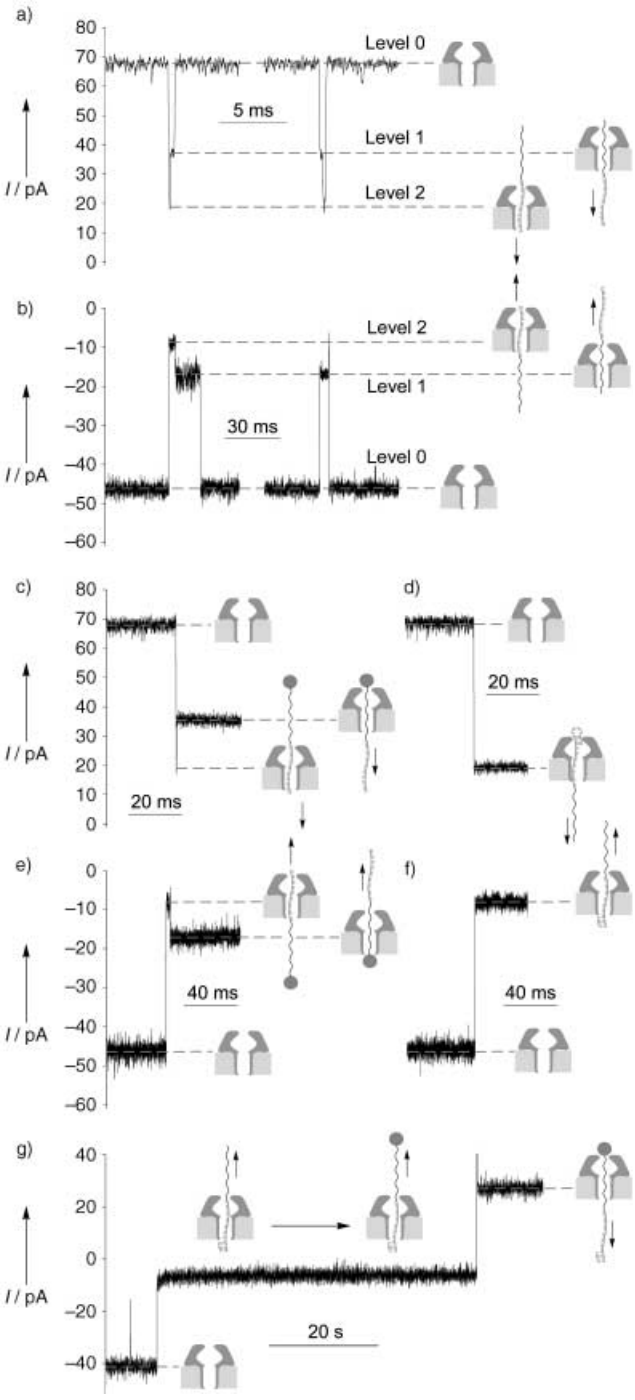

Figure 2. Blocking events caused by thread molecules. Typical events shown are from ion conductance traces recorded at +140 mV when the following thread molecules were added to the *cis* chamber: a) 1 μM **1**, c) 1 μM **1** in the presence of 10 μM streptavidin, and d) 1 μM hairpin thread **2**. Events were also observed at −140 mV with thread molecules added to the *trans* side: b) 1 μM **1**, e) 1 μM **1** and 10 μM streptavidin, and f) 1 μM **1** and 5 μM complementary oligonucleotide strand. g) Rotaxane formation at −120/+120 mV transmembrane potential with 1 μM **1** and 5 μM complementary oligonucleotide strand on the *trans* side, as well as 10 μM streptavidin on the *cis* side. Traces were recorded under symmetric conditions, KCl (500 mM) and morpholinopropanesulfonic acid (MOPS; 5 mM) at pH 7.5, bessel-filtered at 5 KHz, and sampled at 6 μs (a) and 100 μs (b–f), or bessel-filtered at 2 KHz and sampled at 200 μs (g).

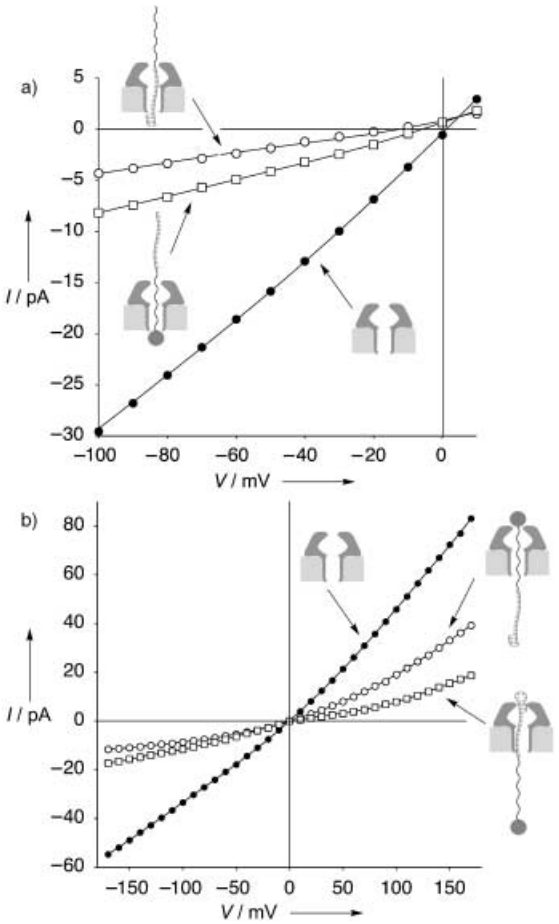

Figure 3. a) Current versus voltage relationships obtained under asymmetric conditions (300 and 500 mM KCl in the *cis* and *trans* chambers, respectively, both buffered with 5 mM MOPS to pH 7.5) for α-HL (●) and for α-HL threaded from the *trans* side with the streptavidin complex of **1** (□) or with **1** hybridized with the complementary oligonucleotide (○). b) Current versus voltage for α-HL (●), α-HL rotaxane with thread **1** (○), and α-HL rotaxane with strand **2** (□) in KCl (500 mM) and MOPS (5 mM) at pH 7.5.

performed under asymmetric conditions (Figure 3a). This selectivity can be accounted for by the presence of a high density of DNA phosphate groups inside the pore.

Formation of a single-molecule α-HL/DNA–PEG rotaxane is a two-step process. The first step involves formation of a pseudorotaxane, as described above. This process is driven by an applied transmembrane potential, which forces threading of the free tail of a polymer capped at one end (double-stranded DNA sequence or streptavidin) through the transmembrane pore and holds it stably in that configuration. The second step (locking) requires efficient capture of the protruding end of the thread on the other side of the bilayer. We found that the best approach for rotaxane formation was to employ a prehybridized strand on the *trans* side of the pore, with streptavidin present on the *cis* side (Figure 1a). We observed that, once the pseudorotaxane was captured (typically in less than a minute under the conditions employed), the α-HL conductance could no longer be returned to its native conductance state, even when the sign of the applied transmembrane potential was changed (Figure 2g). Instead, the α-HL/DNA–PEG rotaxane displayed the conductance value corresponding to the presence of the PEG moiety in the pore at positive potentials, and the conductance level corresponding to the assembly with the DNA segment residing inside the pore at negative potentials. The rotaxane structure proved to be stable over the range of transmembrane potentials studied (−170 to +170 mV) and could be switched multiple times between the DNA and PEG states (Figure 2g).

The DNA–PEG polymer **2**, which has a double-stranded 3′-DNA stem-loop structure, was used to construct a corresponding rotaxane structure of opposite strand orientation to that described above (Figure 1b). The DNA stem-loop structure is known to prevent entry into the vestibule of the α-HL channel when the polymer is applied to the *cis* side of the pore.[2c] Addition of **2** to the *cis* side of the α-HL pore resulted in the formation of a pseudorotaxane, as shown by the observed blocking of the pore when a high positive transmembrane potential was applied. The residual conductance corresponded to the blocking effect expected when DNA is inside the pore (Figure 2d). The α-HL native conductance could be fully recovered by switching back and forth between negative and positive transmembrane potentials (threading/dethreading). However, when streptavidin was added to the *trans* side of the pore, the thread was permanently trapped in the α-HL channel as a result of the formation of a rotaxane structure. This new rotaxane showed a conductance level at negative holding potentials typical of the channel when the PEG segment is lodged inside, which signifies the formation of a rotaxane of the opposite thread orientation to that described above for **1**. This second rotaxane structure was also shown to be stable over a wide range of applied transmembrane potentials (−170 to +170 mV; Figure 3b).

In summary, we have described a methodology for the controlled formation of individual rotaxanes of desired strand orientation with tunable conformational states. We expect this methodology to open up new possibilities for studying nucleic acids and other polymers at the single-molecule level. In addition, this study provides a direct method for trapping DNA strands of defined lengths and sequences inside the α-HL channel structure. Thereby, this method might be useful to studies aimed at establishing the scope and limitations of the nucleobase recognition capacity of α-HL and its utility in DNA sequencing. Our results also suggest that DNA–α-HL rotaxanes might provide a means for multiple-pass single-molecule DNA sequencing, which is likely to have significantly lower sequencing error rates than single-pass analyses.

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[1] a) H. Bayley, C. R. Martin, *Chem. Rev.* **2000**, *100*, 2575; b) S. M. Bezrukov, *J. Membr. Biol.* **2000**, *174*, 1; c) H. Bayley, P. S. Cremer, *Nature*, **2001**, *413*, 226.
[2] a) J. J. Kasianowicz, E. Brandin, D. Branton, D. W. Deamer, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 13770; b) M. Akeson, D. Branton, J. J. Kasianowicz, E. Brandin, D. W. Deamer, *Biophys. J.* **1999**, *77*, 3227; c) W. Vercoutere, S. Winters-Hilt, H. Olsen, D. Deamer, D. Haussler, M. Akeson, *Nat. Biotechnol.* **2001**, *19*, 248; d) S. Howorka, S. Cheley, H. Bayley, *Nat. Biotechnol.* **2001**, *19*, 636.
[3] L. Song, M. R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, J. E. Gouaux, *Science* **1996**, *274*, 1859.
[4] a) L.-Q. Gu, O. Braha, S. Conlan, S. Cheley, H. Bayley, *Nature* **1999**, *398*, 686; b) J. Sánchez-Quesada, M. R. Ghadiri, H. Bayley, O. Braha, *J. Am. Chem. Soc.* **2000**, *122*, 11756; c) L.-Q. Gu, S. Cheley, H. Bayley, *Science* **2001**, *291*, 636; d) L.-Q. Gu, S. Cheley, H. Bayley, *J. Gen. Physiol.* **2001**, *118*, 481.
[5] a) Y. E. Korchev, C. L. Bashford, G. M. Alder, J. J. Kasianowicz, C. A. Pasternak, *J. Membr. Biol.* **1995**, *147*, 233; b) S. M. Bezrukov, I. Vodyanov, R. A. Brutyan, J. J. Kasianowicz, *Macromolecules* **1996**, *29*, 8517; c) S. Howorka, L. Movileanu, X. Lu, M. Magnon, S. Cheley, O. Braha, H. Bayley, *J. Am. Chem. Soc.* **2000**, *122*, 2411.
[6] a) L. Movileanu, S. Howorka, O. Braha, H. Bayley, *Nat. Biotechnol.* **2000**, *18*, 1091; b) L. Movileanu, S. Cheley, S. Howorka, O. Braha, H. Bayley, *J. Gen. Physiol.* **2001**, *117*, 239.
[7] a) A. Meller, L. Nivon, E. Brandin, J. Golovchenko, D. Branton, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1079; b) S. H. Henrickson, M. Misakian, B. Robertson, J. J. Kasianowicz, *Phys. Rev. Lett.* **2000**, *85*, 3057; c) A. Meller, L. Nivon, D. Branton, *Phys. Rev. Lett.* **2001**, *86*, 3435; d) J. J. Kasianowicz, S. E. Henrickson, H. H. Weethall, B. Robertson, *Anal. Chem.* **2001**, *73*, 2268; e) S. Howorka, L. Movileanu, O. Braha, H. Bayley, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12996.
[8] F. M. Raymo, J. F. Stoddart, *Chem. Rev.* **1999**, *99*, 1643.
[9] Lipid bilayers were formed from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine in a teflon planar bilayer apparatus: M. Montal, P. Mueller, *Proc. Natl. Acad. Sci. USA* **1972**, *69*, 3561. The *cis* chamber was at virtual ground, and a positive potential refers to a higher potential in the *trans* chamber. A positive current is produced by cations flowing from the *trans* to the *cis* side of the pore, or by anions flowing in the opposite direction. α-HL is an asymmetric channel; its orientation in planar lipid bilayers was controlled by adding pre-assembled heptamers to the *cis* chamber. α-HL is incorporated into lipid bilayers through the stem domain, which ends up spanning the bilayer, while the cap remains on the *cis* side.
[10] Permeability ratios were calculated from reversal potential measurements and ionic concentrations (converted into ionic activities) by using the Goldman–Hodgkin–Katz equation: B. Hille, *Ionic channels of excitable membranes*, 3rd ed., Sinauer, Sunderland, **2001**.

