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A Protein Pore with a Single Polymer Chain Tethered within the Lumen

Stefan Howorka,*,† Liviu Movileanu,† Xiaofeng Lu,† Melissa Magnon,† Stephen Cheley,† Orit Braha,† and Hagan Bayley*,†,‡

Contribution from the Department of Medical Biochemistry & Genetics, Texas A&M Health Science Center, College Station, Texas 77843-1114, and Department of Chemistry, Texas A&M University, College Station, Texas 77843-3255

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Abstract: A transmembrane protein pore with a single 5000 Da poly(ethylene glycol) (PEG) molecule attached covalently within the channel lumen has been constructed from seven staphylococcal α-hemolysin subunits. The modified heptamer is stable and can be purified by electrophoresis in sodium dodecyl sulfate, without dissociation of the subunits. The properties of the modified pore were studied by single channel current recording. The PEG molecule reduces the mean conductance of the pore by 18%, as would be predicted from the effects of PEG on the conductivity of bulk electrolytes. The recordings also reveal a variety of low amplitude current fluctuations on a time scale of seconds, which are tentatively ascribed to the reorganization of the PEG molecule within the channel lumen and associated movements of the polypeptide chain. Another class of events, comprising uniform high-amplitude negative fluctuations in current with durations of milliseconds, is ascribed to motions of the PEG molecule into one of the channel entrances, thereby producing more extensive channel block. When instead a 3000 Da PEG is attached within the channel lumen, the single channel properties are changed in keeping with the lower mass of the polymer. For example, the high-amplitude fluctuations occur more frequently and are of shorter duration suggesting that the 3000 Da PEG is more mobile than the 5000 Da chain. With further development, the approach taken here should be useful for the indirect monitoring of polymer dynamics at the single molecule level. By using polymers that respond to analytes, it should also be possible to make biosensors from the covalently modified pores.

Introduction

Staphylococcal α-hemolysin (αHL) has been a useful model system with which to test new approaches for engineering membrane proteins and indeed proteins in general. The αHL monomer of 293 amino acids assembles on lipid bilayers to form a mushroom-shaped heptameric pore.[1] The opening of the channel at the cis side of the bilayer measures ∼29 Å in diameter and broadens into a cavity ∼41 Å across (Figure 1a). In the transmembrane domain the channel lumen narrows to form a 14-stranded β-barrel with an average internal diameter of 20 Å (Figure 1a). The αHL pore allows the passage of molecules of up to ∼2000 Da across the bilayer[2−5][1] and is only weakly

* Address correspondence to these authors. Howorka: Texas A&M Health Science Center. E-mail: howorka@medicine.tamu.edu. Bayley: Texas A&M Health Science Center. E-mail: bayley@tamu.edu.
† Texas A&M Health Science Center.
‡ Texas A&M University.

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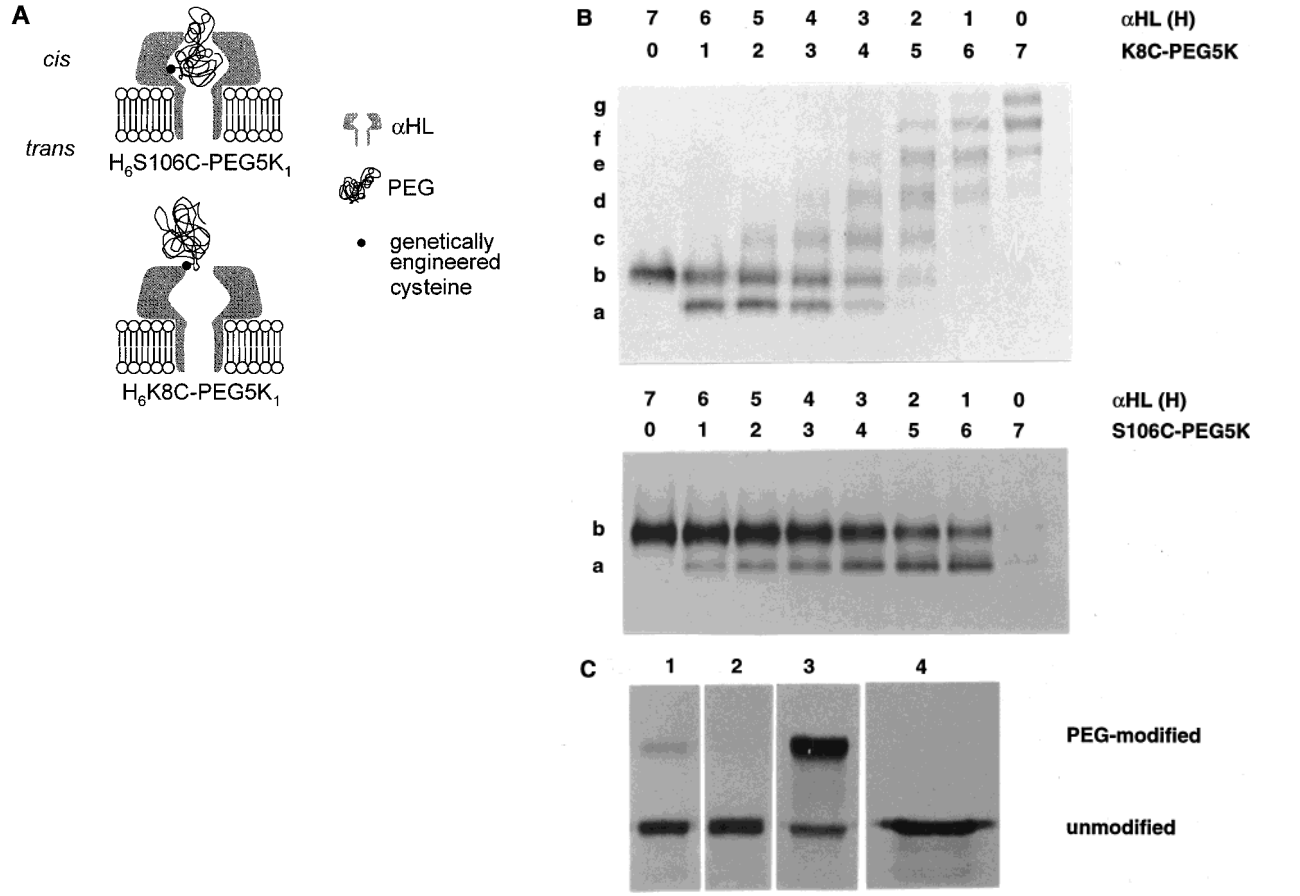

Figure 1. (a) Schematics of $H_6S106C\text{-}PEG5K_1$ and $H_6K8C\text{-}PEG5K_1$ shown as sagittal sections. In each engineered pore only one of the seven subunits is modified. In this work, the mutant K8A was used as the unmodified αHL subunit (H), so that the net charge at the cis channel entrance would not be altered in heteromers containing K8C-PEG5K. (b) SDS-polyacrylamide gel electrophoresis of αHL heptamers formed with unaltered subunits and K8C-PEG5K (*upper* panel) or S106C-PEG5K (*lower* panel). The subunits were ^{35}S-labeled during in vitro expression. K8C and S106C were reduced with 0.5 mM DTT and reacted with 10 mM MePEG5K-OPSS for 20 min at 25 °C. Heptamerization was carried out on rabbit erythrocyte membranes. An autoradiogram is shown. The ratios of the two subunits in the initial mix are shown above each lane. The number of PEG-modified subunits present in the heptamers in each band is as follows: K8C a, 1; b, 0 and 2; c, 3; d, 4; e, 5; f, 6; g, 7; S106C a, 1 and 2; b, 0. (c) Ratio of subunits in the heptamer in band a obtained from the unaltered (H) and S106C-PEG5K subunits. Protein extracted from band a generated with a low fraction of S106C-PEG5K subunits (see panel b) was heated to 95 °C for 5 min and separated in a second SDS-polyacrylamide gel. The bands were quantified by phosphorimager analysis. Lane 1, heated band a ($H_6S106C\text{-}PEG5K_1$); lane 2, heated and DTT-treated band a ($H_6S106C\text{-}PEG5K_1$); lane 3, PEG-modified S106C monomer before heptamerization; lane 4, PEG-modified S106C monomer after DTT treatment.

selective for the charge of transported ions.[6] Besides being the object of a wide variety of studies using mutagenesis,[7−10] αHL has been subjected to protein engineering by targeted chemical modification. These studies include the attachment of photocleavable protecting groups to block assembly,[11] the restoration of activity to an inactive mutant by site-specific alkylation,[12] and the formation of channel blocker sites with noncovalent molecular adapters.[13] Here, we introduce a new targeted modification, the attachment of a synthetic polymer chain *within* the lumen of the pore. We have also been able to use single channel electrical recording to observe current fluctuations associated with the attachment of the polymer, a 3000 or 5000 Da poly(ethylene glycol) (PEG) molecule.

In addition to the protein engineering, the present work is of interest in two areas: single molecule detection and the development of biosensors. The examination and manipulation of individual molecules is a thriving area of research. Single molecule detection methods, which include electrical recording,[14,15] optical spectroscopy,[16,17] and force measurements,[18] can provide structural and functional information that is often difficult or impossible to obtain by conventional techniques, which measure the properties of large ensembles of molecules. Recent accomplishments include observations of the movement of individual atoms and small molecules,[19] the movement of

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linear and rotary motor proteins,[18] the turnover of individual enzymes,[20] and the unfolding and refolding of proteins.[18]

In the area of biosensors, significant progress has been made in developing protein channels and pores as sensor elements.[21−24] According to this concept, analyte molecules modulate the ionic current passing through the pores under a transmembrane potential. For example, binding sites can be engineered into pores expressly for capturing analyte molecules, which act as partial channel blockers. Stochastic sensing, which uses currents from single pores, is an especially attractive prospect.[9,13,23,24] The approach yields both the concentration and identity of an analyte, the latter from its distinctive current signature. By using αHL as a stochastic sensing element, we have succeeded in detecting divalent metal ions[9] and a variety of organic molecules.[13] The work presented here represents a major step toward using responsive polymers[25−27] for stochastic sensing.

Experimental Section

Proteins. The mutant αHL S106C gene was obtained by cassette mutagenesis of the semisynthetic gene αHL-RL2.[10] S106C also contains the mutation Lys-8 → Ala, and four conservative replacements: Val-124 → Leu, Gly-130 → Ser, Asn-139 → Gln, and Ile-142 → Leu. These changes, which were introduced to prevent adventitious proteolysis[28] and to facilitate cassette mutagenesis,[10] do not alter the electrical properties of the pore.[10] The K8A and K8C constructs have already been described.[8,28] ^{35}S-labeled αHL polypeptides K8A, K8C, and S106C were obtained by expression in vitro.[29] To increase the yield of protein, unlabeled methionine was included in the translation mix.[10,30] K8A was used as the unmodified αHL subunit (H) in heteroheptameric pores. Therefore, when a PEG-modified K8C subunit is included in the heptamer, the charge at position 8 is not altered as it would be if the wild-type protein were used.

Chemical Modification of Single-Cysteine Mutants. K8C or S106C monomers were diluted 6-fold from the translation mix into a buffer containing 10 mM MOPS·NaOH, pH 7.4, 150 mM NaCl, 0.5 mM EDTA and reduced with 0.5 mM DTT for 10 min, before modification with 10 mM monomethoxy-PEG5000-*o*-pyridyl disulfide (MePEG5K-OPSS, Shearwater Polymers, Huntsville, AL) for 20 min at 25 °C. Treatment with MePEG3K-OPSS was performed in the same way.

PEG-Modified Heteroheptameric Pores. The modified K8C or S106C monomers were mixed with unmodified K8A αHL monomers (H) in various initial ratios and the mixed subunits were allowed to assemble on rabbit erythrocyte membranes.[29] To analyze which heteromers had been formed with PEG5000-modified subunits, the membranes were recovered by centrifugation, dissolved in gel loading buffer and loaded, without heating, onto a 6% SDS-polyacrylamide gel (5 cm long, 0.75 mm thick, Miniprotean II, Bio-Rad, Hercules, CA). To determine subunit ratios, the samples were heated and analyzed in a 10% SDS-polyacrylamide gel. The dried gels were subjected to phosphorimager or autoradiographic analysis. To prepare the heteromers H_6S106C-PEG5K$_1$ and H_6K8C-PEG5K$_1$ for bilayer recording, subunits were assembled in the following ratios: H:S106C-PEG5K, 3:4, and H:K8C-PEG5K, 6:1. Samples corresponding to a total of 12 μL of translation mix per lane were loaded, without heating, onto 6% SDS-polyacrylamide gels (35 cm long, 1.5 mm thick), which were run for 18 h at 150 V. The unfixed gels were vacuum dried at 50 °C onto Whatman 3MM paper and the protein bands located by autoradiography. The desired bands were cut from the gel and rehydrated in water (300 μL per lane). After removal of the paper, the gel was crushed in the water and the mixture was left to stand for 10 h at 4 °C. A solution of PEG-modified heptamers was then obtained by removal of the acrylamide with a cellulose acetate filter (0.2 μm diameter, Rainin, Woburn, MA). PEG3000 produced no gel shift in heteroheptamers modified at position 106. Therefore, to make H_6S106C-PEG3K$_1$, unmodified subunits (H) and S106-PEG3K were assembled together in a ratio of 4:3. After preparative SDS-polyacrylamide gel electrophoresis, as described above, the major band contained ∼40% H_6-S106C-PEG3K$_1$ and ∼60% H_7, as deduced from the subunit ratio determined by analytical SDS-polyacrylamide gel electrophoresis and by the results of single channel recording.

Planar Bilayer Recordings. Planar lipid membrane recordings were carried out at 24 ± 1 °C.[9,13,31] The cis and trans chambers, each of 2 mL, were separated by a 25-μm-thick Teflon septum (Goodfellow Corporation, Malvern, PA). An aperture in the septum (∼150-μm diameter) was pretreated with 10% (v/v) hexadecane (Aldrich Chemical Co., Milwaukee, WI) in *n*-pentane (Burdick & Jackson, Muskegon, MI). The electrolyte in both chambers was 300 mM KCl, 5 mM Tris·HCl, pH 7.0, containing 100 μM EDTA. A bilayer membrane was formed[31] with 1,2-diphytanoyl-*sn*-glycerophosphocholine (Avanti Polar Lipids, Alabaster, AL). αHL pores were introduced by adding gel-purified heptamers (1 to 6 μL) to the cis chamber, to give a final protein concentration of 0.05−0.3 ng/mL. The cis solution was stirred for 5−30 min until a single pore inserted (8−10 pA step at −40 mV).

Currents were recorded by using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) connected to the chambers by Ag/AgCl electrodes, and monitored with an oscilloscope (Model TAS250, Tektronix, Heenveen, Netherlands). The cis chamber was grounded and a negative current (downward deflection) represents positive charge moving from the cis to the trans side. A Pentium PC equipped with a DigiData 1200 A/D converter (Axon Instruments, Foster City, CA) and a strip chart recorder (BD112, Kipp & Zonen, Bohemia, NY) were used for data acquisition. For most of the experiments, the current traces were low-pass filtered with a built-in 4-pole Bessel filter at a frequency of 5 kHz and stored by using a digital audio tape recorder (DAS-75, Dagan Corporation, Minneapolis, MN). For computer analysis, the data were further filtered with a 8-pole Bessel filter at frequencies in the range 100−3000 Hz and sampled at 10 kHz. For display and statistical analysis, we used the FETCHAN and pSTAT programs, both from the software package pCLAMP7 (Axon Instruments) and Origin (Microcal Software, Northampton, MA). In the case of H_6S106C-PEG3000$_1$, a different protocol was used to allow the examination of rapid events. The signal, filtered at 10 kHz, was recorded on digital audio tape. For analysis, the signal was filtered at 7 kHz with a low-pass Bessel filter and sampled at 333 kHz for computer acquisition using a threshold protocol in the CLAMPEX program from pCLAMP7.

Current amplitudes and lifetimes of the various conductance states are given as mean values (± standard deviations). The value of "*n*" denotes the number of experiments analyzed or, when indicated, the number of events examined.

Results and Discussion

Engineering an αHL Pore with a Single PEG Molecule Tethered within the Central Cavity. In previous work, we have modified the lumen of the heptameric αHL pore by direct genetic engineering[9] and by noncovalent modification with molecular adapters.[13] A principal goal has been to create protein pores that respond to various analytes and can thus be employed as components of biosensors, especially stochastic sensors in which single molecule detection is used.[24] An additional way to modify the interior of the protein would be by the covalent attachment of responsive molecules.[25−27] Responsive polymers attached at specific sites in proteins have demonstrated potential.

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Table 1. Conduction Properties of Unaltered (H_7) and PEG-Modified αHL Pores[a]

channel	mean conductance (pS)[b]	substates (pS)[c]	after PEG cleavage (pS)[d]
H_7	268 ± 5 (11)	none	n.a.
H_6K8C-PEG5K$_1$	244 ± 19 (8)	17 ± 4, d (8)	270 ± 5 (8)
H_6S106C-PEG5K$_1$	221 ± 9 (7)	10 ± 1, d; 120 ± 7, s (7)	267 ± 7 (4)
H_6S106C-PEG3K$_1$	237 ± 4 (5)	128 ± 3, s (5)[e]	272 ± 3 (5)

[a] Experiments were performed at a transmembrane potential of +100 mV with 300 mM KCl, 5 mM Tris·HCl (pH 7.00), 100 μM EDTA in both chambers. The number of experiments analyzed is shown in parentheses. [b] The mean (± standard deviation) of the mean conductance values from the major peaks of all-points histograms (e.g. Figure 2a) was calculated. [c] The mean change in conductance (Δg ± standard deviation) between the most common substates. d, discrete low amplitude events; s, negative current spikes. [d] The conductance was determined after treatment with DTT as described in the text. When a step to an increased steady current was observed, the PEG was assumed to have left the cavity. In the case of H_6S106C-PEG5K$_1$, this took 18−25 min with 10−15 mM DTT. [e] In the case of H_6S106C-PEG3K$_1$ low amplitude events were seen on one occasion in six experiments and are not recorded in the table.

They have, for example, been used to modulate the affinity of streptavidin for biotin.[32,33]

To ascertain the difficulties that might be involved in assembling a structure with an internal polymer, we decided to place a 5000 Da PEG molecule within (or largely within) the central cavity of the cap domain of the αHL pore. The calculated volume of PEG5000 based on the experimental hydrated radius[3,34] is comparable to the volume of the cavity, which is 36 000 Å^3, assuming a sphere of diameter 41 Å. The Flory dimension (R_F) of PEG5000 of 60 Å ($R_F = aN^{0.6}$, where N is the number of polymer repeat units and a is the effective repeat length (3.5 Å)),[35] which has been variously interpreted as a radius or diameter,[36,37] gives a larger volume for PEG5000, but this may be unrealistic.[37]

Heptamers Containing Up to Seven PEG Molecules Can Be Made by Derivatization at an External Site on the αHL Pore. The PEG conjugation chemistry and a means to analyze the assembly of derivatized subunits were tested with the αHL single-cysteine mutant K8C. In this case, the PEG chains would end up located near the surface of the heptamer, at the cis mouth, and therefore be unlikely to interfere with assembly (Figure 1a).[38,39] ^{35}S-labeled K8C polypeptides were obtained by expression in vitro[29] and modified with monomethoxy-PEG5000-*o*-pyridyl disulfide (MePEG5K-OPSS). The PEG is attached to the protein through a disulfide bond that is readily cleaved with dithiothreitol (DTT) (Figure 1c). The modified K8C polypeptides were mixed with unmodified ^{35}S-labeled αHL monomers (H) in various initial ratios and the mixed subunits were allowed to assemble on rabbit erythrocyte membranes.[29] Analysis of H/K8C-PEG5K heteroheptamers by SDS-polyacrylamide gel electrophoresis and autoradiography revealed seven bands, a−g (Figure 1b). The protein in each band was eluted and heated to dissociate the subunits. Further electrophoresis (not shown) revealed the ratio of subunits in each band, which showed that all eight possible combinations[9] of unmodified αHL (H) and K8C-PEG5K subunits had been formed (both H_7 and H_5K8C-PEG5K$_2$ are in band b, Figure 1b). Interestingly, the electrophoretic mobility of heptamers containing a single PEG5000 molecule was increased (band a), while the mobilities of heptamers containing three or more attached PEG5000s (bands c−g) were decreased. Heptamers with two to five PEGs exist in more than one form: the outcome of permutation about the central 7-fold axis.[9] Bands c and d were distinctly broadened, most likely reflecting an incomplete separation of the five permutations each of H_4K8C-PEG5K$_3$ and H_3K8C-PEG5K$_4$. These experiments show that heptamers containing PEG5000 in an external location can be assembled and all eight combinations of subunits can be identified by analytical SDS-polyacrylamide gel electrophoresis.

A Single PEG Chain Can Be Attached at a Point within the Central Cavity of the αHL Pore. Next, we attempted to use the same approach to make αHL heptamers containing PEG5000 attached covalently to a cysteine residue within the large central cavity (position 106 in the polypeptide chain). Modified S106C monomers were mixed with unmodified αHL monomers (H) and allowed to assemble on rabbit erythrocyte membranes.[29] By contrast with the results with K8C-PEG5K, the analysis of H/S106C-PEG5K heteroheptamers revealed only two major bands (Figure 1b), suggesting that the formation of SDS-resistant heptamers containing several modified S106C subunits is disfavored due to crowding of the PEG chains within the central cavity. The measured ratio of unaltered αHL (H) to S106C-PEG5K subunits in band a was 5.9:1, and therefore the oligomer in the band must contain six unmodified subunits (H) and one S106-PEG5K subunit, namely H_6S106C-PEG5K$_1$. At high S106C-PEG5K:H ratios in the assembly mix, a faint band was seen above band a and may represent H_5S106C-PEG5K$_2$.

This experiment shows that H_6S106C-PEG5K$_1$ can be formed and that it is stable as a heptamer at room temperature in the denaturing detergent SDS. Therefore, the cavity might be large enough to contain a PEG5000 molecule. Any hydration pressure that develops in packing the PEG internally would have to be insufficient to dissociate the heptamer. Alternatively, unfavorable interactions would be reduced if part of the PEG chain were extruded through the cis or trans entrance. Experiments with PEG3000 support the latter interpretation. The electrophoretic mobility of H_6S106C-PEG3K$_1$ is the same as that of the unmodified heptamer (H_7) (data not shown) suggesting that the hydrodynamic properties of the heptamer are unaltered and the PEG3000 chain is largely contained within the cavity. By contrast, the altered electrophoretic mobility of H_6S106C-PEG5K$_1$ (Figure 1b) suggests that part of the PEG5000 chain is exposed to solvent.

Ionic Currents Through Individual PEG-Modified αHL Pores. The cavity in the αHL pore lies on the conductive pathway and so the incorporation of a PEG molecule at position 106 would be expected to alter the current that flows through the pore in response to an applied potential. This possibility was tested by performing single channel current measurements on H_6S106C-PEG5K$_1$ eluted from preparative gels.[9] The control homoheptamer (H_7) exhibits a uniform unitary conductance state (Table 1 and refs 9, 10, and 13). By contrast, the PEG-modified αHL pore showed dynamic gating behaviors centered around a

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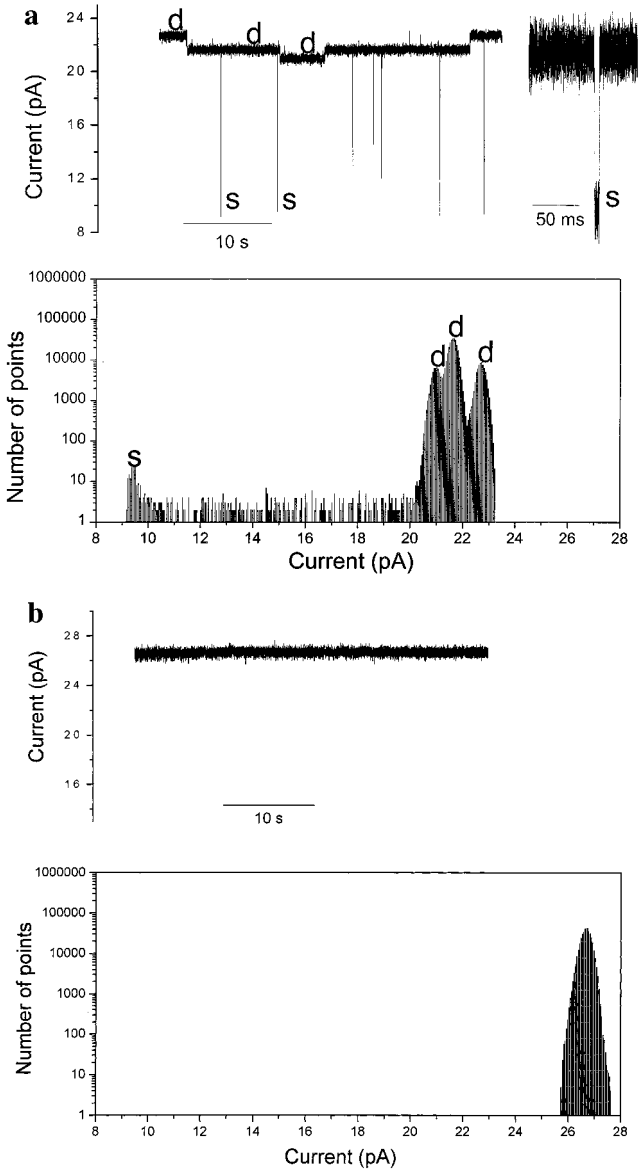

Figure 2. (a) A representative current trace and semilogarithmic amplitude histogram for a single channel current from H_6S106C-PEG5K_1. The current was recorded at +100 mV under symmetrical buffer conditions: 300 mM KCl, 5 mM Tris·HCl (pH 7.00), 100 μM EDTA. The bilayer lipid was 1,2-diphytanoyl-*sn*-glycerophosphocholine. Protein was added to the cis chamber, which was at ground. A positive potential indicates a higher potential in the trans chamber and a positive current is one in which cations flow from the trans to the cis chamber. The current was low-pass filtered at 100 Hz and sampled at 10 kHz. An expanded view of a high-amplitude substate (the last spike in the trace) is shown filtered at 3 kHz. d, low amplitude subconductance state; s, short-lived spike. (b) Signal from the same channel after treatment with 12 mM DTT in the cis chamber, filtered at 100 Hz.

main conductance state of diminished amplitude compared with H_7 (Figure 2a, Table 1). The mean of the main peaks in conductance histograms for H_6S106C-PEG5K_1 was 221 ± 9 pS (n = 7) at +100 mV, in symmetric 300 mM KCl, 5 mM Tris·HCl (pH 7.00), 100 μM EDTA, a reduction of 18% over the value for H_7 (Table 1). Two distinct subconductance behaviors were observed: relatively long-lived low amplitude fluctuations (mean lifetime, 14.5 ± 1.7 s, n = 27 events) and short-lived higher amplitude negative spikes (mean lifetime, 13.7 ± 2.2 ms, n = 87 events; frequency of occurrence 0.20 ± 0.02 s^{-1}). Typically there were three to five low-amplitude states separated by Δg = 10 ± 1 pS. The excess current noise of the low-amplitude states over H_7 single channel noise was modest, ≤5% broadening at half-width of the individual peaks in current histograms (filtered at 5 kHz), denoting an absence of unusual higher frequency events within these states. In two cases (out of seven that were analyzed), the typical low-amplitude behavior (Figure 2a) was preceded by two-state behavior with faster kinetics (Δg = 7 ± 1 pS; mean lifetime of lower conductance state 709 ± 81 ms, n = 19 events; frequency of occurrence 0.32 s^{-1}). The faster transitions lasted for 5 and 8 min before irreversible (>15 min) conversion to the typical behavior. When the PEG was cleaved from the pore, by reduction of the disulfide bond with DTT, the current increased to a value similar to that observed with H_7 (Figure 2b), after a lag period of 18−25 min (n = 4). Long-lived low-amplitude fluctuations were also observed with H_6K8C-PEG5K_1, centered around a mean conductance of 244 ± 19 pS (n = 8) (Table 1), which is higher than the value for H_6S106C-PEG5K_1. There were typically three to five substates with lifetimes ranging from a few tens of milliseconds to hundreds of milliseconds. Δg values (8 pS to 50 pS) were often larger than those of the substates of H_6S106C-PEG5K_1. Strikingly, the short-lived high amplitude spikes were completely absent.

Single channel current measurements were also performed on H_6S106C-PEG3K_1. The preparation was contaminated with H_7 channels and bilayers containing them were disregarded. The mean unitary conductance of H_6S106C-PEG3K_1 was 237 ± 4 pS (n = 5), somewhat higher than that of H_6S106C-PEG5K_1. Low-amplitude events were seen in only one of the six single channels that were observed. The short-lived higher amplitude negative spikes (mean lifetime, 132 ± 10 μs, n = 5) were shorter than those seen with H_6S106C-PEG5K_1, were of a similar amplitude (128 ± 3 pS, n = 5), and occurred more often (26 ± 10 s^{-1}, n = 5). After treatment with 10 mM DTT, the PEG3000 molecule exited the cavity after 15 s to 4 min (n = 5), far more rapidly than PEG5000.

Tentative Interpretation of Current Fluctuations in PEG-Modified αHL Pores. The current fluctuations observed when a PEG molecule of 5000 Da is anchored within the central cavity of αHL are remarkable, compared for example with the single invariant conductance state observed when a more rigid cyclodextrin is bound noncovalently within the channel lumen.[13] While switching between defined conductance states, rather than a continuum of states, was unexpected, plausible explanations can be proposed for the four main behaviors that we have observed with H_6S106C-PEG5K_1.

First, the reduction in current carried by the main conductance states (Figure 2a) most likely arises from changes in the properties of the electrolyte in the cavity caused by the presence of the PEG molecule. The unaltered H_7 pore is ohmic and only weakly ion selective, suggesting that ion transport is through a channel filled with electrolyte with properties close to that of bulk solution. The volume of the cavity is ~36 000 Å^3. Were the entire PEG5000 molecule within the cavity, its "concentration" would be ~23%. At this concentration, the conductivity of a solution of 100 mM KCl would be reduced by 48%,[3,40] far greater than the 18% decrease in single channel conductance observed by us. Nevertheless, the result is reasonable given that a hydrated PEG molecule cannot occlude the entire conductive pathway, from one entrance to the other, and that the PEG chain may lie partly outside the lumen.

Second, the slow low-amplitude fluctuations in current can be tentatively ascribed to rearrangements of the PEG5000 molecule within the cavity correlated with associated movements of the protein (the fluctuations do not occur with unmodified

H_7). Protein motions can occur over a wide range of time scales[41] and recently they have been observed at the single molecule level. For example, substrate fluorescence revealed fluctuations in a rate constant of cholesterol oxidase with a correlation time of about 1 s,[20,42] and FRET measurements revealed fluctuations in the conformation of staphylococcal nuclease with an average time constant of 41 ms, which was increased to 133 ms with substrate bound.[43] The third phenomenon, the very slow (minutes) interconversion between related states, is also likely to be related to rearrangement of the PEG and an associated adjustment of the protein. Long-lived conformational states in proteins have been encountered previously.[20,44,45] Alternative explanations are that the current fluctuations arise entirely from movements of the PEG chain or, at the other extreme, that the fluctuations can be ascribed solely to movements of the protein destabilized by the presence of the PEG. It may be possible to distinguish these possibilities experimentally. For example, if the motion of the PEG were uncoupled from the motion of the protein, the frequency and duration of the fluctuations would be independent of the point of attachment of the PEG within the central cavity.

The fourth phenomenon, the short-lived, high-amplitude, negative current spikes, may represent the partial looping of the PEG5000 chain into the transmembrane barrel or into the cis opening. The millisecond duration of the states is far longer than the dwell time of free PEG molecules within the αHL pore,[4,5,46] but of the same magnitude as relaxation times of PEGs tethered to supported bilayers.[47,48] The uniform amplitude of these events (Figure 2a, histogram peak "s") suggests that one or the other of the two possible looping events predominates. The pore always returns to the conductance state from which it undergoes a high-amplitude excursion ($n = 55$ events), further suggesting that the low-amplitude events involve protein conformational changes as well as PEG reorganization. If instead the low-amplitude events purely represented states of the PEG molecule, the PEG would have to retain "memory" of them during the larger excursions.

The results obtained with PEG3000 are consistent with the interpretation of the behavior of H_6S106C-PEG5K_1 as outlined above. The mean conductance of H_6S106C-PEG3K_1 is only 12% lower than the unmodified pore (Table 1), in keeping with the lower mass of PEG3000 compared with PEG5000. The lower "concentration" of PEG within the H_6S106C-PEG3K_1 pore might also explain the faster release of the PEG chain by DTT. The high-amplitude spikes occur about 100 times more often with H_6S106C-PEG3K_1, compared to H_6S106C-PEG5K_1, and are about 100 times shorter in duration, suggesting that PEG3000 is more mobile than PEG5000 within the cavity. Finally, although the interpretation of the low-amplitude events remains speculative, their rarity in the case of H_6S106C-PEG3K_1 suggests that polymer motion is less strongly coupled to protein movement than it is in H_6S106C-PEG5K_1.

Conclusions and Prospects. In summary, we have shown that a multisubunit protein, a heptameric transmembrane pore, can be constructed with a synthetic polymer tethered within an internal cavity. We do not know whether the entire polymer chain is encapsulated. Certainly, the fluctuations of current passing through a single pore in a transmembrane potential suggest that the PEG chain is flexible and may therefore sample the external solvent. While a detailed interpretation of the current fluctuations will require additional experimentation, it seems possible that current recording will become a useful tool for monitoring the dynamic properties of PEG and other polymers, including oligopeptides and oligonucleotides, at the single molecule level. Further, by using polymers that respond to analytes, it should be possible to make biosensors[9,13] based on this new class of engineered pores. This possibility does not depend on a detailed interpretation of the current fluctuations, only that they would be modulated by analytes in a concentration-dependent manner and at the same time provide analyte-specific signatures.[9,13,24] Systematic studies involving the attachment of a variety of polymers at different sites on the inner wall of the αHL pore have been initiated.

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