Temperature-Responsive Protein Pores

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Abstract: We describe temperature-responsive protein pores containing single elastin-like polypeptide (ELP) loops. The ELP loops were placed within the cavity of the lumen of the α-hemolysin (αHL) pore, a heptamer of known crystal structure. The cavity is roughly spherical with a molecular surface volume of about 39 500 Å³. In an applied potential, the wild-type αHL pore remained open for long periods. In contrast, the ELP loop-containing αHL pores exhibited transient current blockades, the data suggest that the transient current blockades are caused by excursions of ELP into the transmembrane β-barrel domain of the pore. Below its transition temperature, the ELP loop is fully expanded and blocks the pore completely, but reversibly. Above its transition temperature, the ELP is dehydrated and the structure collapses, enabling a substantial flow of ions. Potential applications of temperature-responsive protein pores in medical biotechnology are discussed.

Introduction

With the inspiration of examples from nature, a variety of polymeric materials have been designed that respond to external physical and chemical stimuli such as temperature, pH, electric fields, light, ionic strength, and various chemicals.1—4 For example, the elastin-like polypeptides (ELPs) undergo a sharp inverse temperature transition and become more ordered as the temperature increases.5—15 Below its transition temperature Tc, an ELP is soluble in water, but as the temperature is raised above Tc, it aggregates. This inverse temperature transition has been attributed to the hydrophobic collapse of individual ELP molecules with the expulsion of waters that contact the hydrophobic side chains of the polypeptide in the expanded form.

Here, we describe temperature-responsive pores containing a single ELP loop. The ELP loop is engineered within the cavity that forms part of the lumen of the staphylococcal α-hemolysin (αHL) pore, a mushroom-shaped heptameric protein of known structure (Figure 1A).16 The cavity measures ~45 Å in diameter, and its molecular surface volume is ~39 500 Å³, as determined from the X-ray crystal structure of the pore.16 Recently, tandem repeats of a Gly/Ser-containing sequence have been placed in the cavity.17 When αHL pores were assembled into heteroheptamers comprising wild-type subunits and Gly/Ser-loop-containing subunits, up to 175 exogenous residues were accommodated within the cavity of the pore.

In this work, we show a dramatic change in the functional properties of the αHL pore when the engineered ELP loop acts as a temperature-responsive gating mechanism. In an applied transmembrane potential, the wild-type αHL pore maintains its open state for long periods.18,19 In contrast, the ELP loop-containing αHL pore exhibits an open state decorated by...
transient current blockades. Below its transition temperature $T_c$, the ELP loop is in an expanded conformation and produces full current blockades. Above its transition temperature $T_c$, the ELP loop is dehydrated due to the hydrophobic collapse and causes partial current blockades. We demonstrate that the features of both the transient ELP-induced current blockades and the open-state currents are dependent on the peptide length as well as its sequence.

With further development, we anticipate that such an engineered αHL pore with a built-in temperature-responsive gating mechanism will be useful in biotechnological applications. For example, temperature-responsive protein pores might be used in drug delivery, to release the contents of capsules such as liposomes, or as biotherapeutics, to permeabilize cells to cytotoxic drugs.

### Experimental Methods

#### Generation of a Subcloning Vector

The endogenous Earl sites of pT7-L5-D4 were removed by polymerase chain reaction (PCR) and in vivo recombination (Supporting Information, Figure S1A). The resulting plasmids pYH1-L5-D4 were digested with AscI, followed by replacement of the small fragment with the DNA cassette $5'$-TCGGGGTTAGAAGAGGACAGATGCATGGC-3' (sense) and $5'$-CCGAGCCATGCACTGCTCTTCATACCC-3' (antisense) to introduce an Earl site (underlined) in between the two sites. The resulting subcloning vectors were denoted as pYH1-EC-D4 (Supporting Information, Figure S1B). Replacements were verified by DNA sequencing.

#### Generation of a Subcloning Vector Encoding Monomeric ELP Repeat Unit

The plasmids containing the gene that encodes the ELP repeat unit (pYH1-EM-D4) were generated by ligation of two double-stranded oligonucleotides formed by the single-stranded oligonucleotides, $5'$-TATGAAACTTCCGTACCCGAGAGCTCCACGAGGCATGGC-3' (EM001), $5'$-GGTGGCGGAATCCAGTTGCGGCAGTCGTCGCGG-3' (EM002), $5'$-GCTACCTGGAACGCCACCCCTCTCGACGGCCG-3' (EM003), and $5'$-AGCTTAGCTCTCATACACCTCGCCAGGGACGCTCCAC-3' (EM004), into the pYH1-L5-D4 from which the central Ndel–HindIII fragment was removed (Supporting Information, Figure S1C). The Earl sites, which flank the repeat unit, are underlined. Prior to ligation, the 5' and 3' ends of EM002 and EM003 were phosphorylated by T4 polynucleotide kinase (New England BioLabs, Beverly, MA) followed by enzyme inactivation at 65 °C for 20 min. The repeat unit encoded five tandem repeats of VPQGG. The ligated product was transformed intoSURE2 supercompetent *Escherichia coli* cells (Stratagene, La Jolla, CA) to prevent in vivo recombination. The replacement was verified by DNA sequencing.

#### Construction of αHL Genes with DNA Inserts Encoding ELP Repeats

Concatamers of the repeat unit were prepared by ligation of purine repeated units from pYH1-EM-D4 plasmid by use of Earl sites and an unphosphorylated “cap DNA” (1:20 molar ratio of cap/repeat unit) at 16 °C for 16 h (Supporting Information, Figure S1C). The cap DNA is designed to control the extent of concatamerization and to provide the 5' and MluI site for further cloning. The cap DNA encodes $\text{GGGGS/IDTKEYA/5'-GAGGAGGCTCCTCGGGTGGTAGATA-3'}$ (sense) and $\text{5'-CCGCTACTTGGTGTCAATCGAGCCGAGGGA/3'}$ (antisense). The italic-type letters indicate the exogenous residues, whereas the other letters show the endogenous L5 αHL residues (S11IDTKEYA19). Head-to-tail ligated dimer and tetramer repeats were purified from a preparative 2.5% agarose gel by use of a Qiaex II gel extraction kit (Qiagen, Valencia, CA), followed by ligation to the large Earl–MluI fragment of pYH1-EC-D4 and transformation into SURE2 supercompetent *E. coli* cells (Stratagene, La Jolla, CA). The final constructs consisted of five residues...
(GGSSG) upstream of ELPs, the ELP repeats (25, 50, or 100 residues) and six residues downstream (GGSSG) of ELPs (Figure 1B). The flexible Gly/Ser linker sequences were placed between ELP repeats and \( \alpha \) HL to provide minimal structural perturbation. The modified \( \alpha \) HL genes were sequenced by Lone Star Labs Co. (Houston, TX).

Synthesis, Oligomerization, and Purification of the \( \alpha \) HL Protein Pores. The \( \alpha \) HL polypeptides were synthesized and oligomerized by in vitro coupled transcription and translation in the presence of purified rabbit red blood cell membranes and then purified by SDS-PAGE, as described previously.\(^{17,24}\)

Electrical Recordings in Planar Bilayers. Electrical recordings were carried out with planar bilayer lipid membranes (BLMs).\(^ {25-27}\) The cis and trans chambers of the apparatus were separated by a 25-\( \mu \)m thick Teflon septum (Goodfellow Corp., Malvern, PA). A 1,2-dipalmitoyl-sn-glycerophosphocholine (Avanti Polar Lipids, Alabaster, AL) bilayer was formed across a 60-\( \mu \)m wide aperture in the septum. The electrolyte in both chambers was 2 M KCl and 10 mM potassium phosphate buffer, pH 7.4. The \( \alpha \) HL pores were introduced by adding gel-purified heteroheptamers (0.5–2.0 \( \mu \)L) to the cis chamber, to give a final protein concentration of 0.05–0.3 ng/mL. Single-channel currents were recorded by using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) connected to Ag/AgCl electrodes through agar bridges. The cis chamber was grounded and a positive current (upward deflection) represents positive charge moving from the trans to cis side. A Pentium PC was equipped with a Digidata 1322A A/D converter (Axon) for data acquisition. The signal was low-pass-filtered with an 8-pole Bessel filter at a frequency of 20 kHz and sampled at 50 kHz. For data acquisition and analysis, we used the pClamp 9.2 software package (Axon).

Temperature Controller for Single-Channel Electrical Recordings with Planar Lipid Bilayers. The temperature-control experiments were carried out by using a Dagan HCC-100A controller (Dagan Corp., Minneapolis, MN), which was adapted to planar bilayer recordings. The HCC-100A heats and cools an aluminum thermal stage through Peltier elements. The temperature was computer-controlled through an external command connection via the Digidata 1322A (Axon). Temperature was simultaneously monitored in the aluminum stage and in the bilayer chamber with thermocouple probes.

Molecular Graphs. The ELP loop-containing subunit (E10) was generated by Swiss model.\(^ {28}\) The \( \alpha \) HL model (7ahl) was generated with the SPOCK 6.3 software package.\(^ {29}\)

Results

Preparation of an ELP Loop-Containing \( \alpha \) HL Pore. We have generated \( \alpha \) HL pores with a single ELP loop. These ELP loop-containing \( \alpha \) HL pores were consisted of one ELP-containing \( \alpha \) HL (E) subunit and six wild-type \( \alpha \) HL (W) subunits (Figure 1A). Each E subunit was generated by placing an ELP with Gly/Ser-based linkers upstream of the 106 position of the \( \alpha \) HL protein (Figure 1B). To examine the effect of the length of the inserted loop, we have chosen three ELPs with the Gly/Ser-based linkers upstream of the 106 position of the \( \alpha \) HL polypeptides were synthesized and oligomerized by in vitro transcription and translation (IVTT) in the presence of [\(^ {35}\)S]methionine, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1C; 35, 37, and 45 kDa, respectively), were in accord with the calculated molecular masses (36.4, 38.2, and 41.9 kDa, respectively).

The ELP loop-containing \( \alpha \) HL pores were prepared by IVTT in the presence of [\(^ {35}\)S]methionine and purified rabbit erythrocyte membranes.\(^ {30,31}\) Heteroheptamers with one ELP loop-containing subunit, E\(_5\)W\(_6\), were separated from other heptamers (W\(_7\), E\(_5\)W\(_5\), E\(_6\)W\(_4\), E\(_7\)W\(_3\), E\(_8\)W\(_2\), and E\(_9\)W\(_1\)) by SDS-PAGE (Figure 2A). The four-aspartate-residue tail (D4), which was placed on the C terminus of the E subunits (Figure 1B), allowed identification of the number of the E subunits present in each heteroheptamer by the electrophoretic shifts, as shown in previous studies.\(^ {17,32}\) Heteroheptamers with E5 (or E10) and W subunits showed downward electrophoretic shifts in accord with the number of oligoaspartate tails (Figure 2A, starred). However, the E20W\(_6\) proteins (Figure 2A, dotted) showed an anomalously fast electrophoretic mobility compared with the

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\(^{28}\) Guex, N.; Peitsch, M. C. Electrophoresis 1997, 18, 2714–2723.

\(^{29}\) Christopher, J. A. SPOCK: the structural properties observation and calculation kit; Center for Macromolecular Design, Texas A&M University: College Station, TX, 1998.


WD₆W₆ proteins, where WD is a wild-type subunit with a D4 tail placed on the C terminus. To confirm the purity and stoichiometry of the E₁W₆ heteroheptamers, the proteins were isolated and heated to dissociate the subunits and then further analyzed by a second analytical round of 10% SDS PAGE (Figure 2B). The relative intensity of E₁₀ to W subunit was approximately 1:6 (1:(5.8 ± 0.5), n = 3). For the E₅₁W₆ and E₂₀₁W₆ proteins, the relative intensity of E subunit to W subunit was also 1:6 (E₅₁W₆, 1:(6.3 ± 0.8), n = 3; E₂₀₁W₆, 1:(6.5 ± 0.7), n = 3). The E₁₀₁W₆ and E₂₀₁W₆ samples were exposed for 20 days to obtain satisfactory signals, whereas the E₅₁W₆ samples were exposed for 16 h.

Electrical Currents through Individual ELP Loop-Containing αHL Pores at Room Temperature. E₅₁W₆, E₁₀₁W₆, and E₂₀₁W₆ were further examined by single-channel electrical recordings at room temperature (23 ± 0.5 °C). Currents flowing through individual αHL pores were recorded at +80 mV in 2 M KCl and 10 mM potassium phosphate buffer at pH 7.4. The current flowing through a wild-type αHL pore (W₇) was 164 ± 6 pA (e.g., the open-state current amplitude, n = 7 experiments, Figure 3A). Under these conditions, the open state was of long duration (~hours). The W₇ pore remained opened for an indefinite period of time even at elevated temperatures, as also observed recently. The open-state current amplitude through individual ELP loop-containing αHL pores was significantly reduced and decorated by transient current blockades, the nature of which depended on the ELP length (Table 1, Figure 3B–D) and temperature (see below).

For the engineered αHL pore with a short ELP [E₅₁W₆, molecular mass (ELP) = 2.6 kDa, 36 amino acids, Table 1], we observed an open-state current amplitude of 139 ± 8 pA (n = 4), which is a reduction of 15% ± 2.5% compared to the open state of the W₇ pore (Figure 3B). The very short-lived negative spikes (Figure 3B) are characterized by a mean duration, current amplitude, and frequency of 108 ± 12 µs (n = 4), 98 ± 11 pA (70.5% of the open-state current amplitude, n = 4), and 140 ± 9 s⁻¹ (n = 4), respectively (Table 1).


Figure 3. Representative single-channel electrical recordings of (A) WT-αHL (W₇), (B) E₅₁W₆, (C) E₁₀₁W₆, and (D) E₂₀₁W₆ recorded at room temperature (23 ± 0.5 °C) in 2 M KCl and 10 mM potassium phosphate buffer, pH 7.4. The transmembrane potential was +80 mV.
Table 1. Electrical Signatures of αHL Protein Pores with Single Polymers Attached within the Cavity at Room Temperature

<table>
<thead>
<tr>
<th>modified pore</th>
<th>no. of amino acids</th>
<th>molecular mass (kDa)</th>
<th>ΔI/I WT(^b)</th>
<th>(I/I_{WT})(^c)</th>
<th>event frequency (s(^{-1}))</th>
<th>event duration (µs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αHL-PEG-3 kDa(^d)</td>
<td>N/A</td>
<td>3.0</td>
<td>0.14 ± 0.02 (5)</td>
<td>0.45 ± 0.04 (5)</td>
<td>26 ± 10 (5)</td>
<td>132 ± 10 (5)</td>
</tr>
<tr>
<td>αHL-PEG-5 kDa(^e)</td>
<td>N/A</td>
<td>5.0</td>
<td>0.18 ± 0.03 (8)</td>
<td>0.42 ± 0.03 (8)</td>
<td>20 ± 0.02 (8)</td>
<td>13 700 ± 2200 (8)</td>
</tr>
<tr>
<td>E5(_{W6})</td>
<td>65</td>
<td>4.7</td>
<td>0.12 ± 0.02 (3)</td>
<td>0.14 ± 0.03 (3)</td>
<td>155 ± 12 (3)</td>
<td>65 ± 7 (3)</td>
</tr>
<tr>
<td>E10(_{W6})</td>
<td>36</td>
<td>2.6</td>
<td>0.15 ± 0.03 (4)</td>
<td>0.29 ± 0.04 (4)</td>
<td>140 ± 9 (4)</td>
<td>108 ± 12 (4)</td>
</tr>
<tr>
<td>E10(_{W6})</td>
<td>61</td>
<td>4.4</td>
<td>0.27 ± 0.04 (14)</td>
<td>0.04 ± 0.02 (14)</td>
<td>61 ± 7 (14)</td>
<td>2600 ± 280 (14)</td>
</tr>
<tr>
<td>E20(_{W6})</td>
<td>111</td>
<td>8.1</td>
<td>0.39 ± 0.5 (3)</td>
<td>0.01 ± 0.01 (3)</td>
<td>16.0 ± 2.7 (3)</td>
<td>45 000 ± 5300 (3)</td>
</tr>
</tbody>
</table>

\(^a\) Values are means ± SD for at least three separate single-channel experiments. Room temperature was 23 ± 0.5 °C. \(^b\) ΔI/I WT = (I WT - I 0)/I WT is the reduction in single-channel current amplitude, brought about by the anchored polymer, and normalized to the value of the open state of the wild-type αHL pore (WT). I WT and I 0 are the open-state current amplitudes of the WT pore and the polymer-containing αHL pore, respectively. \(^c\) I/I WT is the residual current associated with polymer-induced current blockades, and normalized to the open-state current amplitude. \(^d\) Values from Movileanu et al.\(^25\) \(^e\) Values from Howorka et al.\(^36\) \(^f\) Values from this work.

Figure 4. Temperature dependence of the single-channel current through the temperature-responsive pore E10\(_{W6}\) recorded at (A) 20, (B) 40, and (C) 60 °C. The transmembrane potential was +80 mV. The other experimental conditions were the same as those presented in Figure 3.

[E10\(_{W6}\), molecular mass (ELP) = 4.4 kDa, 61 amino acids, Table 1, Figure 3C], the open-state current amplitude is further reduced to 123 ± 5 pA (n = 14). This result is consistent with the increase in ELP molecular mass, since a longer polymer is expected to produce a greater obstruction in the ionic flux through the αHL pore. The transient current blockades were also augmented (117 ± 4 pA, 95% ± 2% of the open-state current amplitude, n = 14), less frequent (61 ± 7 s\(^{-1}\), n = 14) and long-lived (2.6 ± 0.28 ms, n = 14) (Table 1, Figure 3C).

In the case of a long ELP [E20\(_{W6}\), molecular mass (ELP) = 8.1 kDa, 111 amino acids, Table 1, Figure 3D], we found a substantial reduction in the open-state current amplitude to 104 ± 5 pA (n = 3). The transient full current blockades (102 ± 5 pA, 99% ± 1% of open-state current amplitude, n = 3) were very long-lived (45.0 ± 5.3 ms, n = 3) and much less frequent (16 ± 2.7 s\(^{-1}\), n = 3) (Table 1, Figure 3D).

Temperature Dependence of an ELP Loop-Containing αHL Protein Pore. The E5\(_{W6}\) pore [molecular mass (ELP) = 2.7 kDa, Table 1] produced short-lived transient spikes at room temperature, with an average duration of 108 ± 12 µs (n = 4), but they were hardly resolvable at elevated temperatures (data not shown). In contrast, the very long-lived current blockades observed with the E20\(_{W6}\) pore were presumably caused by small available volume per ELP length. The E10\(_{W6}\) pore showed full current blockade events of millisecond timescale duration at 20 °C (Figure 4A), and they were resolvable throughout the temperature range examined in this work (20–60 °C). Therefore, the temperature dependence of the features of the E10\(_{W6}\) pore was further examined.

The open-state current amplitude and individual current blockades of E10\(_{W6}\) were highly temperature-sensitive (Figure 4). For example, the open-state current amplitude of the ELP loop-containing αHL pore increased with the temperature, from 119 ± 7 pA (n = 8) at 20 °C to 203 ± 11 pA (n = 5) at 40 °C and 291 ± 12 pA (n = 5) at 60 °C (Figure 4). This finding was consistent with the change in the conductivity of the buffer solution (2 M KCl and 10 mM potassium phosphate, pH 7.4) from 179 mS/cm at 20 °C to 240 mS/cm at 40 °C and 302 mS/cm at 60 °C (Supporting Information, Figure S2). Using the log likelihood ratio (LLR) test\(^18,35\), we found two types of blockades with the same temperature-dependent current amplitude but with distinct temperature-dependent durations and probabilities. For example, at 20 °C, we observed full transient current blockades with two durations: \(t_{off-1} = 390 ± 36 \mu s\),
with a probability $P_1 = 0.05 \pm 0.02$ (the short-lived states), and $t_{\text{off}^{-2}} = 4.20 \pm 0.24$ ms, with a probability $P_2 = 0.95 \pm 0.02$ (the long-lived states) ($n = 8$, Table 2). The mean durations of the short- and long-lived states were temperature-sensitive. At 40 °C, the mean duration of the short-lived states was $t_{\text{off}^{-1}} = 231 \pm 27 \mu s$ ($P_1 = 0.67 \pm 0.03$, $n = 5$), whereas the mean duration of the long-lived states was $t_{\text{off}^{-2}} = 1.8 \pm 0.16$ ms ($P_2 = 0.33 \pm 0.03$, $n = 5$) (Table 2). At 60 °C, the mean duration of the short-lived states was $t_{\text{off}^{-1}} = 120 \pm 16 \mu s$ ($P_1 = 0.95 \pm 0.02$, $n = 5$), whereas the mean duration of the long-lived states was $t_{\text{off}^{-2}} = 720 \pm 83 \mu s$ ($P_2 = 0.05 \pm 0.02$, $n = 5$) (Table 2). The total event frequency increased substantially with the temperature from 53.7 ± 5.7 s$^{-1}$ at 20 °C to 185 ± 17 s$^{-1}$ at 40 °C and 495 ± 38 s$^{-1}$ at 60 °C.

Temperature Dependence of a Gly/Ser-Based Peptide-Containing αHL Protein Pore. We examined whether the single-channel electrical signature of the peptide loop-containing αHL pores is dependent on the sequence of the exogenous peptide. We carried out single-channel recordings with a Gly/Ser-based peptide loop-containing αHL pore, L651W6, under conditions similar to those used for the E101W6 pore. The Gly/Ser-rich peptide, which has the sequence GGGGSGGGSGGSGSG, is similar in length (~65 residues) to the ELP peptide from the E101W6 pore (61 amino acids). At room temperature, in contrast to the data recorded for the E101W6 pore, the single-channel electrical recordings of the L651W6 pore revealed very short-lived (55 ± 7 ms, $n = 3$), but highly frequent (155 ± 12 s$^{-1}$) current spikes (Figure 5). At 20 °C, the open-state current amplitude of the L651W6 pore was 135 ± 7 pA ($n = 3$), whereas the maximum amplitude of the transient current blockades was 118 ± 10 pA ($n = 3$) (Figure 5). At 60 °C, these amplitudes were 312 ± 12 pA ($n = 3$) and 268 ± 12 pA ($n = 3$, respectively). The values of the spike amplitudes were not significantly affected by the rise time of the filter (~15 μs).36,37

Discussion

Features of the ELP Loop-Containing αHL Pores. The engineered αHL pore with a short ELP loop [molecular mass (ELP) ~2.6 kDa] showed short-lived and highly frequent current blockades (Figure 3B, Table 1). We found similar results with a highly flexible poly(ethylene glycol) (PEG-3 kDa) chain covalently attached to the central part of the cavity at position 106 (Table 1).38 On the basis of this previous work, we interpret the short-lived negative spikes as excursions of the ELP loop into the β-barrel part of the αHL pore. In the case of experiments with the PEG-modified αHL pores, this interpretation has been bolstered by observations made with the untethered end of the polymer locked at either side of the membrane.25

The amplitude of the PEG-induced spikes was similar to the value of the permanent current blockade when the untethered end of PEG was locked at the trans side of the bilayer. The short-lived spikes produced by the short ELP loop suggested a high mobility of the polypeptide within the cavity (~39 500 Å$^3$). In contrast, the millisecond time-scale current blockades obtained with the E101W6 pore were consistent with a less flexible ELP loop.

The very long ELP loop from the E201W6 pore was strikingly less mobile than those from the E51W6 and E101W6 pores, as judged by long-lived, large-amplitude, and infrequent current blockades. The high entropic cost for the excursions of the ELP loop into the β barrel is also suggested by a substantially increased amplitude of the noise for the closed states, most likely because of the strong interactions between the polypeptide and the pore walls (Figure 3D). The dwell-time histograms of the ELP loop-induced transient current blockades were fitted by a two-exponential distribution (Table 2). One possible explanation is that the ELP loop partitions into the β-barrel constriction in different conformations. Interestingly, we noticed that the ELP loop-induced current blockades have uniform amplitude. A uniform amplitude has also been observed for the transient current blockades produced by PEGs when permanently anchored within the cavity of the αHL pore.25,38

The engineered αHL pores containing peptide loops similar in length, but different in sequence, exhibited various single-channel electrical signatures. The E10-induced events were large-amplitude current blockades of millisecond time-scale duration. In contrast, the experiments with the L651W6 pore [molecular mass (loop) = 4.7 kDa, Table 1] showed very short-lived and highly frequent current blockades (Figure 5), which is in accord with increased flexibility of the Gly/Ser-based peptide loop because of the absence of bulky side chains at Gly residues.

Comparison of the ELP Loop-Containing αHL Pores with PEG-Modified αHL Pores. It is instructive to compare the single-channel electrical signature of the ELP loop-containing

<table>
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<th>$P_2$</th>
<th>$t_{\text{off}^{-1}}$ (ms)</th>
<th>$t_{\text{off}^{-2}}$ (ms)</th>
<th>$t_{\text{off}^{-1}+}$ (ms)</th>
<th>$t_{\text{off}^{-2}+}$ (ms)</th>
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<td>20</td>
<td>0.05 ± 0.02</td>
<td>0.95 ± 0.02</td>
<td>19 ± 1.7</td>
<td>390 ± 56</td>
<td>4.2 ± 0.24</td>
<td>4.0 ± 0.38</td>
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<tr>
<td>25</td>
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<td>0.91 ± 0.02</td>
<td>14.8 ± 1.8</td>
<td>310 ± 42</td>
<td>2.8 ± 0.18</td>
<td>2.6 ± 0.28</td>
</tr>
<tr>
<td>30</td>
<td>0.42 ± 0.03</td>
<td>0.58 ± 0.03</td>
<td>9.7 ± 1.1</td>
<td>291 ± 32</td>
<td>2.6 ± 0.21</td>
<td>1.6 ± 0.19</td>
</tr>
<tr>
<td>35</td>
<td>0.52 ± 0.03</td>
<td>0.48 ± 0.03</td>
<td>7.5 ± 1.5</td>
<td>240 ± 25</td>
<td>2.3 ± 0.19</td>
<td>1.2 ± 0.11</td>
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<td>0.33 ± 0.03</td>
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<td>1.8 ± 0.16</td>
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<td>0.44 ± 0.076</td>
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<td>0.17 ± 0.02</td>
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<td>156 ± 18</td>
<td>1.2 ± 0.14</td>
<td>0.32 ± 0.063</td>
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<td>0.89 ± 0.12</td>
<td>0.22 ± 0.018</td>
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<tr>
<td>60</td>
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<td>0.05 ± 0.02</td>
<td>1.9 ± 0.4</td>
<td>120 ± 16</td>
<td>0.72 ± 0.083</td>
<td>0.15 ± 0.021</td>
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</tbody>
</table>

*The values are means ± SD for at least five different single-channel experiments. $b$ $P_1$ and $P_2$ are the probabilities of the short- and long-lived states, respectively. $t_{\text{off}}$, the mean duration of interevent intervals, was determined directly from the "on" dwell-time histograms. $t_{\text{off}^{-1}} = t_{\text{off}^{-1}+} + t_{\text{off}^{-2}+}$, respectively. The values of the spike amplitudes were not significantly better, as judged by the log likelihood ratio (LLR) value. 18,35 $t_{\text{off}^{-1}}$ and $t_{\text{off}^{-2}}$, the mean durations of the short- and long-lived states, respectively, were determined directly from the "off" dwell-time histograms. $t_{\text{off}}$, the overall mean duration of the transient current blockade events, was calculated from the formula $t_{\text{off}} = P_1 t_{\text{off}^{-1}} + P_2 t_{\text{off}^{-2}}$. 

αHL pores with those of the PEG-modified αHL pores, which feature a single poly(ethylene glycol) (PEG) anchored within the cavity of the lumen (Table 1).²⁵,³⁸ In both cases, an increment in molecular mass of about 2 kDa (i.e., αHL-PEG-3 kDa to αHL-PEG-5 kDa or E5,W₆ to E10,W₆, Table 1) enhanced the duration of transient current blockades from about 100 μs to several milliseconds, suggesting that the long polymers [PEG-5 kDa and (VPGGG)₁₀] are less mobile than the short ones [PEG-3 kDa and (VPGGG)₅, respectively]. The frequency of the transient current blockades decreased substantially in the case of the PEG-modified αHL pores (αHL-PEG-3 kDa to αHL-PEG-5 kDa, a decrease of 2 orders of magnitude; Table 1), whereas little change in frequency was found for the ELP loop-containing αHL pores (E₅,W₆ to E₁₀,W₆, a decrease of ~57%; Table 1). At room temperature, the amplitude of the transient current blockades made by ELPs was significantly greater than that made by PEGs with similar molecular mass [i.e., (VPGGG)₅ versus PEG-3 kDa and (VPGGG)₁₀ versus PEG-5kDa, Table 1]. For example, at room temperature, a medium-sized ELP loop (molecular mass (ELP) ~ 4.4 kDa) produced a current blockade of 96% ± 2% of a full open state, whereas the PEG-5 kDa produced a current blockade of 58% ± 3% of a full open state (Table 1). These dissimilarities between the features of the ELP loop-containing αHL pores and PEG-modified αHL pores are determined by differences in the local flexibility of the anchored polymers. Also, the PEG chain has a single point of attachment,²⁵,³⁸ while the ELP loop does not have a free end.¹⁷

Recently, Kong and Muthukumar³⁹ have performed Langevin dynamics and Poisson–Nernst–Plank (PNP) calculations to simulate the current fluctuations caused by a single PEG covalently anchored within the αHL cavity.²⁵ The PNP calculations confirmed that the short-lived and large-amplitude negative spikes are due to the obstruction of the β barrel by the tethered PEG. A detailed mechanistic understanding of the transient current blockades produced by the ELP loop might be addressed by using a similar computational methodology.³⁹,⁴⁰ These simulations, for example, might explain why the amplitudes of the transient current blockades are uniform or to what extent the ELP loop partitions into the β-barrel domain of the αHL pore. An interesting question to be addressed is whether the two categories of transient current blockades are caused by ELP partitioning into the barrel while in different conformations or by the interaction of the ELP loop with different binding sites. Understanding this mechanistic problem would certainly ease the interpretation of the values of thermodynamic and kinetic parameters, such as entropies and enthalpies that can be derived from Arrhenius–van’t Hoff-type temperature-dependence plots⁴¹,⁴² of the rate constants of association (kₐ) and dissociation (kₐ₋₁). We plan to carry out such a detailed

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Figure 5. Temperature dependence of the single-channel current through the L65,W₆ pore, which contains a Gly/Ser-based loop, recorded at (A) 20, (B) 40, and (C) 60 °C. The transmembrane potential was +80 mV. The other conditions were the same as those presented in Figure 3.

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strikingly, the normalized conductance for the open and ELp-induced blockades showed a two-state profile with the midpoints located at 40.0 ± 0.4 and 42.2 ± 0.4 °C (n = 5, Figure 6B).

We also normalized the residual current (I_r) associated with the ELp-induced current blockades to the value of the open-state current amplitude (I_0) of the ELp loop-containing αHL pores. For the E10 W_6 pores, this value increased gradually from 0 (i.e., full current blockades) at 20 °C to a maximum value of 0.27 ± 0.02 at 60 °C (i.e., partial current blockades) (Figure 6C). The normalized residual current (I_r/I_0) showed a temperature-dependent two-state profile, with the midpoint located at 38.1 ± 0.4 °C (n = 5, Figure 6C). We interpret this result as a temperature-induced hydrophobic collapse of the anchored ELp. Spectroscopic,9,12,14,43 thermodynamic,5,12,44 and atomic force microscopic measurements10,11,45 and molecular dynamics simulations15,20–22,46 have established that an ELp behaves as a two-state system that undergoes an inverse temperature folding transition. We conclude that below its transition temperature, T_r, the ELp loop is in expanded conformation7,8,10,20,21 and produces full current blockades. Above T_r, the ELp loop is dehydrated due to hydrophobic collapse,7–15,20–22 producing partial current blockades.

Because the thermal properties of ELp are dependent on the peptide features (e.g., the sequence of the repeat unit, its length) and the environment (e.g., salt concentration, pH, temperature), we examined the transition temperature of the mid-sized ELp [(VPGG)_(10)Y-NH_2] by light scattering assay and CD spectroscopy. A solution of 100 µM (VPGG)_(10)Y-NH_2 in 2 M KCl and 10 mM potassium phosphate buffer, pH 7.4, showed temperature transitions with midpoints at 39.6 °C ± 0.2 (n = 3) and 38.4 °C ± 0.26 (n = 5), according to a light scattering assay (350 nm) and CD spectroscopy (molar ellipticity at 222 nm), respectively (Supporting information, Figure S3). The (VPGG)_(10)Y-based ELps exhibit a transition temperature of ~55 °C in phosphate-buffered saline solution.8 According to previous studies in Urry’s group,5 the salt concentration decreases T_r by 14 °C/[c], where [c] is the molar NaCl concentration. If we assume that NaCl and KCl have similar effects on the transition temperatures of ELps, the estimated T_r of (VPGG)_(10)Y-NH_2 in 2 M KCl and 10 mM phosphate buffer, pH 7.4, would be about 27 °C. Recently, Meyer and Chilkoti have shown that T_r increases by decreasing the ELp molecular mass.47 Since the molecular mass of the ELp loop used in this study is much smaller than that in Urry’s work,5,6 we qualitatively conclude that the inverse transition temperature of the VPGG-based ELps should be
greater than 27 °C, which is in accord with our bulk measurements (Supporting Information, Figure S3).

A simplified kinetic model with four states, which is based on the observed temperature-induced changes in the open- and closed-state current amplitudes of the E101 W6 pore, is presented in Figure 7. The ELP loop undergoes a reversible folding temperature transition at ~40 °C. States 1 and 3 represent the open and closed states, respectively, of the ELP loop-containing αHL pore at temperatures below Tc. In these states, the peptide is fully hydrated and expanded. States 2 and 4 indicate the open and partly closed states, respectively, at temperatures above Tc. In these states, the ELP loop is dehydrated, and its structure is collapsed. The excursions of a low-temperature expanded ELP into the β-barrel part of the pore produce a full current blockade (state 3). In contrast, the excursions of a high-temperature collapsed ELP produce a partial current blockade (state 4).

Conclusions. In previous studies from other groups, stimulus-responsive polymers have been attached to proteins to modulate ligand-binding2 and enzymatic activities48 and to produce temperature-induced transitions in protein-based hydrogels.49 In the present work, we have used genetic engineering to place a temperature-responsive gating mechanism within the cavity of the lumen of the αHL pore. The ELP loop-containing αHL pore is stable in SDS–polyacrylamide gels and functional, as judged by its pore-forming ability.

Engineered αHL pores, with functional polymers placed within the cavity, have been already developed.25,32,38,42,50 There is no fundamental technical problem that would prevent replacing an ELP by other functional polypeptide loops that respond to external physical or chemical stimuli, including pH, divalent metal ions, light, ionic strength, or chemical denaturants. The design of channels and pores with stimulus-activated gating mechanisms is a potentially fertile area.51,52 Recently, a light-activated nanovalve has been engineered by the attachment of synthetic photosensitive compounds to a mechanosensitive channel (MscL).53 Kramer and colleagues54 have also designed a light-responsive K+ ion channel. The gating mechanism consists of a pore blocker and a photoisomerizable azobenzene. The azobenzene assumes a trans configuration in long-wavelength light, enabling the blocker to reach the pore. In contrast, the azobenzene assumes a cis configuration in short-wavelength light. In the latter case, the blocker cannot reach the pore, thus allowing ion conduction.

Finally, knowing the relationships between structure and the functional characteristics of temperature-responsive peptides confined in nanocavities may contribute significantly to the rational design of “intelligent” biomaterials. For example, ELP sequences within αHL pores might be engineered further to enable the temperature-controlled release of drugs from lipid vesicles or the permeabilization of mammalian cells for the introduction of cryoprotectants.55,56

Acknowledgment. The experimental part of this paper was done at Texas A&M University System Health Science Center. We thank Jim Abbott (Dagan Corp.) for help concerning a customized noise-free thermal stage for bilayer recordings, Steve Cheley (Texas A&M University) for the pT7-αHL-D4 plasmid, and Aaron Wolfe for the values of the conductivity of KCl solutions at various temperatures. This work was supported by grants from the National Institutes of Health, the Office of Naval Research, and the Medical Research Council. H.B. is the holder of a Royal Society–Wolfson Research Merit Award. L.M. thanks Syracuse University for start-up funds.

Supporting Information Available: Detailed diagrams for the construction of the ELP-containing αHL genes, temperature dependence of the conductivity of the buffer solution, and transition temperature of (VPGGG)10 Y-NH2 in 2 M KCl. This material is available free of charge via the Internet at http://pubs.acs.org.

JA065827T