Aberrantly Large Single-Channel Conductance of Polyhistidine Arm-Containing Protein Nanopores

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ABSTRACT: There have been only a few studies reporting on the impact of polyhistidine affinity tags on the structure, function, and dynamics of proteins. Because of the relatively short size of the tags, they are often thought to have little or no effect on the conformation or activity of a protein. Here, using membrane protein design and single-molecule electrophysiology, we determined that the presence of a hexahistidine arm at the N-terminus of a truncated FhuA-based protein nanopore, leaving the C-terminus untagged, produces an unusual increase in the unitary conductance to ~8 nS in 1 M KCl. To the best of our knowledge, this is the largest single-channel conductance ever recorded with a monomeric β-barrel outer membrane protein. The hexahistidine arm was captured by an anti-polyhistidine tag monoclonal antibody added to the side of the channel-forming protein addition, but not to the opposite side, documenting that this truncated FhuA-based protein nanopore inserts into a planar lipid bilayer with a preferred orientation. This finding is in agreement with the protein insertion in vivo, in which the large loops face the extracellular side of the membrane. The aberrantly large single-channel conductance, likely induced by a greater cross-sectional area of the pore lumen, along with the vectorial insertion into a lipid membrane, will have profound implications for further developments of engineered protein nanopores.

There is a pressing demand for the design and creation of novel protein nanopores in medical bionanotechnology. The β-barrel remains one attractive protein scaffold, because of the remarkable tractability to major modifications in its biophysical, biochemical, and structural features. The fundamental basis of this tractability and versatility of the β-barrel proteins is their unusually high thermodynamic stability. These traits are determined by the rich network of stabilizing hydrogen bonds among the antiparallel β-strands. Monomeric β-barrels are advantageous for protein redesign, because multimeric proteins require further purification steps for separating the targeted modified oligomer from other products of the oligomerization reaction. Therefore, several groups pursued the engineering of these proteins for their use as a single-chain, pore-forming polypeptide for the precise alteration of their structure, chemical function, and folding dynamics at single-molecule resolution. For example, outer membrane protein G (OmpG) of Escherichia coli has received an increasing level of interest in the examinations of gating fluctuations and development of stochastic sensing elements. Moreover, studies of structure–function relationships were extended to other monomeric β-barrel proteins, such as outer membrane protein A (OmpA) of E. coli, outer membrane carboxylate channels (Occ) of Pseudomonas aeruginosa, ferric hydroxamate uptake component A (FhuA) of E. coli, and mitochondrial voltage-dependent anion channel (VDAC).

In this work, we demonstrate the unusual enhancement of the single-channel conductance of a monomeric β-barrel pore by fusing a hexahistidine arm to the N-terminus of the polypeptide chain. This β-barrel is a redesigned variant of ferric hydroxamate uptake A (FhuA) of E. coli, a 714-residue protein composed of 22 antiparallel β-strands. These β-strands are connected to each other by 10 short β-turns (T) on the periplasmic side and 11 large extracellular loops (L) on the extracellular side. The X-ray crystal structure of the native FhuA protein reveals a pore length of ~6.5 nm from the periplasm to extracellular side, whereas the cross-sectional diameter varies at different locations of the pore lumen, it being 2.6 nm × 3.9 nm on the periplasmic opening and 2.6 nm × 3.6 nm on the extracellular entrance. These dimensions included the average length of the side chains of the internal residues. FhuA is a TonB-dependent transpor21−23 that mediates the navigation of Fe3+ complexed by the siderophore ferrichrome across the outer membrane (OM) of Gram-negative bacteria.
way, the lumen of the FhuA protein serves as a permeation conduit for the high-affinity, energy-driven uptake of Fe\(^{3+}\) into the bacterial cell, which is facilitated by the TonB complex to unplug the channel.\(^{24}\) However, its primary role of Fe\(^{3+}\) transporter further extends to a number of antibiotics, including albomycin\(^{25-28}\) and rifamycin.\(^ {29}\) Moreover, FhuA functions as a receptor for a number of bacteriophages, such as T1, T5, and \(\phi 80,27-29\) antimicrobial microcin J25 peptide,\(^ {30}\) and colicin M toxin.\(^ {31,32}\)

Only a few studies demonstrated the potential implications of polyhistidine affinity tags on the structure–function relationship of proteins. Prior explorations emphasized the alterations in structure,\(^ {33}\) stability,\(^ {34}\) and dynamics\(^ {35}\) of proteins depending on the location of the polyhistidine arm, fused to the C- or N-terminus of the recombinant protein. Some reports shared legitimate concerns that polyhistidine arms a C- or N-terminus of the recombinant protein. Some reports depending on the location of the polyhistidine arm, fused to the receptor for a number of bacteriophages, such as T1, T5, and \(\phi 80,27-29\) antimicrobial microcin J25 peptide,\(^ {30}\) and colicin M toxin.\(^ {31,32}\)

**EXPERIMENTAL METHODS**

**Design and Mutagenesis of the Expression Constructs.** All the designed genes were constructed using conventional polymerase chain reaction (PCR) and cloned into expression vector pPR-IBA1 using the Bsal restriction site. The \(\texttt{tl-fhua}\) gene was amplified from the \(\texttt{fhuA} \Delta c/\Delta l\) plasmid\(^ {36}\) using the forward primer \(\texttt{S'-CGG TCT CCA ATG CTG AAA GAA GAT TT-3'}\) and the reverse primer \(\texttt{S'-GGA GGT CTC CGC GCT TTA AAA ACG AAA AAG-3'}\). \(\texttt{fhuA} \Delta c/\Delta l\) lacked the regions encoding the core domain (\(C\)) and extracellular loops L3−L5, L10, and L11, simply named tagless FhuA (TL-FhuA), showed a single-channel conductance of \(\sim 8\) nS in 1 M KCl (Figure 1).\(^ {37}\) In this way, FhuA was converted positively charged peptide with the FhuA pore lumen confirmed its monomeric nature. Moreover, we were able to probe transient captures of the hexahistidine tag mononclonal antibody (mAb) was added to the side of the channel-forming protein addition, but not to the opposite side. This finding suggests that the hexahistidine arm was indeed accessible to the aqueous phase. In addition, this aberrant conductance of \(\sim 8\) nS was also noted when the hexahistidine arm was fused to the C-terminus of the TL-FhuA protein, which is within \(\sim 0.7\) nm of the N-terminus.

**Protein Expression and Purification.** All the \(\texttt{fhuA}\) gene variants were transformed into \(\text{E. coli}\) BL21 (DE3) cells. The ampicillin-resistant transformed cells were grown in 1 L of Luria-Bertani (LB) medium at 37 °C until OD\(_{600}\) reached \(\sim 0.5\), at which time the protein expression was induced by 0.5 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG). Cells were further grown for an additional 3−4 h until the cell growth plateaued, as measured by OD\(_{600}\). Then, cells were harvested by centrifugation at 3700g for 30 min at 4 °C. Cell lysis was accomplished using a model 110L microfluidizer (Microfluidics,
Newton, MA). Cell lysates were centrifuged at 3700g for 20 min at 4 °C to separate the insoluble pellets from the supernatant. Next, the pellets were washed by being resuspended in 300 mM KCl, 50 mM Tris-HCl, and 5 mM EDTA (pH 8) and spun down at 11200g for 20 min at 4 °C. This process was repeated, followed by two washes with 300 mM KCl, 50 mM Tris-HCl, 5 mM EDTA, and 0.5% Triton (pH 8) and a final wash with 50 mM Tris-HCl (pH 8). To purify TL-FhuA and [PA]FhuA, washed pellets were solubilized in the denaturing buffer, containing 50 mM Tris-HCl (pH 8) and 8 M urea, and loaded onto an anion exchange column (Bio-Rad, Hercules, CA), which was equilibrated with the same denaturing buffer. Proteins were eluted using a linear gradient of 1000 mM KCl, 50 mM Tris-HCl (pH 8), and 8 M urea. Washed H₆[PA]FhuA and H₆FhuA pellets were solubilized in the denaturing buffer containing 500 mM KCl, 50 mM Tris-HCl (pH 8), and 8 M urea.

Protein Refolding. Lyophilized protein samples were solubilized in 200 mM KCl, 8 M urea, and 50 mM Tris-HCl (pH 8) for 2 h at a final concentration of 30–50 μM. Then, all denatured proteins were refolded through rapid dilution. In addition, TL-FhuA was also refolded through a dialysis protocol. In the case of rapid dilution, denatured protein samples were quickly diluted into the cold refolding buffer, containing 200 mM KCl, 50 mM Tris-HCl (pH 8), 8 M urea, and 6 mM imidazole. The proteins were eluted with 500 mM KCl, 50 mM Tris-HCl (pH 8), 8 M urea, and 300 mM imidazole. All the fractions were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis for purity levels of >95%. Pure fractions were pulled out and dialyzed extensively against doubly distilled H₂O. Dialyzed fractions were lyophilized and stored at −80 °C.

Single-Channel Electrical Recordings. All single-channel electrical recordings were conducted at room temperature using planar lipid bilayers with 1,2-diphytanoyl-sn-glycero-phosphocholine (Avanti Polar Lipids, Alabaster, AL), as previously described. The lipid bilayers were formed across a 100-μm diameter aperture in a 25-μm thick Teflon film (Goodfellow Corp., Malvern, PA), which separated the cis and trans compartments. The aperture was pretreated with hexadecane (Sigma-Aldrich, St. Louis, MO) dissolved in highly purified pentane (high-performance liquid chromatography grade, Fisher, Fair Lawn, NJ) at a concentration of 10% (v/v). The refolded proteins were added to the cis compartment at a final concentration of 0.3–1.3 ng/μL. The cis compartment was grounded. The single-channel currents were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The electrolyte solution contained 1000 mM KCl and 10 mM Tris-HCl (pH 8). The single-channel currents were low-pass filtered with an eight-pole Bessel filter (model 900, Frequency Devices, Ottawa, IL) at a frequency of 10 kHz and sampled at 50 kHz, unless otherwise stated. A desktop personal computer was equipped with a DigiData 1440A A/D converter (Axon) for the acquisition of data. For both acquisition and analysis of data, we used the pClamp 10.5 software package (Axon). In the case of the mouse mAb binding experiment, the cis compartment buffer was washed out with the fresh buffer before the addition of mAb to the chamber. The anti-hexahistidine tag mAb was purchased from GenScript (Piscataway, NJ).

RESULTS

Discovering an Unusual High-Conductance Open State of a Monomeric β-Barrel. Recent single-channel recordings demonstrated that a truncation FhuA mutant lacking the 160-residue cork domain and five extracellular loops (L3–L5, L10, and L11) exhibits an open-state conductance of ~4 nS. Indeed, we confirmed that the unitary conductance histogram of TL-FhuA, a truncation FhuA derivative with no tags, showed two major peaks, a low-conductance peak centered at ~2.5 ± 0.1 nS (N = 41 distinct single-channel insertions) and an open-state conductance peak centered at ~4.0 ± 0.1 nS (N = 43) (Figure 2A and Figure S1A). For the sake of clarity in this section, we denote the numbers of distinct single-channel insertions and independent single-channel experiments with N and n, respectively. Surprisingly, we...
noted that the single-channel insertion of $H_6[PA]\_3FhuA$, a truncation FhuA mutant containing a six-His tag and a $[PA]\_3$ linker at the N-terminus, into a planar lipid bilayer displayed a substantial increase in open-state conductance, from $\sim$4 nS for TL-FhuA to $7.8 \pm 0.1$ nS ($N = 53$) (Figure 2B and Figure S1B). In addition, this engineered protein nanopore exhibited another low-conductance peak centered at 4.4 S1B). In addition, this engineered protein nanopore exhibited with respect to TL-FhuA could be due to the presence of TL-FhuA to 7.8 truncation FhuA mutant containing a six-His tag and a $[PA]\_3$.

On the basis of these findings, we judged that the observed significant increase in the unitary conductance of $H_6[PA]\_3FhuA$ with respect to TL-FhuA could be due to the presence of $[PA]\_3$, $H_6$ or both. To investigate this further, we developed two truncation FhuA variants: $H_6[PA]\_3FhuA$, featuring the fusion of $H_6$ to the N-terminus of TL-FhuA, and $[PA]\_3FhuA$, featuring the fusion of $[PA]\_3$ to the N-terminus of TL-FhuA. Indeed, the $H_6[PA]\_3FhuA$ single-channel conductance histogram displayed a peak centered at 8.0 $\pm$ 0.1 nS ($N = 36$) (Figure 2C and Figure S1C). As a distinctive property of $H_6[PA]\_3FhuA$ with respect to $H_6[PA]\_3FhuA$, these protein nanopores often showed long-lived current blockades $I/I_0 = 43 \pm 11\%$ ($n = 4$) at applied transmembrane potentials of at least $\pm 20$ mV (Figure S3D). On the other hand, $[PA]\_3FhuA$ failed to show a uniform single-channel conductance, despite numerous attempts. Specifically, the single-channel conductance histogram showed a broad distribution, in the range of 1.5–7.0 nS ($n = 12$) (Figure 2D).

The protein nanopore with the most reproducible electrical signature had a conductance of $\sim$5.5 nS ($n = 3$). Moreover, in contrast to TL-FhuA, which showed no single-channel current blockades at 20 mV, the most reproducible electrical signature of $[PA]\_3FhuA$ displayed a noisy electrical signature with transient current blockades having $I/I_0$ values between 30 and 85% (Figure S4). Therefore, we conclude that $H_6$ is critical for forming a high-conductance open-state conformation of the truncated FhuA protein within a lipid bilayer.

Far-ultraviolet circular dichroism (CD) experiments were conducted to ensure proper folding of all truncation FhuA mutants under detergent solvation conditions. Indeed, the CD spectra of all truncation FhuA variants looked quite similar to each other, displaying minima at a wavelength of $\sim$218 nm, which is indicative of a folded $\beta$-sheet structure (Figure S5). Because $\theta$ is dependent on the reciprocal of protein concentration and the number of peptide bonds, distinctions in these numbers among all truncation FhuA derivatives produced small alterations in the molar ellipticity from one mutant to another.

Moreover, we also performed urea-induced protein unfolding experiments to determine whether these truncation FhuA variants show any distinction in terms of their thermodynamic stability, which could be related to their differences in pore forming activity. The chemical denaturant-induced unfolding data were obtained by exciting the protein at a wavelength of 280 nm and monitoring the tryptophan fluorescence in the range of 310–420 nm (Figure S6 and Table S1). The unfolding of individual FhuA derivatives was monitored by the change in the wavelength of maximum emission ($\lambda_{max}$). The equilibrium unfolding curves for all the FhuA variants followed a two-state model. The fit of the unfolding curves with a symmetrical sigmoidal function permitted the determination of urea concentration at the midpoint of the unfolding transition, $C_o$, and the cooperativity parameter, $m$. The thermodynamic stability of the truncation FhuA derivatives in proteomicrobes was expressed in terms of the free energy of unfolding, $\Delta G^u$ where $\Delta G^u = mC_o$. Notably, all truncation FhuA derivatives exhibited closely similar thermodynamic stability parameters in the aqueous phase with a midpoint of the unfolding transition, $C_o$, located at $\sim$4 M and an unfolding free energy, $\Delta G^u$ of $\sim$2.6 kcal/mol.

$H_6[PA]\_3FhuA$ Is a Monomeric Protein Nanopore. Here, we were interested in examining whether $H_6[PA]\_3FhuA$ is a dimer, because it shows a double conductance of that exhibited by TL-FhuA, or is a new high-conductance open substate. TL-FhuA always inserted into a lipid bilayer in a single orientation, as judged by its asymmetric values of the current amplitudes at positive and negative biases. For example, at a transmembrane potential of 40 mV, the unitary current difference of TL-FhuA $\Delta I = I_+ - I_-$ was $\sim$5 $\pm$ 1 pA ($n = 8$), where $I_+$ and $I_-$ are the current amplitudes at positive and negative transmembrane potentials, respectively. The unitary current values at positive and negative biases were $\sim$162 and $\sim$167 pA, respectively ($n = 8$). If $H_6[PA]\_3FhuA$ was a dimer, then this current asymmetry of the negative voltage with respect to positive voltage would have increased. Instead, the $H_6[PA]\_3FhuA$ protein nanopore showed an opposite current asymmetry with respect to that of TL-FhuA at the same transmembrane potential [$\Delta I = 4 \pm 1$ pA ($n = 8$)]. The unitary current values at positive and negative biases were $\sim$318 and $\sim$314 pA, respectively ($n = 8$). We further explored the interaction of Syn B2, a short positively charged polypeptide, with both TL-FhuA and $H_6[PA]\_3FhuA$. Syn B2 is a 23-residue polypeptide with five positive charges and, therefore, is prone to interact with the acidic lumen of FhuA.20 When inserted into a planar lipid bilayer, TL-FhuA showed no events at an applied transmembrane potential of 20 mV (Figure S3A). In contrast, $H_6[PA]\_3FhuA$ displayed transient current blockades at a rate of 1.5 $\pm$ 0.7 s$^{-1}$ ($n = 3$) with $I/I_0$ being between 10 and 65% (Figure 3A).

In the presence of 10 $\mu$M Syn B2 added to the trans side, TL-FhuA displayed infrequent current blockades at a frequency of 0.32 $\pm$ 0.08 s$^{-1}$ ($n = 3$) with $I/I_0$ being between 10 and 35% (Figure 3B). In contrast, when $H_6[PA]\_3FhuA$ was employed, 10 $\mu$M Syn B2 added to the trans side produced very frequent current blockades at a rate of 186 $\pm$ 167 pA ($n = 3$) with $I/I_0$ being between 10 and 100% (Figure 3C). This finding indicates that there was a drastic alteration in the nature of the attractive electrostatic interactions between positively charged Syn B2 and the dense negative charge distribution within the FhuA pore lumen.21 If $H_6[PA]\_3FhuA$ were a dimer, then the frequency of current blockades would have been double that frequency of individual monomers. Instead, we discovered a 580-fold increase in the event frequency. Moreover, the amplitude of these Syn B2-produced current blockades was in a range of $>50\%$ of the unitary current, which is in accord with a monomeric structure of $H_6[PA]\_3FhuA$ in a lipid bilayer. Otherwise, Syn B2 would have produced current blockades in two steps for a dimer.
Is the Hexahistidine Arm Tightly Bound to the β-Barrel Scaffold of H₆[PA]₃FhuA? Here, we asked if the hexahistidine arm strongly interacted with the FhuA scaffold, thereby providing some mechanical stability for this high-unitary conductance, open-state conformation. Therefore, we examined the ability to capture the six-His tag outside the pore lumen via a transient binding with an anti-His tag monoclonal antibody (mAb). This experiment also targeted the question of whether the six-His tag was accessible to the aqueous phase on either the periplasmic or cytoplasmic side of the protein. If the six-His tag were exposed at least in part, it would have been sampled by the presence of mAb in solution. In the absence of mAb, H₆[PA]₃FhuA displayed events with the following three distinct dwell times: $\tau_1 = 0.02 \pm 0.01$ ms ($p = 0.45 \pm 0.17$; $I/I_0$ ranging from 10 to 30%), $\tau_2 = 1.0 \pm 0.4$ ms ($p = 0.47 \pm 0.22$; $I/I_0$ ranging from 30 to 50%), and $\tau_3 = 21 \pm 8$ ms ($p = 0.09 \pm 0.05$; $I/I_0$ ranging from 30 to 50%) (for all values, $n = 4$) (Figure 4). The very short dwell time, $\tau_1$, was extrapolated from the fitting curve and assuming the contribution of missing events because of limited time resolution.51,52 The addition of 67 nM mAb to the cis compartment (e.g., the side of the channel-forming protein addition) resulted in reversible and very long-lived current blockades (Figure 5 and Figure S7). The very long-lived current blockades were on the order of one minute to several minutes, with a normalized current ($I/I_0$) of 83.78 ± 8.54% ($n = 3$), indicative of a strong binding interaction of the mAb—six-His tag complex.53 It is worth mentioning that these long-lived events were not noted in the absence of mAb.
Reversible current blockades resulted in five distinct dwell times, two of which were closely similar to the $H_6[PA]_3FhuA$ events, namely, $\tau_1^c = 0.02 \pm 0.01$ ms ($p = 0.67 \pm 0.07$; $I/I_0$ ranging from 10 to 30%) and $\tau_3^c = 2.7 \pm 0.1$ ms ($p = 0.11 \pm 0.02$; $I/I_0$ ranging from 40 to 60%), whereas the other three event types were likely due to the mAb–six-His tag interaction. Their mean durations were as follows: $\tau_1^t = 0.15 \pm 0.12$ ms ($p = 0.15 \pm 0.09$; $I/I_0$ ranging from 40 to 80%), $\tau_2^t = 61 \pm 11$ ms ($p = 0.05 \pm 0.01$; $I/I_0$ ranging from 50 to 60%), and $\tau_3^t = 1800 \pm 690$ ms ($p = 0.02 \pm 0.01$; $I/I_0$ ranging from 50 to 60%) (for all values, $n = 3$). Because the $\tau_3^t$ dwell time was very short, this event likely resulted from the frequent collisions of mAb with the pore opening, but without a specific mAb–six-His tag binding interaction. The $\tau_4^t$ population appears to be close to the $\tau_3^t$ value, although these two events are different in their electrical signature (Figures 4B and 5B). For instance, the $\tau_3^t$ event was decorated by short-lived recovery current transitions to the fully open state, whereas $\tau_4^t$ did not exhibit this feature, which was indicative of a continuous mAb–six-His tag binding event. Because the anti-His tag mAb can also sample binding interactions with the four-His and five-His stretches of the hexahistidine arm, this property explains, at least in part, why we noted multiple binding dwell times. In other words, the very long-lived current blockade likely occurred because of the binding of the hexahistidine arm, whereas $\tau_4^t$ and $\tau_4^t$ were potentially determined by the binding of four-His and five-His stretches of the hexahistidine arm, respectively.

The binding of mAb to the six-His tag in the cis compartment points out the specific orientation of $H_6[PA]_3FhuA$ in the planar lipid bilayer. $H_6[PA]_3FhuA$ inserts into the bilayer in a single orientation via extracellular loops first. This hypothesis was also confirmed with a control experiment in which $H_6[PA]_3FhuA$ was examined in the presence of 67 nM mAb added to the trans side. Indeed, no significant change in the electrical signature, which pertained to the specific mAb–six-His tag binding, was observed (Figure 6). The three observed types of events were closely similar to the current blockades of $H_6[PA]_3FhuA$ in the absence of mAb, with the following average dwell times: $\tau_1^t = 0.02$ ms ($p = 0.67$; $I/I_0$ ranging from 10 to 30%), $\tau_2^t = 1.1$ ms ($p = 0.23$; $I/I_0$ ranging from 25 to 50%), and $\tau_3^t = 8.5$ ms ($p = 0.1$; $I/I_0$ ranging from 35 to 50%). The absence of the short-lived collision events noticed when the mAb was added to the cis side can be explained by the long protrusion of the $\beta$-barrel into the aqueous phase, so these events were electrically silent in this case. The outcomes of these experiments suggest that the hexahistidine arm was exposed to only the cis side and not permanently bound to the $\beta$-barrel scaffold of $H_6[PA]_3FhuA$.

Is the N-Terminus Essential for Attaining the Unusually High Single-Channel Conductance of Hexahistidine Arm-Containing FhuA? Given that the $\sim 8$ nS conductance open state was noted using the two independent hexahistidine arm-containing truncation FhuA mutants, we asked whether this outcome was dependent on the choice of the N-terminus. The homology structure of the truncation TL-FhuA mutant (Figure 1) indicates a relatively short distance of $\sim 0.7$ nm between the C- and N-termini. Therefore, a fusion site at the C-terminus would introduce the hexahistidine arm almost at the same location of the pore lumen near its periplasmic opening. Interestingly, the reconstitution of the FhuA(GGS)$_4H_6$ protein into a planar lipid membrane again revealed a high-conductance open state with a unitary value of $7.7 \pm 0.2$ nS ($n = 5$) (Figure S8), indicating that this unusual outcome was independent of the type of terminus. Moreover, the use of a (GGS)$_4$ polypeptide substantially longer and more flexible than (PA)$_3$ confirmed that the linker between the

Figure 5. Transient captures of the mouse monoclonal anti-six-His tag antibody (mAb) using $H_6[PA]_3FhuA$. (A) Schematic model of $H_6[PA]_3FhuA$ in the presence of mAb. (B) Representative single-channel electrical recording of $H_6[PA]_3FhuA$ in the presence of 67 nM mAb added to the cis side of the membrane. The representative reversible mAb–six-His binding event is colored dark blue, along with an expanded trace and its normalized all-point current amplitude histogram, whereas the very long-lived mAb–six-His binding event is shown with a dotted blue line. (C) Semilog scatter plot of $I/I_0$ vs dwell time of the events of $H_6[PA]_3FhuA$ in the presence of mAb added to the cis side. (D) Dwell time histogram in a semilogarithmic representation. The transmembrane potential was $-20$ mV. Other experimental conditions were the same as those in Figure 2. For the sake of clarity, all the electrical traces were low-pass Bessel filtered at a frequency of 7 kHz.
hexahistidine arm and terminus does not preclude the attainment of this high-conductance open state of the truncation FhuA mutant. The high-conductance open states of ∼8 nS, regardless of the choice of the terminus for the hexahistidine arm fusion, can be explained at least in part by the proximal location of the N- and C-termini.

**DISCUSSION**

In this work, we provide compelling experimental evidence of the existence of the aberrant, high-conductance open state of a hexahistidine arm-containing FhuA protein lacking the N-terminal cork domain and multiple extracellular loops. To the best of our knowledge, this is the highest single-channel conductance ever observed with a monomeric β-barrel OM protein. Such an unusually high single-channel conductance was noted with three independently prepared truncation FhuA derivatives, which differed from each other by both the terminus at which the hexahistidine arm was fused and the nature of the linker, a stiff (PA)₃ versus a highly flexible (GGS)₄ polypeptide. We noted a substantial increase in the channel conductance to values near ∼8 nS, indicating that the hexahistidine arm-containing truncation FhuA mutants likely exhibited a change in the cross-sectional area of the pore lumen.

The X-ray crystal structure of the homologous truncated FhuA protein reveals a variable internal diameter between 2.6 and 3.9 nm.²⁰ If the β-barrel of FhuA would maintain its lumen dimensions in the absence of the cork domain, with an average internal radius of ∼3.3 nm, and we assume that the voltage drop occurs across the entire length of the pore of ∼6.5 nm, then we obtain a unitary conductance of ∼9.9 nS in 1 M KCl. This value was inferred by assuming the contribution of the access resistance to the total resistance of the pore.⁵₆,⁵₇ Such a unitary conductance is even greater than the high conductance of the new open state, as obtained by the fusion of the hexahistidine arm to one of the termini of the truncated TL-FhuA protein. Because the dimensions mentioned above were crystallographically determined using a native FhuA protein, it is likely that the internal cork domain of the protein has a stabilizing effect on the conformation and shape of the β-barrel scaffold.

Very recently, using cryo-electron microscopy and two-dimensional crystallization, we showed that the c-ring complex of the Vₒ sector of V-ATPase forms a transmembrane protein pore with a cylindrical shape, whose internal diameter is ∼3.5 nm, whereas the pore length measures ∼6.5 nm.⁴⁵ Interestingly, this protein complex, reconstituted into a planar lipid membrane, exhibited channel forming ability with a unitary conductance of ∼8.3 nS. The Delcour group has extensively examined the wild type and various truncation mutants of the PapC usher of *E. coli*, a dimeric pore complex, whose individual monomers consist of a 24-stranded β-barrel channel, along with an occluding, 76-residue cork domain, as well as globular N- and C-terminal domains located on the periplasmic side.⁵₆,⁵⁹ The inner dimensions of the cross-sectional area of the 24-strand β-barrel protein pore are ∼2.5 nm × 4.5 nm. It was discovered that the removal of the plug domain of the PapC dimer, leaving a blocking α-helix domain, resulted in an increase in the single-channel conductance with two individual peaks of ∼3.0 and ∼7.3 nS for the monomer and dimer open states, respectively.²⁰

Single-channel electrophysiology, along with solid-state nuclear magnetic resonance spectroscopy and molecular dynamics (MD) simulations, showed that the mitochondrial voltage-dependent anion channel (VDAC), a 19-strand monomeric β-barrel, undergoes cross-sectional shape fluctuations that were affected by the presence of an N-terminal α-helix.¹⁸ The removal of this N-terminal α-helix led to an intermediate single-channel electrical signature affecting both the open and closed substrates of VDAC. Interestingly,
computational electrophysiology determinations via MD simulations demonstrated that the deletion of the N-terminal α-helix of VDAC, while the overall structure remained unaltered, produced a substantial increase in single-channel conductance from ~4.2 to ~6.7 nS. The electrophysiology measurements revealed that such a truncation mutant exhibited a reduced unitary conductance at the closed-state levels of the wild-type VDAC protein and an increased current noise, indicating that the N-terminal α-helix has a rather stabilizing effect on the cross-sectional shape of the pore lumen.

The biophysical mechanism for driving this high-conductance state of hexahistidine arm-containing FhuA remains unclear. We postulate that one of the two possibilities can explain this finding. First, the expanded β-barrel structure corresponding to the high-conductance open substrate might be inherited from the very early stages of the refolding process, which was mediated by the solubilizing detergents. TL-FhuA might be prone to forming a semicollapsed structure with a ~4 nS conductance, which would be prevented, otherwise, by the presence of a polyhistidine cluster at the N- or C-terminus of TL-FhuA. Breathing conformational fluctuations of the monomeric β-barrel scaffold either in a reversible or irreversible fashion were reported previously. Second, at least one histidine residue might strongly interact with the cluster of negatively charged aspartic acids located on β-turn T1, which is in the proximity of the N- and C-termini (Figure S9).

The free energy of unfolding, $\Delta G^\ddagger_u$, for all truncation FhuA derivatives in proteomicelles was surprisingly small, given the expectation that β-barrel membrane proteins exhibit high thermodynamic stability. However, this unexpected outcome is not unique among the examinations of β-barrel proteins. For example, Tamm and colleagues61 have reported that the free energy of unfolding of OmpA in lipid bilayer is only ~4.5 kcal/mol at pH 7, which is on the same order of magnitude with water-soluble proteins that are closely similar in size. Of course, this small value includes the free energy required for the transfer of all residues into the lipid bilayer (~1 kcal/mol).62 Moreover, it was recently shown that the 19-strand hVDAC-2 barrel exhibits a relatively low free energy of unfolding in detergent micelles, in the range of 2–5 kcal/mol.63 The composition of $\Delta G^\ddagger_u$ likely encompasses large thermodynamic factors that ultimately offset each other, resulting in a much smaller free energy of unfolding. It is worth mentioning that the interfacial forces at the detergent-protein and lipid-protein interfaces64 play a pivotal role in modulating these contributions to the free energy of unfolding.

The single-channel electrical recordings involving the transient captures of the N-terminally fused hexahistidine arm by a monoclonal antibody, mAb,57 demonstrated that this peptide stretch is accessible to the aqueous phase on the periplasmic side of the pore; therefore, it does not undergo excursions from the periplasmic to extracellular sides. Otherwise, these excursions would completely block the pore lumen, closely similar to those current blockades observed with the positively charged Syn B2. The experiments involving the transient long-lived captures of the anti-six-His tag mAb demonstrated the vectorial insertion of truncation FhuA variants with the extracellular loops exposed to the trans side and short β-turns exposed to the cis side. The hydrophilic tip of the extracellular loops might stochastically partition into transient, very short-lived pores of the lipid bilayer,65 initiating protein insertion. This finding is in agreement with those of other OM proteins, such as the monomeric mammalian VDAC66 and trimeric maltoporin (Lamb)67 of E. coli, which feature unidirectional insertion into a lipid bilayer. On the other hand, there are other OM proteins, including the monomeric Omp68 and trimeric OM protein F (OmpF)69 of E. coli, that insert into a lipid bilayer with no preferred orientation.

We exploited the asymmetric single-channel electrical signature of the hexahistidine arm-containing FhuA in the form of a current–voltage profile and voltage-induced current gating (e.g., uneven current amplitudes and gating fluctuations noted at both transmembrane voltage polarities). These features were coupled with the ability to capture mAb on the cis side, but not on the trans side. Previously, an engineered disulfide bond between two cysteines on the extracellular loops of OmpG enabled the modulation of the gating fluctuations using a reducing agent added to specific sides of the membrane.68 Very recently, engineered peripheral cysteines of OmpF were used to produce reversible or nonreversible disulfide reactions with thiol-directed methoxy-poly(ethylene glycol), a reagent previously employed to probe the interior of transmembrane protein pores.70,71 In both cases, the addition of a sulphydryl-reactive reagent to a specific side of the chamber revealed the preferred orientation of the protein. Udho et al.15 found that the osmotic gradient across the lipid bilayer catalyzed the insertion of the FhuA protein into the membrane, but with no preferred orientation. These distinctions between their outcomes and our findings can be explained by the use of different conditions (e.g., with or without an osmotic gradient) as well as different proteins (e.g., FhuA vs heavily truncated hexahistidine arm-containing FhuA protein). Finally, the vectorial insertion of cark-free FhuA variants into a planar lipid bilayer is in excellent accord with its orientation in the OM of E. coli, where the large loops face the cytoplasmic compartment of the membrane.

Our experiments with Syn B2 indirectly support the possibility that the 8 nS conductance open state is likely due to a change in the β-barrel conformation. We added the Syn B2 polypeptide to the trans side of the chamber and observed an aberrantly increased frequency in the Syn B2-induced current blockades with H6[PA]3FhuA, as compared with data acquired with TL-FhuA under similar conditions. We rule out the possibility that this effect is due to a direct interaction between Syn B2 and the six-His tag, because of its positioning near the periplasmic side of the pore. Moreover, in the past, we showed that no change in the frequency of binding interactions between a positively charged Syn B2 polypeptide and a protein pore occurs when there is a change in the charge on the opposite side of the polypeptide addition side.72 Therefore, our experiments with Syn B2 also suggest volumetric changes in the β-barrel conformation.

In summary, the hexahistidine arm-containing truncation FhuA mutant, acquiring an aberrantly high-conductance open state and inserting with a preferred orientation, has prospects for protein engineering in medical molecular biotechnology. Our single-channel electrical recordings with the positively charged Syn B2 polypeptide clearly indicated a poor interaction with TL-FhuA, but a substantially increased frequency of the binding events with H6[PA]3FhuA, more than likely because of the expanded cross-sectional area of the pore lumen of the latter protein, enabling an increased level of exposure of Syn B2 to more internal acidic residues. Moreover, this study raises awareness among investigators in membrane chemical biology of the potential of a major impact of the fusion of polyhistidine...
affinity tags at the terminus side on the pore forming activity of transmembrane proteins. In the future, the preferred orientation will be pivotal for the design of FhuA protein nanoparticles with controllable gating occlusions that regulate transmembrane transport across lipid vesicles and nanocapsules for drug delivery and biotherapeutics.

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