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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 their relatively short size, they are often assumed to have little or no effect on the conformation and 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 up to ~8 nS in 1 M KCl. To 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.

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Keywords

FhuA; Single-molecule detection; Ion channel; Membrane protein engineering; Peptide-antibody interactions; Protein dynamics

There is a pressing demand for the design and creation of novel protein nanopores in medical bionanotechnology.¹ The β barrel remains one attractive protein scaffold,^{2, 3} 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 anti-parallel 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.^{4, 5} 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, the outer membrane protein G (OmpG) of *Escherichia coli* has received an increasing interest for the examinations of gating fluctuations^{6, 7} and development of stochastic sensing elements.^{4, 8, 9} Moreover, studies on structure-function relationships were extended to other monomeric β -barrel proteins, such as the outer membrane protein A (OmpA) of *E. coli*,¹⁰ outer membrane carboxylate channels (Occ) of *Pseudomonas aeruginosa*,^{11–14} ferric hydroxamate uptake component A (FhuA) of *E. coli*,^{5, 15–17} and mitochondrial voltage-dependent anion channel (VDAC).^{18, 19}

In this work, we demonstrate the unusual enhancement in 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 anti-parallel β strands.²⁰ 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 protein²⁰ reveals a pore length of ~ 6.5 nm from the periplasmic to extracellular side, whereas the cross-sectional diameter varies at different locations of the pore lumen, with 2.6×3.9 nm on the periplasmic opening and 2.6×3.6 nm on the extracellular entrance. These dimensions included the average length of the side chains of the internal residues. FhuA is a TonB- dependent transporter^{21–23} that mediates the navigation of Fe^{3+} complexed by the siderophore ferrichrome across the outer membrane (OM) of Gram-negative bacteria.^{15, 17} In this way, the lumen of the FhuA protein serves as a permeation conduit for the high-affinity, energy-driven Fe^{3+} uptake into the bacterial cell, which is facilitated by the

TonB complex to unplug the channel.²⁴ However, its primary role of Fe³⁺ transporter further extends to a number of antibiotics, including albomycin²⁵ and rifamycin.²⁶ Moreover, FhuA functions as a receptor for a number of bacteriophages, such as T1, T5, and ϕ 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 on the alterations of structure,³³ stability,³⁴ and dynamics³⁵ of proteins depending on the location of the polyhistidine arm, either fused to the C- or N-terminus of the recombinant protein. Some reports shared legitimate concerns that polyhistidine arms affect the enzymatic activity of proteins.^{36–38} To our knowledge, this is the first single-molecule examination of the major changes in the electrical signature of a transmembrane protein pore by a fused polyhistidine arm. An extensive truncation FhuA mutant, featuring the deletion of an N-terminal, 160-residue cork domain and most of the five large extracellular loops, L3, L4, L5, L10, and L11, simply named tagless FhuA (TL-FhuA), showed a single-channel conductance of ~4 nS in 1 M KCl (Fig. 1).³⁹ In this way, FhuA was converted from a non-ion conducting OM protein to an ion conducting β -barrel nanopore, whose large unitary conductance was greater than those values previously recorded under different contexts.¹⁵ These situations included other truncation FhuA mutants^{40, 41} as well as single-channel conductance values measured by the interactions of FhuA with bacteriophage T5.⁴²

Here, we show that the fusion of a hexahistidine arm to the N-terminus of the TL-FhuA protein, leaving the C-terminus untagged, produced a unitary conductance of ~8 nS. This aberrant conductance likely resulted from a drastic change in the overall shape of the pore lumen. Interactions of a short and positively charged peptide with the FhuA pore lumen confirmed its monomeric nature. Moreover, we were able to probe transient captures of the hexahistidine arm when an anti-hexahistidine tag monoclonal 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 ~8 nS was also noted when the hexahistidine arm was fused to the C-terminus of the TL-FhuA protein, which is within a location of ~0.7 nm away from the N-terminus.

Experimental Methods

Design and mutagenesis of the expression constructs

All the designed genes were constructed using conventional PCR and cloned into the expression vector pPR-IBA1 using BsaI restriction site. The *tl-fhua* gene was amplified from *fhua* - *c/ 5I* plasmid³⁹ using the forward primer 5'- CGG CGG TCT CCA ATG CTG AAA GAA GTT CAG TT - 3' and the reverse primer 5'- GGA GGT CTC CGC GCT TTA AAA ACG AAAG - 3'. *fhua* - *c/ 5I* lacked the regions coding for the cork domain (C) and extracellular loops L3, L4, L5, L10, and L11, as compared to the wild-type *fhua*. TL-FhuA has no tags at either N- or C-terminus. The *[pa]₃fhua* gene, which encodes 3 repeats of PA at the N-terminus of TL-FhuA, was constructed using the forward primer 5'-TAT ACG GTC TCC AAT GCC TGC TCC CGC CCC AGC ACT GAA AGAA - 3', the reverse primer 5'- GGA GGT CTC CGC GCT TTA AAA ACG AAAG - 3', and the *tl-fhua* plasmid as

template DNA. The *h₆[pa]₃fhuA* gene, which encodes the 6×His tag and [PA]₃ at the N-terminus of TL-FhuA, was constructed using the forward primer 5'-TAT AGG TCT CGA ATG CAC CAT CAC CAT CAC CAT CCT GCT CCC GCC CCA - 3', the reverse primer 5'-GGA GGT CTC CGC GCT TTA AAA ACG AAA G - 3', and the *[pa]₃fhuA* plasmid as template DNA. Similarly, *h₆fhuA* gene, which encodes the 6×His tag at the N-terminus of TL-FhuA, was constructed using the forward primer 5'-TAT ACG GTC TCC AAT GCA CCA TCA CCA TCA CCA TCT GAA A - 3', the reverse primer 5'-GGA GG TCT CCG CGC TTT AAA AAC GAA AG - 3', and the *h₆[pa]₃fhuA* plasmid as template DNA. The *fhuA[ggs]₄h₆* gene encoding the 6×His tag and [GGS]₄ at the C-terminus of TL-FhuA was constructed using two rounds of PCR. First, the *fhuA[ggs]₄* gene was constructed using the forward primer 5'-TAT ACG GTC TCC AAT GCT GAA AGA AGT TC-3', the reverse primer 5'-AAC ACT ACC ACC ACT ACC ACC GCT GCC GCC GCT GCC GCC AAA ACG AAA GGT TGC GG - 3', and the *tl-fhuA* plasmid as template DNA. The second round of PCR was performed using the *fhuA[ggs]₄* gene as template DNA, along with the forward primer 5'-TAT ACG GTC TCC AAT GCT GAA AGA AGT TC-3', and the reverse primer 5'-GAA GAG CGC CGA GAC CTT AAT GGT GAT GGT GAT GGT GAC TAC CAC CAC T - 3'.

Protein expression and purification

All the *fhuA* gene variants were transformed into *E. coli* BL21 (DE3) cells. The ampicillin-resistant transformed cells were grown in 1 L LB media at 37 °C until OD₆₀₀ reached ~0.5, at which time the protein expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were further grown for an additional duration of 3–4 hours until the cell growth plateaued, as measured by OD₆₀₀. Then, cells were harvested by centrifugation at 3,700 *g* for 30 min and at 4°C. The cell lysis was accomplished using a Model 110L microfluidizer (Microfluidics, Newton, MA). Cell lysates were centrifuged at 3,700 *g* for 20 min and at 4°C to separate the insoluble pellets from the supernatant. Further, the pellets were washed by re-suspending them in 300 mM KCl, 50 mM Tris.HCl, 5 mM EDTA, pH 8, and spun down at 11,200 *g* for 20 min and at 4°C. This process was repeated, then followed by two washes with 300 mM KCl, 50 mM Tris.HCl, 5 mM EDTA, 0.5% Triton, pH 8, and a final wash with 50 mM Tris.HCl, pH 8. In order to purify TL-FhuA and [PA]₃FhuA, washed pellets were solubilized in the denaturing buffer, containing 50 mM Tris.HCl, pH 8, 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, 8 M urea. Washed H₆[PA]₃FhuA and H₆FhuA pellets were solubilized in the denaturing buffer containing 500 mM KCl, 50 mM Tris.HCl, pH 8, 8 M urea. The protein-containing solutions were filtered using 0.2 μm filters (Thermo Fisher Scientific, Rochester, NY) and loaded onto a 5 ml Ni-NTA column (Bio-Rad), which was equilibrated with the same denaturing buffer, via a BioLogic DuoFlow chromatography system (Bio-Rad). Then, the column was washed with 500 mM KCl, 50 mM Tris.HCl, pH 8, 8 M urea, 6 mM imidazole. The proteins were eluted with 500 mM KCl, 50 mM Tris.HCl, pH 8, 8 M urea, 300 mM imidazole. All the fractions were analyzed using SDS-PAGE for purity levels greater than 95%. Pure fractions were pulled out and dialyzed extensively against ddH₂O. Dialyzed fractions were lyophilized and stored at -80 °C.

Protein refolding

Lyophilized protein samples were solubilized in 200 mM KCl, 8 M urea, 50 mM Tris.HCl, pH 8, for 2 h at a final concentration of 30–50 μ M. Then, all denatured proteins were refolded through rapid dilution.⁴³ In addition, TL-FhuA was also refolded through a dialysis protocol.³⁹ In the case of rapid dilution, denatured protein samples were quickly diluted into the cold refolding buffer, containing 200 mM KCl, 0.5% n-dodecyl- β -D-maltopyranoside (DDM), 20 mM Tris.HCl, pH 8.0, and incubated for durations longer than 48 h at 4°C. To refold TL-FhuA via dialysis, the denatured protein samples were incubated in 1.5% DDM. They were dialyzed against the refolding buffer containing 200 mM KCl, 20 mM Tris.HCl, pH 8, at 4°C for durations longer than 48 h at 4°C. Then, the refolded protein sample was passed through a Superdex 200 HR size-exclusion chromatography column, which was equilibrated with 200 mM KCl, 20 mM Tris.HCl, pH 8, 0.5% DDM, using a BioLogic DuoFlow chromatography system (Bio-Rad). Protein concentrations were determined by their molar absorptivity at a wavelength of 280 nm.

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 conducted.^{44, 45} The lipid bilayers were formed across a 100 μ m-diameter aperture in a 25 μ m-thick Teflon film (Goodfellow Corporation, 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 (Fisher HPLC grade, 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/ μ l. 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, 10 mM Tris.HCl, pH 8. The single-channel currents were low-pass filtered with an 8-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 PC was equipped with a DigiData 1440A A/D converter (Axon) for data acquisition. For both data acquisition and analysis, 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, L4, L5, L10, and L11) exhibits an open-state conductance of $\sim$ 4 nS.³⁹ 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 $\sim$ 2.5 $\pm$ 0.1 nS (N= 41 distinct single-channel insertions) and an open-state conductance peak centered at $\sim$ 4.0 $\pm$ 0.1 nS (N= 43) (Fig. 2A; Supporting Information, Fig. S1A). For the sake of clarity in the results section, we call the numbers of

distinct single-channel insertions and independent single-channel experiments by N and n, respectively. Surprisingly, we noted that the single-channel insertion of H₆[PA]₃FhuA, a truncation FhuA mutant containing a 6×His tag and a [PA]₃ linker at the N terminus, into a planar lipid bilayer displayed a substantial increase in the open-state conductance, from ~4 nS for TL-FhuA to 7.8 ± 0.1 nS (N= 53) (Fig. 2B; Supporting Information, Fig. S1B). In addition, this engineered protein nanopore exhibited another low-conductance peak centered at 4.4 ± 0.1 nS (N= 32). Notably, the single-channel electrical signatures of the ~4 nS sub-states of TL-FhuA and H₆[PA]₃FhuA were distinctive from each other (Supporting Information, Fig. S2), in which TL-FhuA was quieter than H₆[PA]₃FhuA. At a transmembrane potential up to 20 mV, H₆[PA]₃FhuA exhibited more frequent current blockades at negative potentials (Supporting Information, Fig. S3AB). However, at voltages ± 40 mV this nanopore showed long-lived current blockades with a normalized current blockade, I/I_0 , of 49 ± 14 % (n= 4), where I and I_0 indicate the average current blockade and amplitude of the unitary current, respectively (Supporting Information, Fig. S3C).

Based upon these findings, we judged that the observed significant increase in the unitary conductance of H₆[PA]₃FhuA with respect to TL-FhuA could be due to the presence of [PA]₃, H₆, or both. To investigate this further, we developed two truncation FhuA variants; H₆FhuA, featuring the fusion of H₆ to the N-terminus of the TL-FhuA, and [PA]₃FhuA, featuring the fusion of [PA]₃ to the N-terminus of TL-FhuA. Indeed, H₆FhuA single-channel conductance histogram displayed a peak centered at 8.0 ± 0.1 nS (N= 36) (Fig. 2C; Supporting Information, Fig S1C). As a distinctive property of H₆FhuA with respect to H₆[PA]₃FhuA, these protein nanopores often showed long-lived current blockades ($I/I_0 = 43 \pm 11$ %; n = 4) at applied transmembrane potentials ± 20 mV (Supporting Information, Fig. S3D). On the contrary, [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 1.5 – 7.0 nS (n= 12) (Fig. 2D). The protein nanopore with the most reproducible electrical signature had a conductance of ~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 in a range between 30% – 85% (Supplementary Information, Fig. S4). Therefore, we conclude that H₆ is critical for forming a large-conductance open-state conformation of truncated FhuA protein within a lipid bilayer.

Far-UV 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 closely similar to each other displaying minima at a wavelength of ~218 nm, which is indicative of a folded β -sheet structure (Supporting Information, Fig. S5).^{46, 47} Because θ 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 inquire 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 310 through 420 nm (Supporting Information, Fig. S6, Table S1).¹⁰ The unfolding of individual FhuA derivatives was monitored by the change in the wavelength of maximum emission (λ_{max}). The equilibrium unfolding curves for all the FhuA variants followed a two-state model. Fit of the unfolding curves with a symmetrical sigmoidal function permitted the determination of urea concentration at the midpoint of the unfolding transition, C_m , and the cooperativity parameter, m . The thermodynamic stability of the truncation FhuA derivatives in proteomicelles was expressed in terms of the free energy of unfolding, G_u° , where $G_u^\circ = mC_m$. Notably, all truncation FhuA derivatives exhibited closely similar thermodynamic stability parameters in the aqueous phase with a midpoint of the unfolding transition, C_m , located at ~4 M and an unfolding free energy, G_u° , of ~2.6 kcal/mol.

H₆[PA]₃FhuA is a monomeric protein nanopore

Here, we were interested in examining whether H₆[PA]₃FhuA is a dimer, because it shows a double conductance of that exhibited by TL-FhuA, or is a new large-conductance open sub-state. TL-FhuA always inserted into a lipid bilayer into 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, $I = I_+ - I_- = -5 \pm 1$ 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 ~162 and ~167 pA, respectively ($n = 8$). If H₆[PA]₃FhuA was a dimer, then this current asymmetry of the negative with respect to positive voltage would have increased. Instead, the H₆[PA]₃FhuA protein nanopore showed an opposite current asymmetry with respect to TL-FhuA at the same transmembrane potential, $I = 4 \pm 1$ pA ($n = 8$). The unitary current values at positive and negative biases were ~318 and ~314 pA, respectively ($n = 8$). We further explored the interaction of Syn B2,^{48–50} a short positively charged polypeptide, with both TL-FhuA and H₆[PA]₃FhuA. Syn B2 is a 23 residue-long polypeptide with five positive charges, so prone for interacting 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 (Supporting Information, Fig. S3A). In contrast, H₆[PA]₃FhuA displayed transient current blockades at a rate of 1.5 ± 0.7 s⁻¹ ($n = 3$) with I/I_0 ranging between 10% and 65% (Fig. 3A).

In the presence of 10 μ M Syn B2 added to the *trans* side, TL-FhuA displayed infrequent current blockades at a frequency of 0.32 ± 0.08 s⁻¹ ($n = 3$) with I/I_0 ranging between 10% and 35% (Fig. 3B). In contrast, when H₆[PA]₃FhuA was employed, 10 μ M Syn B2 added to the *trans* side produced very frequent current blockades at a rate of 186 ± 35 s⁻¹ ($n = 3$) with I/I_0 ranging between 10% and 100% (Fig. 3C). This finding indicates that there was a drastic alteration in the nature of the attractive electrostatic interactions between positively charged Syn B2 and dense negative charge distribution within the FhuA pore lumen.²⁰ If H₆[PA]₃FhuA was 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 greater than 50% out of the unitary current, which is in accord with a monomeric structure of H₆[PA]₃FhuA in a lipid bilayer. Otherwise, Syn B2 would have been 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 large-unitary conductance, open-state conformation. Therefore, we examined the ability to capture the 6 $\times$ His tag outside the pore lumen via a transient binding with an anti-His tag monoclonal antibody (mAb).

This experiment also targeted the knowledge whether the 6 $\times$ His tag was accessible to the aqueous phase on either the periplasmic or cytoplasmic side of the protein. If the 6 $\times$ His tag was 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%) (all values; $n = 4$) (Fig. 4). The very short dwell time, τ_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 (Fig. 5; Supporting Information, Fig. S7). The very long-lived current blockades were in the order of one to several minutes, with a normalized current $I/I_0 = 83.78 \pm 8.54\%$ ($n = 3$), indicative of a strong binding interaction of the mAb-6 $\times$ His tag complex.⁵³ It is worth mentioning that these long-lived events were never noted in the absence of mAb.

Reversible current blockades resulted in five distinct dwell times, out of which two were closely similar to H₆[PA]₃FhuA nanopore's events, namely $\tau'_1 = 0.02 \pm 0.01$ ms ($p = 0.67 \pm 0.07$, I/I_0 ranging from 10% to 30%) and $\tau'_2 = 2.7 \pm 0.1$ 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-6 $\times$ His tag interaction. Their mean durations were the following: $\tau'_3 = 0.15 \pm 0.12$ ms ($p = 0.15 \pm 0.09$, I/I_0 ranging from 40% to 80%), $\tau'_4 = 61 \pm 11$ ms ($p = 0.05 \pm 0.01$; I/I_0 ranging from 50% to 60%), and $\tau'_5 = 1800 \pm 690$ ms ($p = 0.02 \pm 0.01$; I/I_0 ranging from 50% to 60%) (all values; $n = 3$). Because the τ'_3 dwell time was very short, this event likely resulted from the frequent collisions of mAb with the pore opening, but without a specific mAb-6 $\times$ His binding interaction. The τ'_4 population appears to be close to the τ_3 value, although these two events are different in their electrical signature (Fig. 4B and Fig. 5B). For instance, the τ_3 event was decorated by short-lived recovery current transitions to the fully open state, whereas τ'_4 did not exhibit this feature, which was indicative of a continuous mAb-6 $\times$ His binding event. Since the anti-His tag mAb can also sample binding interactions with the 4 $\times$ His and 5 $\times$ His stretches of the hexahistidine arm,^{54, 55} 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 due to the binding of the hexahistidine arm, whereas τ'_4 and τ'_5 were potentially determined by the binding of 4 $\times$ His- and 5 $\times$ His-stretches of the hexahistidine arm, respectively.

The binding of mAb to 6×His tag in *cis* compartment points out the specific orientation of H₆[PA]₃FhuA in the planar lipid bilayer. H₆[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 H₆[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-6×His binding was observed (Fig. 6). The observed three types of events were closely similar to the current blockades of H₆[PA]₃FhuA in the absence of mAb, with the following average dwell times: $\tau^*_1 = 0.02$ ms ($p = 0.67$; I/I_0 ranging from 10% to 30%), $\tau^*_2 = 1.1$ ms ($p = 0.23$; I/I_0 ranging from 25% to 50%), and $\tau^*_3 = 8.5$ 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 β barrel into aqueous phase, so these events were electrically silent in this case. The outcomes of these experiments suggest that the hexahistidine arm was only exposed to the *cis* side and was not permanently bound to the β -barrel scaffold of H₆[PA]₃FhuA.

Is the N-terminus essential in attaining the unusually high single-channel conductance of hexahistidine arm-containing FhuA?

Given that the ~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 N-terminus choice. The homology structure of the truncation TL-FhuA mutant (Fig. 1) indicates a relatively short distance of ~0.7 nm between the C and N terminus. 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)₄H₆ protein into a planar lipid membrane again revealed a high-conductance open state with a unitary value of 7.7 ± 0.2 nS ($n = 5$) (Supporting Information, Fig. S8), indicating that this unusual outcome was independent of the terminus type. Moreover, the use of a substantially longer and more flexible (GGS)₄ than (PA)₃ confirmed that the linker in between the 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 terminus choice for the hexahistidine arm fusion, can be explained at least in part due to the proximal location of the N- and C-termini.

Discussion

In this work, we provide a compelling experimental evidence for 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 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 as well as 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 get 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 terminals of the truncated TL-FhuA protein. Since the above-mentioned dimensions 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 2D crystallization, we showed that the c-ring complex of the V_0 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.^{58, 59} The inner dimensions of the cross-sectional area of the 24-stranded β -barrel protein pore are $\sim 2.5 \times 4.5$ 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.⁵⁸

Single-channel electrophysiology, along with solid-state NMR spectroscopy and molecular dynamics (MD) simulations, showed that the mitochondrial voltage-dependent anion channel (VDAC), a 19-stranded 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 sub-states of VDAC. Interestingly, computational electrophysiology determinations via MD simulations demonstrated that the deletion of the N-terminal α -helix of VDAC, but keeping the overall structure unaltered, produced a substantial increase in the 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 sub-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 sub-state might be inherited from the very early stages of the refolding process, which was mediated by the solubilizing detergents. The TL-FhuA might be prone to attain a semi-collapsed structure of ~4 nS conductance, which would be prevented, otherwise, by the presence of a polyhistidine cluster either at the N-terminus or C-terminus of TL-FhuA. Breathing conformational fluctuations of the monomeric β -barrel scaffold either in a reversible⁶ or irreversible^{18, 60} fashion were reported previously. Second, at least one histidine residue might strongly interact with the cluster of negatively charged aspartic acids located on the β -turn T1, which is in the proximity of the N- and C-termini (Supporting Information, Fig. S9).

The free energy of unfolding, G_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 colleagues (2004) have reported that the free energy of unfolding of OmpA in lipid bilayer is only ~4.5 kcal/mol at pH 7,⁶¹ which is in the same order of magnitude with water-soluble proteins of closely similar 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-stranded 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 G_u° likely encompasses large thermodynamic factors that ultimately cancel 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-terminus-fused hexahistidine arm by a monoclonal antibody, mAb,⁵⁵ demonstrated that this peptide stretch is accessible to aqueous phase on the periplasmic side of the pore; thereby, 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 anti-6×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 the protein insertion. This finding is in agreement with 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 contrary, there are other OM proteins, including the monomeric OmpG⁶⁸ and trimeric OM protein F (OmpF)⁶⁹ of *E. coli*, which 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 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 non-reversible disulfide reactions with thiol-directed methoxy-poly(ethylene glycol),⁶⁹ a reagent previously employed to probe the interior of transmembrane protein pores.^{70, 71} In both cases, the addition of sulfhydryl-reactive reagent to a specific side of the chamber pointed out the preferred orientation of the protein. Udho and colleagues (2009) found that the osmotic gradient across lipid bilayer catalyzed the insertion of the FhuA protein into the membrane, but with no preferred orientation.¹⁵ These distinctions between their outcomes and our findings can be explained by the use of different conditions (e.g., with or without osmotic gradient) as well as different proteins (e.g., FhuA versus heavily truncated hexahistidine arm-containing FhuA protein). Finally, the vectorial insertion of cork-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 that the 8 nS-conductance open state is likely due to an alteration 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 that this effect is due to a direct interaction between Syn B2 and the 6×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 charge alteration is on the opposite side of the polypeptide addition side.⁴⁸ Therefore, our experiments with Syn B2 also suggest volumetric changes of 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 due to an expanded cross-sectional area of the pore lumen of the latter protein, enabling an increased exposure of Syn B2 to more internal acidic residues. Moreover, this study raises awareness among investigators in membrane chemical biology for the potentiality of the major impact of the fusion of polyhistidine affinity tags at the terminus side on the pore-forming activity of transmembrane proteins. In the future, preferred orientation is pivotal for designing FhuA protein nanopores with controllable gating occlusions that regulate transmembrane transport across lipid vesicles and nanocapsules for drug delivery and biotherapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Ribbon diagram of tagless truncated FhuA (TL-FhuA) protein derived through homology modeling

(A) The side view of TL-FhuA; (B) The top view of TL-FhuA, which was taken from the periplasmic side. The luminal dimensions of TL-FhuA, as determined from C_{α} to C_{α} , were inferred from the X-ray crystal structure of the native FhuA (FhuA). The N-terminus of the pore was highlighted in black.

Figure 2. Single-channel conductance histogram of four truncation FhuA variants
 (A) TL-FhuA, a truncation FhuA derivative with no tags; (B) $H_6[PA]_3FhuA$, a truncation FhuA mutant containing a 6×His tag and a $[PA]_3$ linker at the N terminus; (C) H_6FhuA , a truncation FhuA variant containing a 6×His tag at the N terminus; (D) $[PA]_3FhuA$, a truncation FhuA variant containing a $[PA]_3$ linker at the N terminus. The measurements were performed using an electrolyte solution containing 1 M KCl, 10 mM Tris.HCl, pH 8.0. The single-channel conductance histograms were derived using data points resulting from several multi-channel insertion experiments. The single-channel conductance values were determined at a transmembrane potential was +20 mV.

Figure 3. Experimental evidence for the monomeric nature of $H_6[PA]_3 FhuA$

(A) Representative single-channel electrical recording of $H_6[PA]_3 FhuA$ without Syn B2 (left), along with its corresponding normalized event-amplitude histogram, I/I_0 (right); (B) Representative single-channel electrical recording of TL-FhuA in the presence of 10 μM Syn B2 added to the *trans* side (left), along with its corresponding normalized event-amplitude histogram, I/I_0 (right); (C) Representative single-channel electrical recording of $H_6[PA]_3 FhuA$ in the presence of 10 μM Syn B2 added to the *trans* side (left), along with its corresponding normalized event-amplitude histogram, I/I_0 (right). Other experimental conditions were the same as those in Fig. 2. The transmembrane potential was +20 mV. For

the sake of clarity, all the electrical traces were low-pass Bessel filtered at a frequency of 7 kHz.

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Figure 4. Single-channel electrical signature of $H_6[PA]_3FhuA$

(A) Schematic model of $H_6[PA]_3FhuA$; (B) Representative single-channel electrical recording of $H_6[PA]_3FhuA$. One representative event was highlighted in green line, along with an expanded trace and its normalized all-point current amplitude histogram; (C) Semi-log scatter plot of I/I_0 versus dwell time of the events of $H_6[PA]_3FhuA$; (D) The dwell-time histogram in a semi-logarithmic representation. The transmembrane potential was -20 mV. Other experimental conditions were the same as those in Fig. 2. For the sake of clarity, all the electrical traces were low-pass Bessel filtered at a frequency of 7 kHz.

Figure 5. Transient captures of mouse monoclonal anti-6xHis tag antibody (mAb) using H₆[PA]₃FhuA

(A) Schematic model of H₆[PA]₃FhuA in the presence of mAb; (B) Representative single-channel electrical recording of H₆[PA]₃FhuA in the presence of 67 nM mAb added to the *cis* side of the membrane. The representative reversible mAb-6xHis binding event was highlighted in bold blue line, along with an expanded trace and its normalized all-point current amplitude histogram, whereas the very long-lived mAb-6xHis binding event was highlighted in dotted blue line; (C) Semi-log scatter plot of I/I_0 versus dwell time of the events of H₆[PA]₃FhuA in the presence of mAb added to the *cis* side; (D) The dwell-time

histogram in a semi-logarithmic representation. The transmembrane potential was -20 mV. Other experimental conditions were the same as those in Fig. 2. For the sake of clarity, all the electrical traces were low-pass Bessel filtered at a frequency of 7 kHz.

Figure 6. Single-channel electrical signature of $H_6[PA]_3FhuA$ when mAb was added to the *trans* side

(A) Schematic model of $H_6[PA]_3FhuA$ in the presence of mAb added to the *trans* side; (B) Single-channel electrical recording of $H_6[PA]_3FhuA$ in the presence of 67 nM mAb added to the *trans* side of the membrane. Representative event of $H_6[PA]_3FhuA$ was highlighted in green line, along with an expanded trace and its normalized all-point current amplitude histogram; (C) Semi-log scatter plot of I/I_0 versus dwell time of the events of $H_6[PA]_3FhuA$ in the presence of 67 nM mAb added to the *trans* side; (D) Dwell-time histogram in a semi-logarithmic representation. The transmembrane potential was -20 mV. Other experimental

conditions were the same as those in Fig. 2. For the sake of clarity, all the electrical traces were low-pass Bessel filtered at a frequency of 7 kHz.

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