

Controlling a Single Protein in a Nanopore through Electrostatic Traps

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Abstract: Protein–protein pore interaction is a fundamental and ubiquitous process in biology and medical biotechnology. Here, we employed high-resolution time-resolved single-channel electrical recording along with protein engineering to examine a protein–protein pore interaction at single-molecule resolution. The pore was formed by *Staphylococcus aureus* α -hemolysin (α HL) protein and contained electrostatic traps formed by rings of seven aspartic acid residues placed at two different positions within the pore lumen. The protein analytes were positively charged presequences (pb₂) of varying length fused to the small ribonuclease barnase (Ba). The presence of the electrostatic traps greatly enhanced the interaction of the pb₂-Ba protein with the α HL protein pore. This study demonstrates the high sensitivity of the nanopore technique to an array of factors that govern the protein–protein pore interaction, including the length of the pb₂ presequence, the position of the electrostatic traps within the pore lumen, the ionic strength of the aqueous phase, and the transmembrane potential. Alterations in the functional properties of the pb₂-Ba protein and the α HL protein pore and systematic changes of the experimental parameters revealed the balance between forces driving the pb₂-Ba protein into the pore and forces driving it out.

Introduction

Protein translocation across biological membranes is a complex, multistep process involving protein–protein and protein–pore interactions.^{1,2} For example, during protein translocation through mitochondrial membranes, a protein–pore interaction constitutes one of the first important steps of protein import into the mitochondrial matrix. Once synthesized in the cytosol, the translocating proteins have to be recognized through their positively charged N-terminal targeting sequence by acidic binding sites of the receptors in the outer mitochondrial membrane.³ Then, the proteins are unfolded and translocated in a linear fashion across the two mitochondrial membranes to reach the matrix.^{4,5} This process is mediated by the transmembrane potential of the inner membrane and the ATP-dependent mitochondrial motor Hsp70. A comprehensive understanding of the protein–pore interaction at single-molecule resolution is

still missing due to the considerable complexity in the translocation machineries, a lack of sufficient structural information, and complications caused by the intrinsic gating substates of most protein translocases.^{2,6,7}

Recent advances in single-molecule technology utilizing transmembrane protein pores demonstrated the possibility to examine biochemical events at high temporal and spatial resolution and detect, explore, and manipulate individual polypeptides.^{8–14} For example, Loic Auvray and co-workers employed the α -hemolysin (α HL) protein pore to study the folding–unfolding transitions of *E. coli* maltose binding protein (MBP) under denaturing conditions.¹⁴ Their work shows that

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into the α HL pore depends on the length of the pb₂ presequence. Three pb₂ presequences of 35 (pb₂(35)), 65 (pb₂(65)), and 95 (pb₂(95)) amino acids in length were employed in this work (Figure 1b,c). When fused to the N-terminus of Ba, these presequences are all exposed to aqueous phase²⁵ and their presence does not alter the stability of the folded Ba domain.^{21,23,26} For all engineered α HL pores, the pb₂-Ba protein with a long pb₂ presequence interacted strongly with the electrostatic traps due to a weaker entropic penalty and steric clash of the Ba domain with the polypeptide fragment partitioning within the pore lumen. Overall, altering the experimental conditions and functional features of the protein pore and interacting protein we were able to identify the mechanisms of protein–pore interaction, revealing the complexity of this process, which encompasses pulling entropic and electrostatic forces.

Methods

Barnase Proteins. The substrate proteins consisted of presequences containing the first 35, 65, or 95 amino acids of yeast pre-cytochrome (pb₂)²⁷ fused to the N terminus of the small ribonuclease barnase (Ba).²⁸ Cysteine, at the 30 position in the presequence, has been mutated to valine (C14V) to prevent disulfide bonds between the fused presequences. Histidine 102 in the Ba domain was also replaced by alanine (H102A) for the inactivation of protein during its expression in *E. coli*. The genes for the three different precursors were inserted under the *lac* promoter into pQE60 plasmids.

Expression and Purification of the pb₂-Ba Proteins. *E. coli* M15 cells were transformed with the plasmids encoding the pb₂-Ba proteins and induced by IPTG. After 20 min, the cells were pulsed with 0.4 mg/mL leucine. Cells were harvested and re-suspended in the breaking buffer (50 mM NaOAc/HOAc pH 5, 1 mM PMSF, 2 mM EDTA, 5 mM benzamidine) and then sonicated on ice and spun down at 4 °C. Pellets were re-suspended in the washing buffer (50 mM NaOAc/HOAc, pH 5, 1% (w/v) Triton, 200 mM NaCl, 1 mM PMSF, 2 mM EDTA, 5 mM benzamidine). This step was repeated three times, and the final pellets were re-suspended in the extraction buffer (50 mM NaOAc/HOAc, 6 M guanidine hydrochloride, 0.1% (w/v) Triton, 0.5 mM PMSF, 2 mM EDTA, 5 mM DTT, 1 μ g/mL leupeptin, 1 μ g/mL antipain, pH 5). Pellets were homogenized, and the resultant solutions were spun down at 130 000g for 1 h and at 4 °C. The supernatants were dialyzed against the dialysis buffer (50 mM NaOAc/HOAc, 1.5 M guanidine hydrochloride, 0.5 mM PMSF, 2 mM EDTA, pH 5) for 3 h at 4 °C. The supernatants were diluted into 50 mL of dilution buffer (50 mM NaOAc/HOAc, 2 mM EDTA, 5 mM DTT, pH 5). The proteins were concentrated by Amicon 50 mL stirred cell at 4 °C (~8–10 mg/L of the cell culture).

Mutagenesis of the α HL Protein. The K131D₇, K147D₇, and K131D₇/K147D₇ mutants were made from the α HL gene in the plasmid that was synthetically constructed to produce unique restriction sites (α HL-RL3). Details of the construction of these mutants are published elsewhere.²⁹ Briefly, K131D and K147D were constructed by PCR-based recombination as previously described.³⁰ K131D/K147D was constructed by cassette mutagenesis.³¹

Expression and Purification of the α HL Protein Pores. The α HL proteins were synthesized *in vitro* by coupled transcription and

translation (IVTT) in the presence of rabbit erythrocyte membranes as previously described.^{31,32} Briefly, the [³⁵S]methionine-labeled polypeptides were purified on an 8% SDS-polyacrylamide gel. The dried gel was autoradiographed, and the bands corresponding to each α HL protein pore were excised and rehydrated in 500 μ L of MilliQ water. Gel fragments were removed with a spin filter, and the resulting filtrate was stored frozen in 50 μ L aliquots at –80 °C.

Electrical Recordings on Planar Lipid Bilayers. Electrical recordings were carried out with planar bilayer lipid membranes (BLMs).^{33,34} The *cis* and *trans* chambers of the apparatus were separated by a 25 μ m thick Teflon septum (Goodfellow Corp., Malvern, PA). A 1,2 diphytanoyl-*sn*-glycerophosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) bilayer was formed across an 80 μ m wide aperture in the septum. The electrolyte in both chambers was 1 M KCl, 10 mM potassium phosphate, pH 7.4, unless otherwise stated. The α HL pores were introduced by adding gel-purified homoheptamers (0.5–2.0 μ L) to the *cis* chamber to give a final protein concentration of 0.05–0.3 ng/mL. Single-channel currents were recorded 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. An Optiplex Pentium PC (Dell Computers, Austin, TX) 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, unless otherwise stated. For data acquisition and analysis, we used the pClamp 9.2 software package (Axon).

Results and Discussion

Dramatic Strengthening of the Protein–Pore Interaction Induced by the Exit Trap. We followed the interaction of the pb₂-Ba proteins with the engineered α HL protein pores in planar lipid bilayers by characterizing transient current blockades of individual channels (Figure 1a). The single-channel experiments were performed with the pb₂-Ba protein added to the *trans* side of the bilayer because the *cis* side of the α HL protein protrudes far in the aqueous phase, about 50 Å away from the bilayer surface, which would lead to complications in the interpretation of the single-channel data (Figure 1a). We measured the residence probability of the pb₂-Ba protein within the α HL pore, defined as the residence time spent by the protein analyte within the pore lumen divided by the total recording time

$$P_r = \frac{t_{\text{occupied}}}{t_{\text{total}}} \quad (1)$$

where t_{occupied} and t_{total} indicate the total residence time of the pb₂-Ba protein within the pore lumen and the total recording time, respectively.

Single channels of wild-type α -hemolysin (WT- α HL) at a transmembrane potential of +40 mV and in 1 M KCl, 10 mM potassium phosphate, pH 7.4 permit a current of ~43 pA (Figure 2a). Addition of 200 nM pb₂(95)-Ba proteins to the *trans* side of the bilayer produced infrequent and short-lived current blockades with an event frequency and dwell time $f = 0.06 \pm 0.01 \text{ s}^{-1}$ and $\tau = 0.31 \pm 0.06 \text{ ms}$ ($n = 3$), respectively (Figure 2a). Presumably, the current blockades were caused by the pb₂-Ba proteins partitioning into the pore lumen and obstructing the ionic flow through the α HL pore. Introducing a ring of

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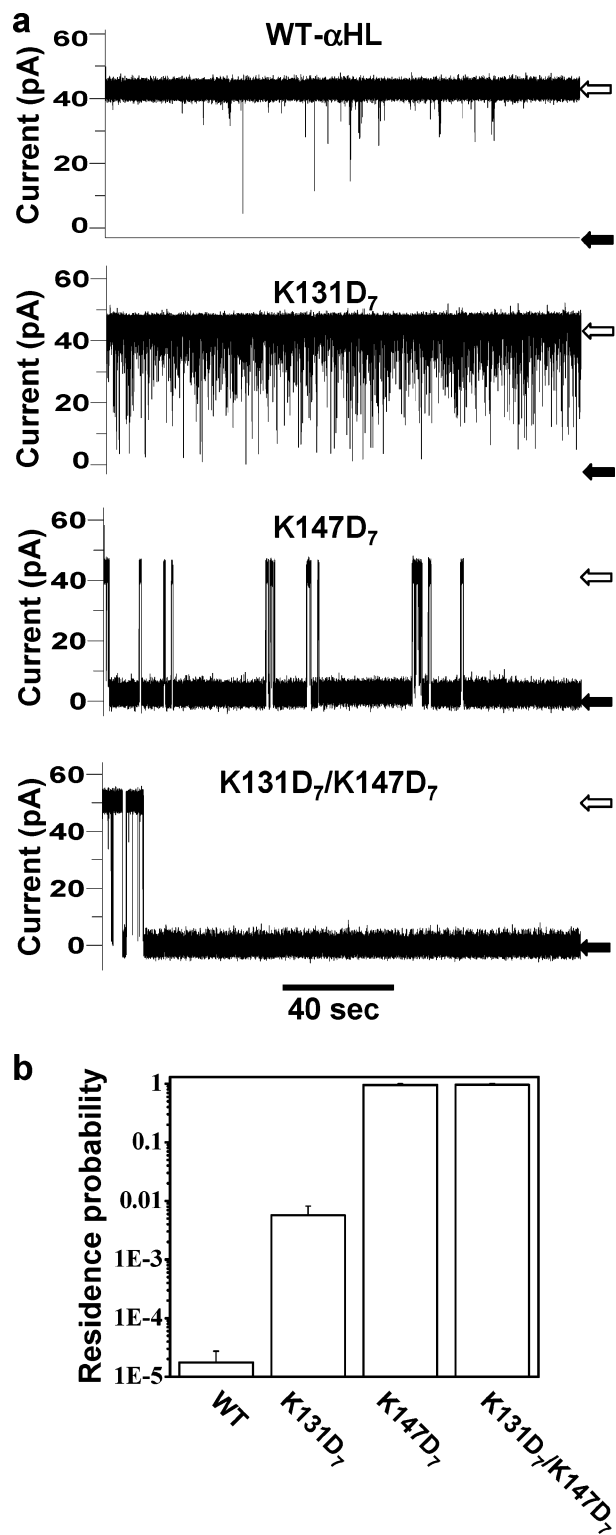


Figure 2. Effect of the pb₂-Ba protein on the single-channel electrical recordings performed with the wild-type (WT) and electrostatic trap-containing α HL pores: (a) Representative single-channel electrical recordings of the WT- α HL, K131D₇, K147D₇, and K131D₇/K147D₇ pores in the presence of 200 nM pb₂(95)-Ba added to the *trans* chamber. (b) A semilogarithmic plot of the residence probability of a single channel in the presence of 200 nM pb₂(95)-Ba added to the *trans* chamber. The residence probability was calculated as the total time for which the channel is in the closed state divided by the total recording time. Single-channel recordings were performed at room temperature in 1 M KCl, 10 mM potassium phosphate, pH 7.4 with a transmembrane potential of +40 mV. The white and black arrows indicate the open and closed states, respectively. The single-channel electrical traces were low-pass Bessel filtered at 2 kHz.

negative charges into the pore near the *trans* surface of the membrane (K131D₇, Figure 1a) increased the frequency of the current blockades almost 2 orders of magnitude, but had only a small effect on the dwell time ($f = 5.9 \pm 0.36 \text{ s}^{-1}$, $\tau = 0.24 \pm 0.03 \text{ ms}$, $n = 3$, Figure 2a). The high event frequency observed in single-channel recordings with K131D₇ was likely caused by the location of the engineered trap, near aqueous phase in the *trans* side of the bilayer, which contains the pb₂(95)-Ba protein (Figure 1a).

The effect of the pb₂(95)-Ba protein on the single-channel current through the α HL pore changed significantly when the electrostatic trap was moved from the *trans* opening of the pore to the *cis* end of the β barrel (K147D₇, Figure 1a). The transient current blockades became long lived, with the duration (τ) in the range of tens of seconds to minutes (Figure 2a) and a high residence probability (0.95 ± 0.01 , $n = 3$), but a moderate frequency ($\sim 0.012 \text{ s}^{-1}$) (Figure 2b). The α HL protein pore that contained both traps (K131D₇/K147D₇, Figure 1a) at the same time was also blocked by the pb₂(95)-Ba protein for long periods with a residence probability of 0.97 ± 0.03 ($n = 3$, Figure 2b). This residence probability is slightly greater than the value recorded with the K147D₇ pore, but the K131D₇/K147D₇ pore produced fewer long-lived current blockades ($\sim 0.006 \text{ s}^{-1}$) than the K147D₇ pore. In the absence of the pb₂-Ba protein, the WT and engineered α HL pores were open for long periods and showed no transient current fluctuations (Supporting Information, Figure S1a), suggesting that the current blockades mentioned above were not caused by intrinsic gating fluctuations of the α HL pore alone.

Tuning the Protein–Pore Interaction by Ionic Strength.

The pb₂ presequence is positively charged, and the traps within the pore lumen are negatively charged. Therefore, it seems likely that the interaction between the two proteins is electrostatic in nature. If this mechanism is correct, it should be possible to tune the interaction by changing the ionic strength of the buffer solution. We found that the transient current blockades, produced by the pb₂-Ba proteins in single-channel traces recorded with the K147D₇ pore, were more frequent and long lived in low-salt buffer than in high-salt buffer (Figure 3). Decreasing the salt concentration from 1 to 0.15 M KCl increases the residence probability by 2-fold and 1.6×10^3 -fold for the pb₂(95)-Ba and pb₂(35)-Ba proteins, respectively, at a transmembrane potential of +20 mV (Figure 3b).

These results confirm that the interaction between the pb₂ presequence and the trap-containing α HL pore is electrostatic in nature and can be manipulated by the ionic strength of the buffer solution. Again, control single-channel electrical recordings with the K147D₇ pore alone in low-salt buffer showed that neither the short-lived nor long-lived current blockades, detected under these experimental conditions, were caused by intrinsic gating events of the trap-containing α HL pore itself (Supporting Information, Figure S1b). In addition, bovine serum albumin (BSA) showed only a weak interaction with the K147D₇ protein pore (Supporting Information, Figure S2), indicating that a property specific to the pb₂-Ba protein is responsible for its interaction with the pore.

Controlling a Single Protein in a Nanopore by the Length of the Presequence. Previous work has shown that the efficiency of protein translocation *in vitro* is dependent on the

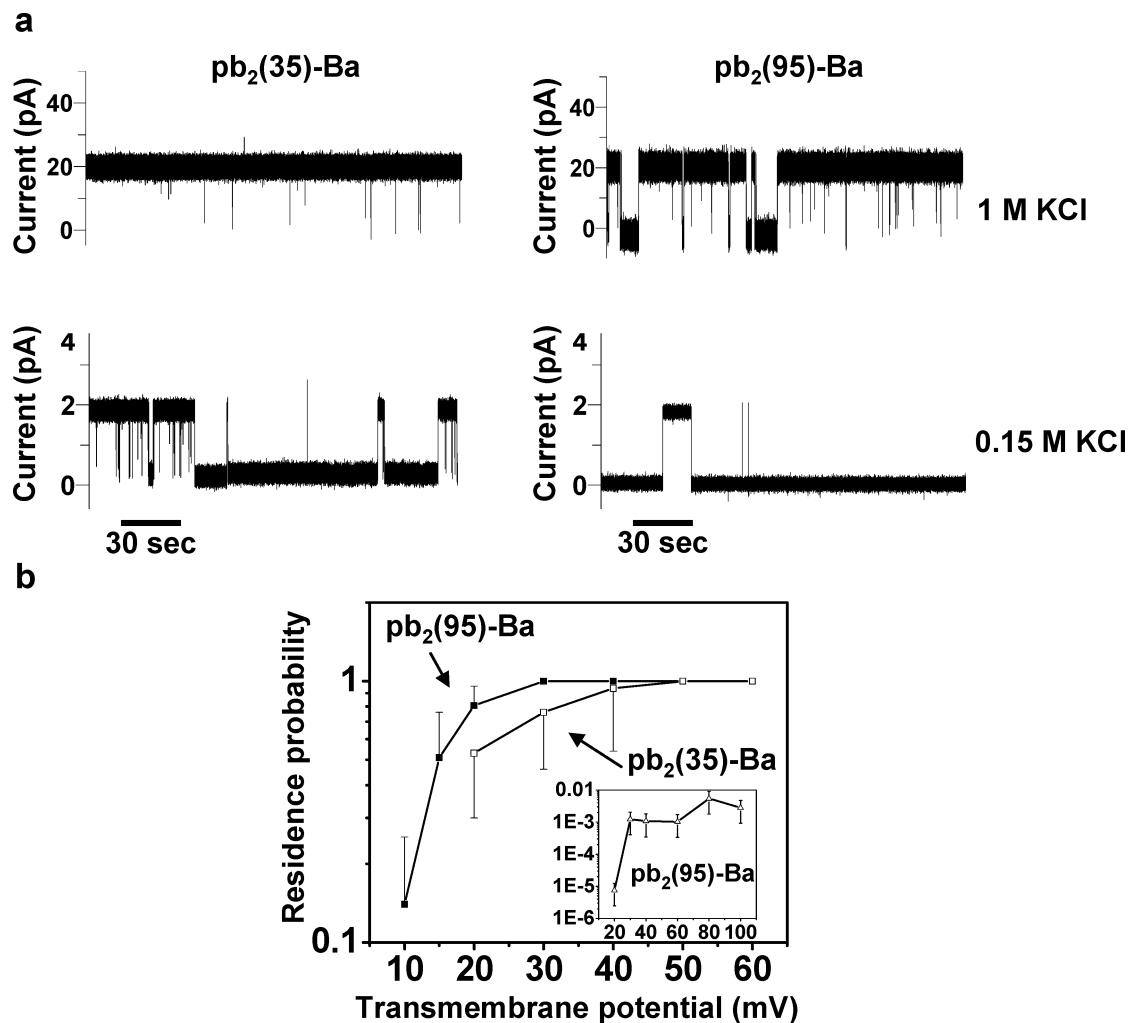


Figure 3. Dependence of the interaction between the pb₂-Ba proteins and the K147D₇ pore on the ionic strength. (a) Single-channel electrical recordings performed at a transmembrane potential of +20 mV and with the buffer solution containing either 1 M KCl (the upper traces) or 0.15 M KCl (the lower traces). (b) The residence probability. The data presented in panel b are calculated from the single-channel electrical recordings with the buffer solution containing 0.15 M KCl. pb₂-Ba (200 nM) was added to the *trans* chamber. The insert represents the control experiment with the WT-αHL pore and the pb₂(95)-Ba protein in buffer solution containing 0.15 M KCl. The other experimental conditions of the single-channel recordings were the same as those in Figure 2.

length of the presequence,^{21,23,25,35} most likely because longer presequences can reach the translocation machinery in the inner mitochondrial membrane to interact with the ATP-dependent motor Hsp70³⁶ or/and the electrical potential across this membrane.²³ To test whether the residence time of the protein in the pore lumen is also dependent on the length of the pb₂ presequence, we compared the residence probabilities of the protein analytes with the pb₂(35), pb₂(65), and pb₂(95) presequences in the K147D₇ pore at transmembrane potentials of +20, +30, and +40 mV (Figure 4). We found that also in the heterologous single-molecule system analyzed here the interaction of the pore with the pb₂(95)-Ba proteins was stronger than that with the pb₂(35)-Ba proteins at all transmembrane potentials (Table 1, Figure 4b).

Mitochondrial presequences have negligible secondary structure in solution,³⁷ and they are exposed to aqueous phase when

fused to the folded Ba domain.²⁵ The pb₂(35), pb₂(65), and pb₂(95) presequences would have a length of ~120, ~224, and ~329 Å, respectively, in a fully extended conformation, which is much longer than the length of the β-barrel part of the pore (~50 Å). All pb₂ presequences share the same first 35 residues and have the same charge density (Figure 1b), which raises the question of why the interaction between the K147D₇ pore and the pb₂-Ba protein depends on the length of the pb₂ presequence. The simplest hypothesis is that the part of the pb₂-Ba protein outside the pore pulls on the part inside the pore due to the entropic repulsion of the folded Ba domain from the pore (Figure 1d).³⁸ Since all protein analytes have the same Ba domain, the apparent difference in the interaction of the protein with the pore should be due to the length of the pb₂ presequence. If this reasoning is correct, the pb₂(95)-Ba protein causes less repulsion than pb₂(35)-Ba because higher transmembrane potential was needed to produce long-lived current blockades by the pb₂(35)-Ba protein with both K131D₇ (Supporting Information, Figure S3) and K147D₇ pores (Figure 4).

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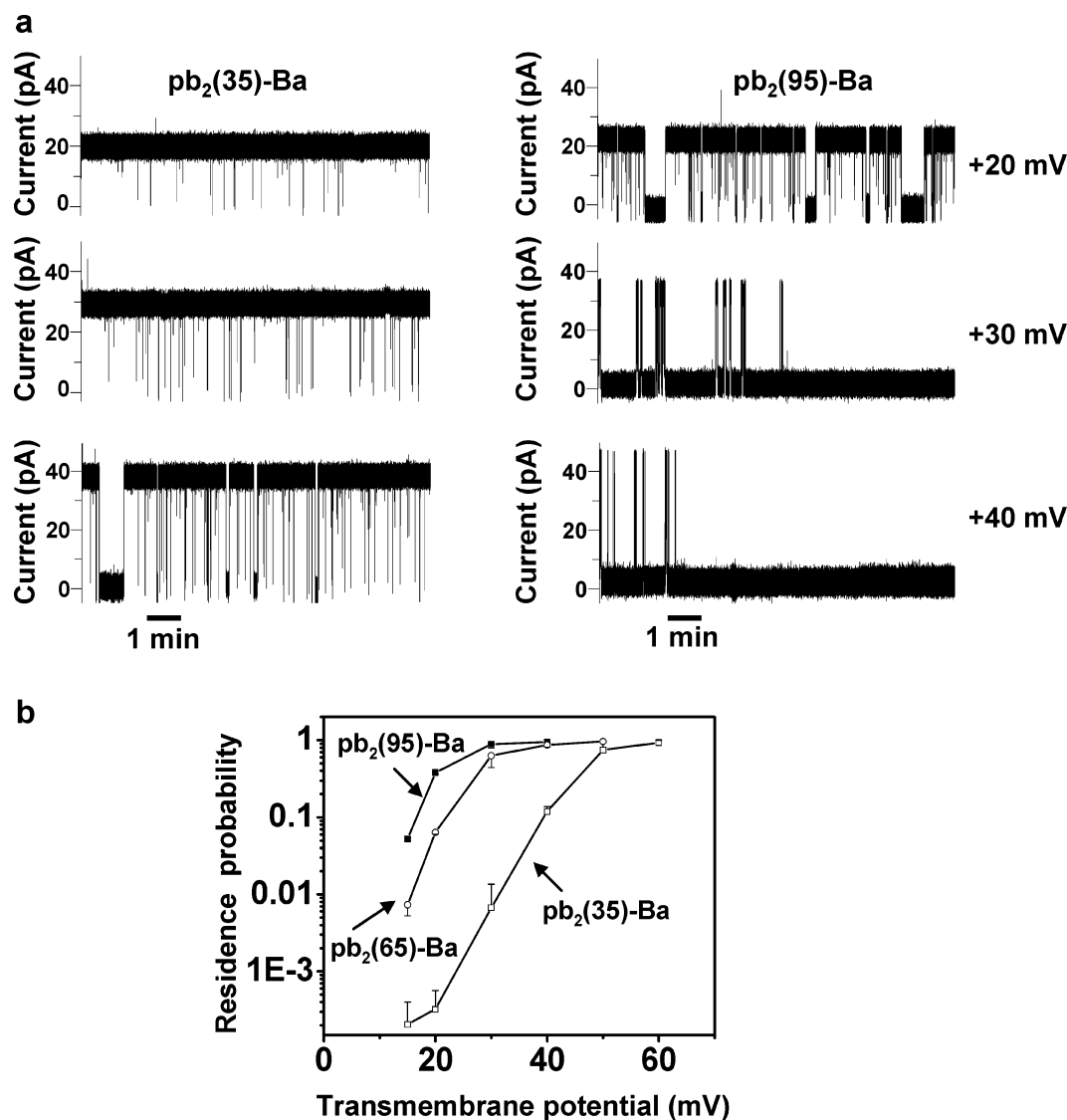


Figure 4. Dependence of the interaction between the pb₂-Ba proteins and the K147D₇ pore on the pb₂ presequence at various transmembrane potentials. (a) Representative single-channel electrical recordings in the presence of the pb₂(35)-Ba and pb₂(95)-Ba proteins. (b) Dependence of the residence probability on the transmembrane potential. pb₂-Ba (200 nM) was added to the *trans* chamber. The other experimental conditions of the single-channel recordings were the same as those in Figure 2.

Table 1. Residence Probabilities (P_r) Measured for the pb₂-Ba Proteins Interacting with a Single Trap-Containing K147D₇ Protein Pore^a

protein	transmembrane potential (mV)	residence probability, P_r
pb ₂ (35)-Ba	+15	0.00021 ± 0.00015
	+20	0.00032 ± 0.00024
	+30	0.006 ± 0.006
	+40	0.12 ± 0.02
pb ₂ (65)-Ba	+15	0.0073 ± 0.0021
	+20	0.063 ± 0.003
	+30	0.63 ± 0.18
	+40	0.86 ± 0.06
pb ₂ (95)-Ba	+15	0.053 ± 0.004
	+20	0.38 ± 0.02
	+30	0.89 ± 0.10
	+40	0.95 ± 0.04

^a The buffer solution contained 1 M KCl, 10 mM potassium phosphate, pH 7.4. The numbers represent means ± SDs.

One way to test that this repulsion has an entropic contribution is to increase the temperature at which the experiments are performed. We investigated the effect of temperature on protein–pore interaction using the K131D₇ pore mutant because

the charge–charge interaction occurs at the mouth of the pore, meaning the interaction between the positively charged presequence and the negatively charged aspartic acids at position 131. The transmembrane potential tilts the free energy landscape with a maximum and minimum driving force when the presequence is trapped at position 147 and 131, respectively. In accord with our hypothesis, single-channel electrical recordings performed at ~55 °C (+80 mV, 50 nM pb₂(95)-Ba added to the *trans* side of the lipid bilayer) showed that the frequency of the long-lived current blockades increased with the temperature, whereas their dwell time decreased (Supporting Information, Figure S4). The temperature dependence experiments revealed that both the association and dissociation rate constants increased by increasing the temperature because the activation free energies for the protein–protein pore interaction decreased. Therefore, the difference in the interaction of proteins of varying pb₂ length with the pore is at least in part due to the entropic repulsion. Control single-channel recordings with the K131D₇ pore alone showed no intrinsic gating events at 55 °C

(data not shown), again confirming the exceptional robustness of this β -barrel protein.

The repulsion forces exerted on the folded Ba domain can be estimated and are in the range of the forces that the import machinery would be expected to produce.^{5,39} At +15 mV, the residence probabilities observed with the pb₂(35)-Ba and pb₂(95)-Ba proteins interacting with the K147D₇ pore were 0.00021 ± 0.00015 and 0.053 ± 0.0035 , respectively (Figure 4b, Table 1). Therefore, the change of the free energy of the protein–protein pore interaction can be calculated from the reduction in the residence probability³⁸

$$E_{\text{entropic}} = kT \ln \left(\frac{P_r^{\text{pb}_2(95)}}{P_r^{\text{pb}_2(35)}} \right) \quad (2)$$

which gives a value of $\sim 5.5kT$. If the hypothetical entropic repulsion is elastic in nature, an extension of ~ 70 Å (~ 20 residues) yields an entropic force of ~ 6.4 pN. The force exerted on the pb₂(35) presequence during protein import into the mitochondrial matrix must be greater than the entropic force exerted by the folded Ba domain. Indeed, recent theoretical studies on the pulling forces required to induce the mechanical unfolding of the pb₂(35)-Ba protein indicated values between 11 and 13.5 pN.³⁹ A transmembrane potential of +40 mV, and assuming a linear voltage drop across the bilayer of ~ 40 Å in length, results in an electrical pulling force on the pb₂ presequence of ~ 6.4 pN. In accord with these calculations, we noticed first long-lived events produced by the pb₂(35)-Ba protein at $\sim +40$ mV (Figure 4a), at which point the pulling force counterbalances the entropic repulsion exerted by the folded Ba domain. In contrast, the pb₂(95)-Ba protein produced long-lived current blockades even at much lower transmembrane potentials, confirming a weak effect of the entropic repulsion of the Ba domain exerted on the partitioned pb₂(95) presequence (Figure 4b).

What other secondary mechanisms might also contribute to the difference in the interaction between the trap-containing α HL pore and the pb₂-Ba proteins with varying length of the pb₂ presequence? One simple interpretation is that this difference reflects the fact that the longer presequence has more positively charged residues that might also interact with the pore opening containing negatively charged aspartic acid residues.²⁰ Previous work employing mitochondrial import assay indicated that altering the residue composition of the presequence led to significant reduction in import rates.²³ Second, the long-lived blockades might be lengthened further by the excursion of the long presequences beyond the restriction site near the position K147, hindering the protein from sliding back to the *trans* side. In this case, the constriction region, near K147 residues,²⁰ can act as an entrapment site. By analogy, it has been proposed that ATP-dependent Hsp70 motor plays a critical role in entrapping the presequence once it passes through the inner membrane.⁴⁰ Both mechanisms are supported by the increased residence time with the increase of the number of charged residues in the pb₂ presequence (Figure 4b).

Although protein translocation is a complex process,² our simplified model using engineered α HL protein pore reproduced

some aspects of the behavior of the translocation machinery in intact mitochondria remarkably closely,³ and as the experimental system is easily manipulated it may make it possible to test mechanistic explanations for the behaviors. For example, the acidic-chain hypothesis states that two binding sites of the protein translocase of the outer membrane (TOM complex) mediate the interaction of the positively charged presequences with the outer membrane translocation machinery.⁴¹ In our model system too the precursor proteins interacted with the pore only when acidic rings were engineered into the translocation channel and two electrostatic traps enhanced the interaction.

How do the binding sites function in protein translocation? The binding site at the entry of the pore may lead to concentrating the pb₂-Ba proteins at the translocation channel (the targeting step).⁴² Indeed, in our studies the electrostatic trap at the entry of the pore increased the frequency of the interaction between the pb₂-Ba proteins and the α HL pore. The second binding site at the exit of translocation channel can provide an additional mechanism to minimize back sliding (the translocation step).⁴² When we explored the electrostatic trap at the exit of the pore, the back sliding to the *trans* side was minimized by virtue of the detected long-lived current blockades. The strongest interaction was observed when two traps were present within the pore lumen, suggesting that coupling the targeting and translocation steps are likely required for efficient protein import.⁴³

Concluding Remarks. In summary, we demonstrated that single-channel recordings with protein nanopores, along with protein engineering, represent a powerful approach to analyze protein–pore interactions. We found that threading a protein through a narrow transmembrane protein pore requires both a leading sequence of the translocating protein and an electrostatic trap (i.e., binding site) located within the pore lumen. Alterations of various factors, including the length of the presequence, location of the trap, transmembrane potential, temperature, and ionic strength, revealed the complexity of the protein–pore interaction encompassing repulsion, driving, and electrostatic forces. This preliminary work can be expanded in the future to other robust β -barrel protein pores or other interacting protein substrates with varying structural and stability features. This methodology makes it possible to test detailed biochemical and biophysical models of the mechanism of protein translocation. With further experimentation, the broader rules inferred from such studies should be directly applicable to different complex β -barrel protein translocases such as those of anthrax toxin,^{44,45} botulinum neurotoxins,^{46,47} and the mitochondrial import machinery,^{3,7} generating more realistic models on protein translocation. Also, with adaptation to recently developed solid-state nanopores,^{48,49} it might be possible in the future to pull folded protein domains through their leading presequences, thus

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unfolding them mechanically. In this way, the nanopore technique would permit characterization of new mechanically induced unfolding pathways in a system more immediately relevant to a natural process such as protein import into mitochondria and protein degradation by ATP-dependent proteases^{4,5} than simple AFM experiments.⁵⁰ A spin off of this work might be investigation of different protein domains through attractive forces between movable polypeptide arms and electrostatic traps engineered within the desired location of the pore lumen. This approach complements previous work that has employed tethered movable arms for detection and exploration of proteins and DNA.^{8,10,51,52}

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Supporting Information Available: Typical single-channel electrical recordings with the WT- α HL and engineered α HL protein pores, interaction between bovine serum albumin and a single trap-containing K147D₇ protein pore, interaction of the pb₂-Ba proteins with the single trap-containing K131D₇ pore, and the effect of temperature on the interaction between the pb₂(95)-Ba protein and a single trap-containing K131D₇ pore. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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