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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 (αHL) 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 (pb_2) of varying length fused to the small ribonuclease barnase (Ba). The presence of the electrostatic traps greatly enhanced the interaction of the pb_2-Ba protein with the αHL 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 pb_2 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 pb_2-Ba protein and the αHL protein pore and systematic changes of the experimental parameters revealed the balance between forces driving the pb_2-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 (αHL) 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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the nanopore technique represents a versatile tool for studying the kinetics of partially folded proteins at high temporal resolution. These single-molecule electrical measurements will likely stimulate new computational and theoretical work on the behavior of polypeptides in β-barrel protein pores.[13,15−17]

In the mitochondrial translocation system, the transmembrane potential of the inner membrane is thought to drive electrophoretically insertion of the targeting presequence into the pore lumen.[18,19] In this hypothesis, the positively charged presequence functions as a leading polypeptide fragment for driving the protein analyte into the pore lumen. If this hypothesis is correct, it may be possible to reconstitute a mitochondrial protein import system artificially using a single protein pore inserted into a planar lipid membrane. In this heterologous experimental system, the transmembrane potential could pull on the presequence, assisting the partitioning of the protein into the pore lumen.

Here, we employed a novel experimental design to investigate the above hypothesis. We analyzed the mechanisms by which a single N-terminal targeting sequence-containing protein interacts with a transmembrane protein pore. We combined high-resolution, single-channel electrical recordings along with protein engineering of the protein analyte and the protein pore to explore the protein−pore interaction at single-molecule resolution. The pore was formed by the *Staphylococcal* αHL protein, a robust and versatile heptameric β barrel whose high-resolution atomic structure is known (Figure 1a).[20] The protein analyte, pb_2-Ba, consisted of the N-terminal region of pre-cytochrome b_2 (pb_2, Figure 1b,c) fused to the N terminus of the small ribonuclease barnase (Ba).[21−23] We expected that the pb_2-Ba protein would be targeted to the pore lumen through attractive electrostatic interactions between the positively charged pb_2 presequence (Figure 1c) and negatively charged traps engineered at strategic positions within the β-barrel part of the αHL pore (Figure 1a). We tested this prediction using single-channel electrical recordings[8,24] to monitor the residence time of the pb_2-Ba protein within the pore lumen, as probed by transient alterations of the open-state, single-channel current (Figure 1d).

We tested three specific questions. First, we explored how electrostatic traps introduced into the αHL pore through mutation of seven amino acids to negatively charged aspartic acid residues at either the trans opening ($K131D_7$, i.e., a single amino acid mutation in one subunit will result in seven single-site mutations in the heptameric pore) or near the constriction region ($K147D_7$) or both (Figure 1a) would affect the residence time of the pb_2-Ba protein within the pore lumen. We found that the interaction between the engineered traps (Figure 1a)

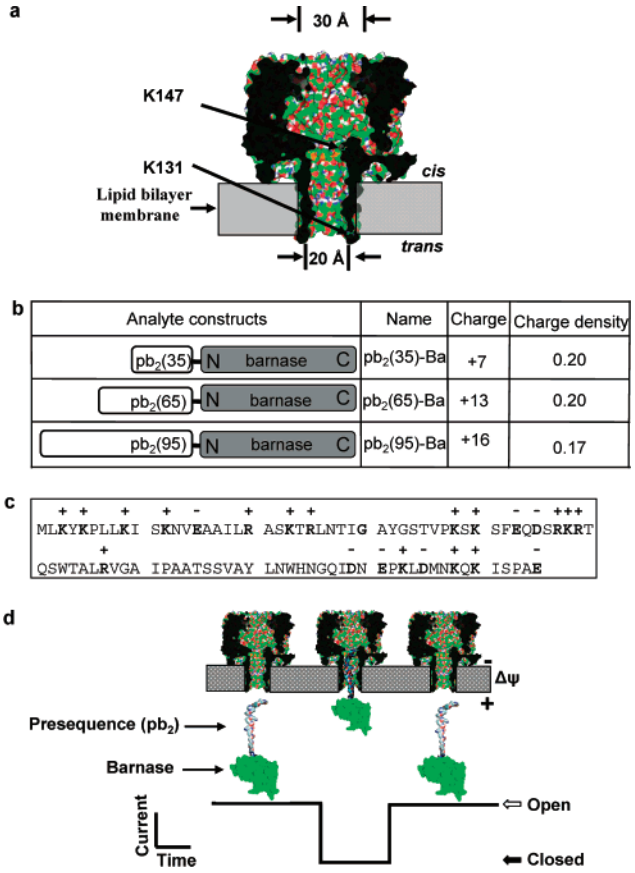

Figure 1. Protein engineering of the αHL pore and the pb_2-Ba proteins: (a) Sectional view of the αHL pore with engineered electrostatic traps shown by arrows. This view was generated by PyMOL[53] using the coordinates from the crystal structure of the αHL pore (7ahl.pdb).[20] K131D and K147D mutations introduced an acidic ring of aspartic acids on either the trans or cis end of the β barrel or both. Planar lipid membrane is indicated by the area in gray. (b) Design of the pb_2-Ba proteins. Different fragments of Yeast pre-cytochrome b_2 (pb_2) were fused to the N-terminus of barnase (Ba) (see Supporting Information, Materials and Methods). The total charge and charge density (i.e., the number of positively charged amino acids divided by the total number of the residues of pb_2) of the pb_2 presequence are indicated in the table. (c) The amino acid sequence of the first 95 residues of the pb_2 presequence. (d) Cartoon that indicates partitioning of the pb_2-Ba protein into the αHL protein pore from the trans side of the bilayer. Partitioning of a single pb_2-Ba protein into the pore lumen is observed by a transient blockade of the single-channel current. Both proteins are represented at the same scale.

and the pb_2 presequence (Figure 1c) drove the partitioning of the pb_2-Ba protein into the αHL pore, permitting us to systematically study the protein−pore interaction (Figure 1d). Second, because we found that the partitioning of the pb_2-Ba protein into a trap-containing αHL pore is strongly dependent on electric charges in the protein and pore, we also expected that the residence time substantially increases by decreasing the ionic strength of the aqueous phase due to a weak charge screening of the pb_2 presequence and the pore interior. We found that one can finely control the partitioning of the pb_2-Ba protein into the αHL pore by the precise tuning of the ionic strength of aqueous phase, facilitating a comprehensive exploration of the protein−pore interaction. Third, we explored the detailed mechanistic interpretation of previously conducted experiments on protein import into intact mitochondria.[21−23]

Earlier work had shown that import rates into purified yeast mitochondria increased with the length of the presequence.[21] Here, we investigated how the partitioning of the pb_2-Ba protein

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into the αHL pore depends on the length of the pb_2 presequence. Three pb_2 presequences of 35 ($pb_2(35)$), 65 ($pb_2(65)$), and 95 ($pb_2(95)$) 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 phase[25] and their presence does not alter the stability of the folded Ba domain.[21,23,26] For all engineered αHL pores, the pb_2-Ba protein with a long pb_2 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 (pb_2)[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 pb_2-Ba Proteins. *E. coli* M15 cells were transformed with the plasmids encoding the pb_2-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 μg/mL leupeptin, 1 μg/mL antipain, pH 5). Pellets were homogenized, and the resultant solutions were spun down at 130 000*g* 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 αHL Protein. The $K131D_7$, $K147D_7$, and $K131D_7/K147D_7$ mutants were made from the αHL gene in the plasmid that was synthetically constructed to produce unique restriction sites (αHL-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 αHL Protein Pores. The αHL 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 [^{35}S]methionine-labeled polypeptides were purified on an 8% SDS-polyacrylamide gel. The dried gel was autoradiographed, and the bands corresponding to each αHL protein pore were excised and rehydrated in 500 μL of MilliQ water. Gel fragments were removed with a spin filter, and the resulting filtrate was stored frozen in 50 μL 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 μm thick Teflon septum (Goodfellow Corp., Malvern, PA). A 1,2 diphytanoyl-*sn*-glycerophosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) bilayer was formed across an 80 μm 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 αHL pores were introduced by adding gel-purified homoheptamers (0.5−2.0 μL) 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 pb_2-Ba proteins with the engineered αHL protein pores in planar lipid bilayers by characterizing transient current blockades of individual channels (Figure 1a). The single-channel experiments were performed with the pb_2-Ba protein added to the *trans* side of the bilayer because the *cis* side of the αHL 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 pb_2-Ba protein within the αHL pore, defined as the residence time spent by the protein analyte within the pore lumen divided by the total recording time

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

where $t_{occupied}$ and t_{total} indicate the total residence time of the pb_2-Ba protein within the pore lumen and the total recording time, respectively.

Single channels of wild-type α-hemolysin (WT-αHL) 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 $pb_2(95)$-Ba proteins to the *trans* side of the bilayer produced infrequent and short-lived current blockades with an event frequency and dwell time $f = 0.06 \pm 0.01\ s^{-1}$ and $\tau = 0.31 \pm 0.06$ ms ($n = 3$), respectively (Figure 2a). Presumably, the current blockades were caused by the pb_2-Ba proteins partitioning into the pore lumen and obstructing the ionic flow through the αHL pore. Introducing a ring of

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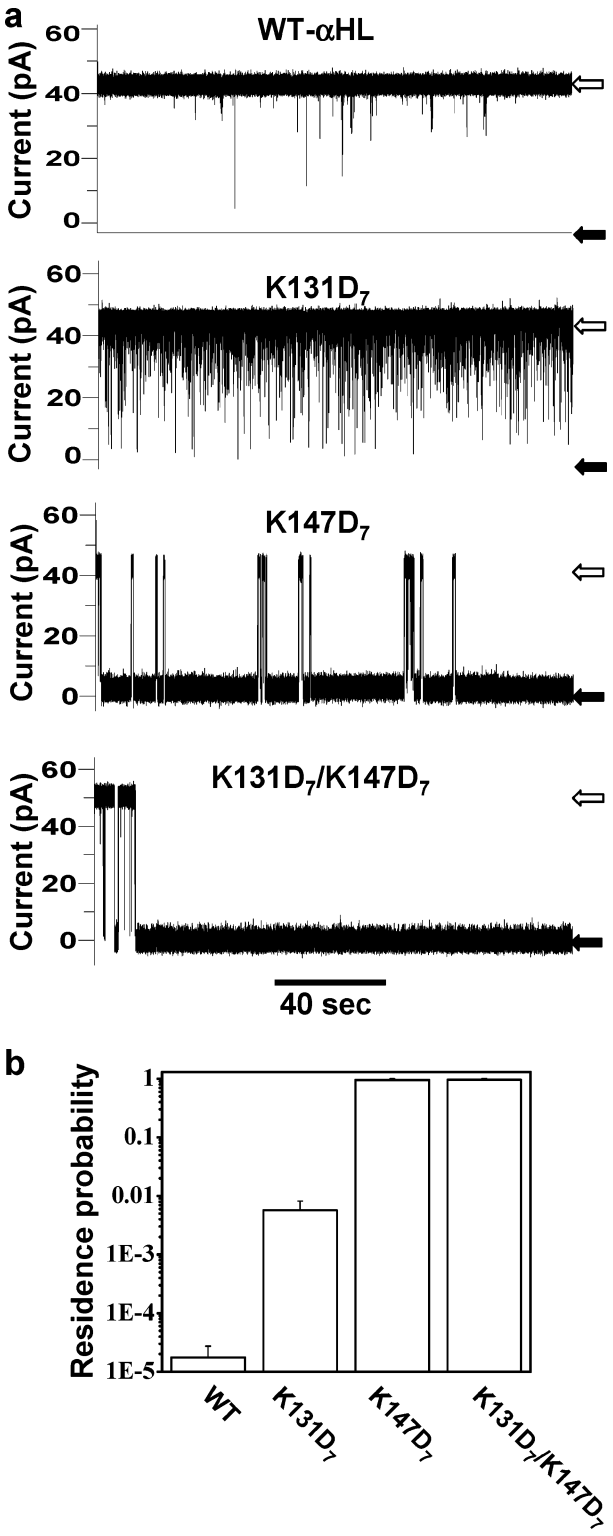

Figure 2. Effect of the pb_2-Ba protein on the single-channel electrical recordings performed with the wild-type (WT) and electrostatic trap-containing αHL pores: (a) Representative single-channel electrical recordings of the WT-αHL, $K131D_7$, $K147D_7$, and $K131D_7/K147D_7$ pores in the presence of 200 nM pb_2(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 pb_2(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 ($K131D_7$, Figure 1a) increased the frequency of the current blockades almost 2 orders of magnitude, but had only a small effect on the dwell time ($f = 5.9 \pm 0.36\ s^{-1}$, $\tau = 0.24 \pm 0.03$ ms, $n = 3$, Figure 2a). The high event frequency observed in single-channel recordings with $K131D_7$ was likely caused by the location of the engineered trap, near aqueous phase in the *trans* side of the bilayer, which contains the pb_2-(95)-Ba protein (Figure 1a).

The effect of the pb_2(95)-Ba protein on the single-channel current through the αHL pore changed significantly when the electrostatic trap was moved from the *trans* opening of the pore to the *cis* end of the β barrel ($K147D_7$, 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 ($\sim 0.012\ s^{-1}$) (Figure 2b). The αHL protein pore that contained both traps ($K131D_7/K147D_7$, Figure 1a) at the same time was also blocked by the pb_2(95)-Ba protein for long periods with a residence probability of 0.97 ± 0.03 ($n = 3$, Figure 2b). This residence probably is slightly greater than the value recorded with the $K147D_7$ pore, but the $K131D_7/K147D_7$ pore produced fewer long-lived current blockades ($\sim 0.006\ s^{-1}$) than the $K147D_7$ pore. In the absence of the pb_2-Ba protein, the WT and engineered αHL 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 αHL pore alone.

Tuning the Protein−Pore Interaction by Ionic Strength. The pb_2 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 pb_2-Ba proteins in single-channel traces recorded with the $K147D_7$ 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 pb_2(95)-Ba and pb_2(35)-Ba proteins, respectively, at a transmembrane potential of +20 mV (Figure 3b).

These results confirm that the interaction between the pb_2 presequence and the trap-containing αHL 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 $K147D_7$ 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 αHL pore itself (Supporting Information, Figure S1b). In addition, bovine serum albumin (BSA) showed only a weak interaction with the $K147D_7$ protein pore (Supporting Information, Figure S2), indicating that a property specific to the pb_2-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

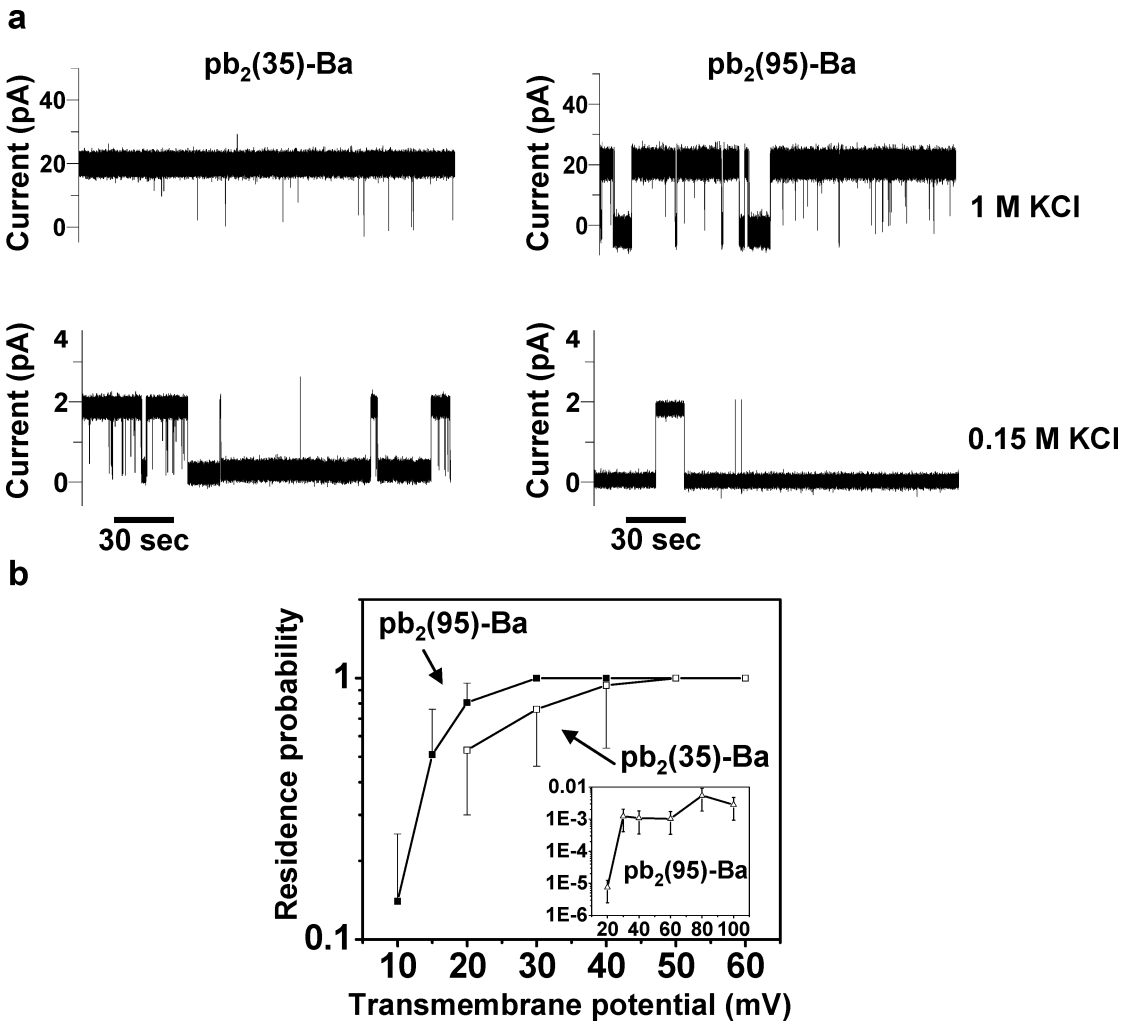

Figure 3. Dependence of the interaction between the pb_2-Ba proteins and the $K147D_7$ pore on the ionic strength. (a) Single-channel electrical recordings performed at a transmembrane potential of +20 mV and with the buffer solution containing either 1 M KCl (the upper traces) or 0.15 M KCl (the lower 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. pb_2-Ba (200 nM) was added to the *trans* chamber. The insert represents the control experiment with the WT-αHL pore and the pb_2(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 Hsp70[36] 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 pb_2 presequence, we compared the residence probabilities of the protein analytes with the pb_2(35), pb_2(65), and pb_2(95) presequences in the $K147D_7$ 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 pb_2(95)-Ba proteins was stronger than that with the pb_2(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 pb_2(35), pb_2(65), and pb_2(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 pb_2 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 $K147D_7$ pore and the pb_2-Ba protein depends on the length of the pb_2 presequence. The simplest hypothesis is that the part of the pb_2-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 pb_2 presequence. If this reasoning is correct, the pb_2(95)-Ba protein causes less repulsion than pb_2(35)-Ba because higher transmembrane potential was needed to produce long-lived current blockades by the pb_2(35)-Ba protein with both $K131D_7$ (Supporting Information, Figure S3) and $K147D_7$ pores (Figure 4).

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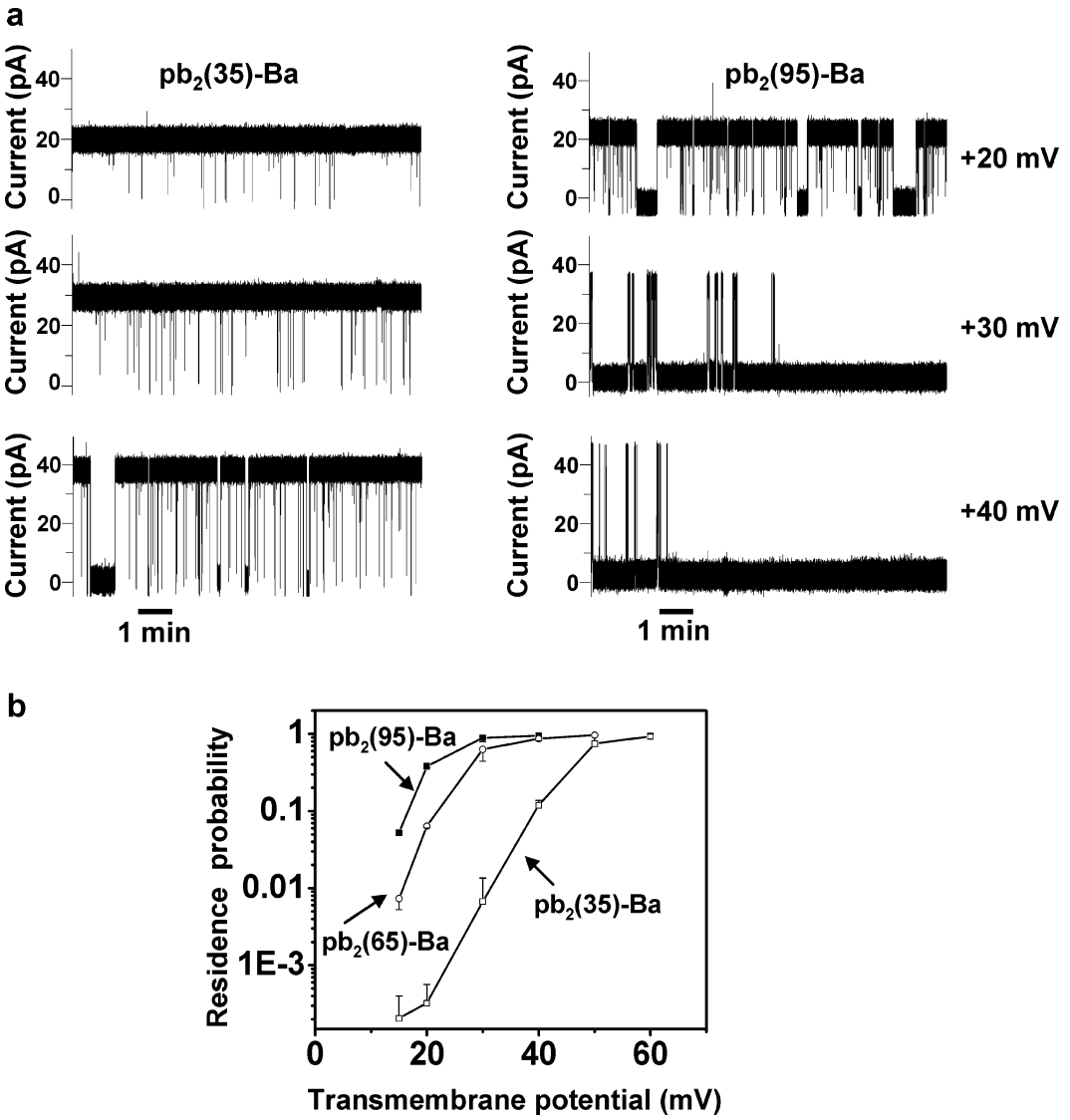

Figure 4. Dependence of the interaction between the pb_2-Ba proteins and the $K147D_7$ pore on the pb_2 presequence at various transmembrane potentials. (a) Representative single-channel electrical recordings in the presence of the pb_2(35)-Ba and pb_2(95)-Ba proteins. (b) Dependence of the residence probability on the transmembrane potential. pb_2-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 pb_2-Ba Proteins Interacting with a Single Trap-Containing $K147D_7$ 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 $K131D_7$ 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 pb_2 length with the pore is at least in part due to the entropic repulsion. Control single-channel recordings with the $K131D_7$ 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 pb_2(35)-Ba and pb_2-(95)-Ba proteins interacting with the $K147D_7$ pore were 0.00021 $\pm$ 0.00015 and 0.053 $\pm$ 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 probability[38]

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

which gives a value of ~5.5*kT*. 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 pb_2(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 pb_2(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 pb_2 presequence of ~6.4 pN. In accord with these calculations, we noticed first long-lived events produced by the pb_2(35)-Ba protein at ~+40 mV (Figure 4a), at which point the pulling force counterbalances the entropic repulsion exerted by the folded Ba domain. In contrast, the pb_2(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 pb_2(95) presequence (Figure 4b).

What other secondary mechanisms might also contribute to the difference in the interaction between the trap-containing αHL pore and the pb_2-Ba proteins with varying length of the pb_2 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 pb_2 presequence (Figure 4b).

Although protein translocation is a complex process,[2] our simplified model using engineered αHL 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 pb_2-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 pb_2-Ba proteins and the αHL 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-αHL and engineered αHL protein pores, interaction between bovine serum albumin and a single trap-containing $K147D_7$ protein pore, interaction of the pb_2-Ba proteins with the single trap-containing $K131D_7$ pore, and the effect of temperature on the interaction between the pb_2(95)-Ba protein and a single trap-containing $K131D_7$ pore. This material is available free of charge via the Internet at http://pubs.acs.org.

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