Squeezing a single polypeptide through a nanopore

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We have already seen a decade of single-molecule science involving protein nanopores, and many lessons have been learned from the extensive studies in this exciting realm. Given the promise of the single-molecule nanopore technique for ultra-rapid sequencing of nucleic acids, most of these investigations have been focused on understanding the transit of single-stranded DNA through a protein nanopore. In contrast, the biophysical and biotechnological applications of polypeptide translocation through a protein nanopore have not been pursued as aggressively. However, recent explorations have shown that a mechanistic understanding of polypeptide translocation at unprecedented single-molecule resolution can be achieved using high-resolution, time-resolved single-channel electrical recordings with nanopores and protein design. Moreover, these efforts have begun to unravel the complexity of the protein–pore interactions that involve various thermodynamic forces. Finally, combining recordings of single-channel electrical currents through nanopores with protein engineering proves to be not only a novel single-molecule analytical tool for the detection, examination, and characterization of polypeptides, but also a critical element for prospective high-throughput screening devices in drug design and proteomics.

1. Introduction

In this Highlight article, we review some recent studies regarding the interaction between polypeptides and protein pores examined by the resistive-pulse method.[1,2] The resistive-pulse technique was first used in Coulter counters. The basic idea of a Coulter counter is that if a non-conducting particle moves across a capillary tube filled with a conducting medium, it decreases the conductance of the capillary tube compared with its conductance in the absence of the non-conducting particle. Thus, the translocation of particles through a capillary tube is revealed by voltage pulses. This principle is ubiquitous in biology. For example, one can imagine that a capillary tube is replaced by a transmembrane protein pore, and a particle is substituted by a translocating polypeptide. If the membrane containing a single protein pore separates two chambers filled with an electrolyte solution, a single-channel current can be readily measured between the two electrodes placed in each of the chambers. A transient partitioning of a single polypeptide into a transmembrane protein pore will be accompanied by a transient single-channel current blockade.

Based upon these ideas, recent advances in single-molecule technology utilizing protein nanopores have provided an opportunity to examine biochemical events at high temporal and spatial resolution, and to detect, explore and manipulate individual polypeptides. Extensive studies in this direction have been performed in Bayley's group during the past decade.[3–5] For example, a stochastic biosensor has been designed by covalently attaching a highly flexible water-soluble polymer within the large vestibule of the α-hemolysin (αHL) protein pore.[4] The polymer was tagged with a ligand at its untethered end.[4] Binding proteins were detected outside the pore lumen either by permanent or transient captures through their specific interaction with the ligand. This construct represented the proof of principle for single-molecule stochastic detection of proteins at high temporal resolution.

In two elegant follow-up papers,[6,7] Bayley and co-workers probed the

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single-molecule interactions of an engineered peptide inhibitor with the catalytic subunit of the cAMP-dependent protein kinase, utilizing the label-free electrical recordings along with membrane protein design of the αHL protein pore. Inspired by prior studies employing nucleic acids,[8–12] several groups began to examine polypeptides using protein pores[13–18] or synthetic nanopores.[19,20] However, single-molecule studies that employ folded protein domains partitioning into a single transmembrane protein pore are much more difficult to perform than those using nucleic acids. The proteins are not uniformly charged, carrying positive, negative, polar and hydrophobic side chains. Furthermore, their folding features, their kinetic signature between folded and partly folded states in aqueous phase, which is correlated with the probability to partition into the pore under different conformations, and the effect of the excluded volume cause complications in data interpretation of single-channel recordings.[21,22]

Despite these experimental challenges, recent studies employing single-channel electrical recordings with protein nanopores point out numerous opportunities for unveiling either the biophysical characteristics of polypeptides or the underlying features of the protein–pore interaction. Very recently, Lee and co-workers have shown that even single-site amino acid mutations in a folded protein can cause significant alterations in the amplitude and duration of transient single-channel current blockades produced by partitioning of the folded protein into an αHL protein pore.[18]

In a different approach, Auvray and co-workers explored the folding–unfolding of maltoporin binding protein (MBP) interacting with the αHL protein pore, under various conditions depending on the concentration of chemical denaturant guanidinium hydrochloride (Gdm-HCl).[17] In the absence of the denaturing agent, no transient current blockades were observed, indicating a large entropic penalty for MBP to partition into the αHL protein pore. Interestingly, current blockades were noticed in the presence of Gdm-HCl. The frequency and duration of transient current blockades was dependent on the concentration of Gdm-HCl in the chamber. The unique single-channel electrical signature encoded by partitioning of the MBP into the αHL protein pore was dependent on the unfolded state of MBP.

Therefore, the protein nanopore represented not only a single-molecule probe for interrogating polypeptides,[4,6,7,14–17,23] but also a suitable model for studying protein translocation *in vitro*.[13,16] We have also identified that tackling protein translocation requires a stable β-barrel pore, a versatile platform for protein design, and a high-resolution single-molecule approach. We strongly relied on the use of staphylococcal α-hemolysin (αHL)[24] reconstituted in a planar lipid bilayer, because this heptameric protein is an excellent model for examining protein translocation through a β-barrel-type pore. This strategy has two fundamental advantages.

First, the αHL protein is similar in structure and size to many protein-conducting β-barrel pores found in the outer membranes of mitochondria, chloroplasts, and Gram-negative bacteria. In the transmembrane domain, the αHL channel narrows to form a β-barrel region with an average diameter of ~20 Å and a length of ~52 Å.[24] This is similar to the effective internal diameter of the protein import channel in the mitochondrial outer membrane, which is ~20 Å.[21,25] Furthermore, studies employing poly(ethylene glycol)s (PEGs) for determining the pore geometry of the reconstituted Toc75 protein translocase from the outer membrane of the chloroplasts indicated an average internal diameter of ~22 Å.[26]

Second, the αHL protein is a robust and tractable β-barrel-type pore. The extraordinary power of the αHL protein lies in the following attributes: (i) the availability of its high-resolution structure,[24] (ii) its unprecedented thermal stability,[27,28] (iii) its biochemical and biophysical properties that are representative of β-barrel proteins,[29,30] (iv) its ease of genetic engineering,[5,31,32] (v) the engineered pore remains open indefinitely under extreme environmental conditions,[17,33] (vi) its protein does not perform a certain function, so it might represent a good blank state for protein translocation,[16,33] (vii) its large single-channel conductance, which facilitates high-resolution electrical recordings.[13,34]

2. Free energy landscape for traversing a protein nanopore

The translocation of a polypeptide through the αHL protein pore induces a current blockade, the nature of which is dependent on the fundamental properties of the polypeptide chain, including its length, diameter, secondary structure, charge, and amino acid sequence.[13–16] In general, this process is governed by a two-barrier, single-well free energy landscape (Fig. 1): the entry barrier for passing from the aqueous phase into the pore lumen, the binding site located within the pore lumen, and the exit barrier for passing from the pore lumen into the aqueous phase.[13] The underlying kinetics are directly dependent on the amplitude of the energetic heights and the depth of the well. The energetic heights and the well are dependent not only on the geometry and surface charge of the pore lumen, but also the biophysical features of the translocating polypeptide, as mentioned above. In addition, the kinetics of the transient current fluctuations made by the polypeptides are dependent on the transmembrane

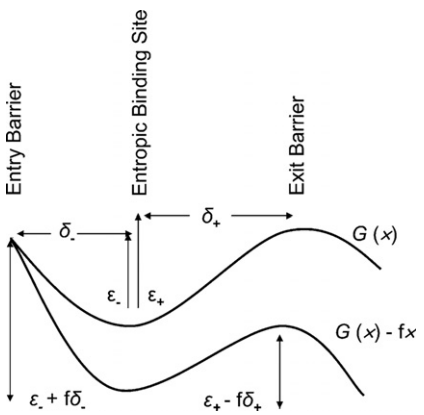

Fig. 1 A simplified two-barrier single-well free energy landscape for the translocation of polypeptides through a single wild-type αHL (WT-αHL) pore.[13] The first and second barriers indicate the entry and exit free energy heights, respectively. The upper line represents the free energy landscape at zero transmembrane potential. The bottom line indicates the hypothetical free energy landscape at a transmembrane potential greater than zero. Here, ε_- and ε_+ indicate the free energy barriers for entry and exit through the αHL protein pore, respectively. δ_- and δ_+ represent the electrical distances from the minimum of the potential to the transition state of the backward and forward reactions, respectively.

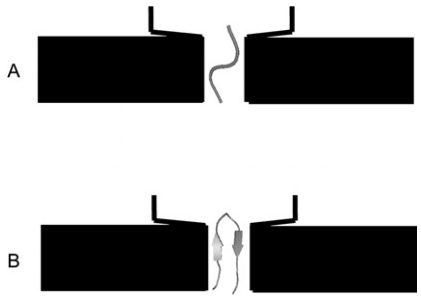

Fig. 2 Translocation of a β-hairpin through a protein nanopore is dependent on the folding features of the polypeptide.[16] Stable β-hairpins have to overcome a greater energetic barrier than unfolded polypeptides. Therefore, the transit time of the peptide within the pore is dependent not only on the geometric and other biophysical characteristics of the protein nanopore, but also on the folding state of the translocating polypeptide.

potential due to the tilting of the free energy landscape (Fig. 1).

The kinetics of the translocation of short β-hairpin peptides though protein nanopores have been recently studied in combination with Langevin molecular dynamics simulations.[16] We performed single-channel electrical recordings with the wild-type αHL (WT-αHL) pore interacting with β-hairpin peptides of varying folding stabilities. Interestingly, the dwell time of the transient current blockades produced by the β-hairpin peptides was dependent on their average conformation in aqueous phase. Highly unfolded peptides entered the pore in an extended conformation, traversing the αHL pore in a fast single-file event (Fig. 2A). In contrast, the folded β-hairpin peptides traversed the αHL pore in a misfolded or fully folded conformation, resulting in a long-lived current blockade (Fig. 2B). The Langevin dynamics suggested that strong hydrophobic contacts between the β-hairpin peptides and the pore lumen result in a dwell time between two and three orders of magnitude longer.

3. Playing with electrostatic interactions: why two traps are better than one

In a very recent study,[33] we were able to control the underlying kinetics of polypeptide translocation through electrostatic traps engineered within the

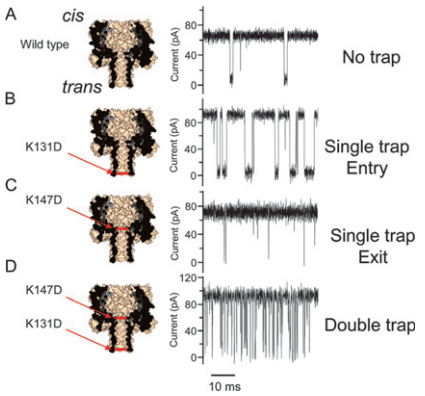

Fig. 3 Dramatic changes of the kinetics of polypeptide translocation are observed in the presence of negatively-charged traps engineered either on the *trans* or *cis* end of the β-barrel part of the pore or both.[33] The right-hand traces represent typical single-channel electrical recordings with the wild-type and engineered αHL protein pores in the presence of 34 μM of the Syn B2 synthetic presequence polypeptide added to the *trans* side: (A) WT-αHL, (B) $K131D_7$, (C) $K147D_7$, (D) $K131D_7$/$K147D_7$. The frequency and duration of the polypeptide-induced current blockades were dependent on the position of the electrostatic trap. All traces were recorded in symmetrical buffer conditions (1 M KCl, 10 mM potassium phosphate, pH 7.4), and at a transmembrane potential of +80 mV. The single-channel electrical traces were low-pass Bessel filtered at 2 kHz.[33] Reproduced with permission from *J. Am. Chem. Soc.* 2007, **129**, 14034–14041. ©2007 American Chemical Society.

pore lumen at the entry and exit of the β-barrel. These traps consisted of negatively-charged heptads of aspartic acids replacing positively-charged lysines either on the *trans* entrance of the pore, the *cis* end of the β-barrel or both (Fig. 3). We have examined the effect of 25-residue-long polypeptides, which resemble positively-charged presequences involved in protein import into mitochondria,[21,26] on the single-channel electrical signatures recorded with wild-type and engineered αHL pores. These polypeptides were added to the *trans* side of the lipid bilayer (Fig. 3A).

The combination of these single-molecule experiments with protein engineering is inspired by protein translocation across the translocase of the outer membrane of the mitochondria (TOM). It is hypothesized that the TOM channel is a β-barrel protein pore[35,36] containing two negatively-charged binding sites located at the entry and exit of the pore lumen.[37] Surprisingly, folded proteins are translocated across the TOM channel in the absence of any ATP-dependent machinery or chemical or energetic gradient. The proteins are targeted into mitochondrial matrices through their positively-charged polypeptide presequences that are fused to the N-terminal of the folded protein domains. These presequences interact with the negatively-charged binding sites that are located within the pore lumen of the TOM channel. Once they are translocated through the TOM channel, the presequences are directed towards the lumen of the translocase of the inner membrane of mitochondria (TIM) and captured by the ATP-dependent Hsp70 motor for further import.

Translocation of a positively-charged polypeptide through a transmembrane protein pore that already contains a single binding site adds to the complexity of the problem of protein traffic across a single protein pore, which is a multi-step process involving protein–protein and protein–pore interactions.[38] Given this added complexity, is there any way that we can anticipate the underlying kinetics of this interaction by altering the charge distribution within the pore lumen? What is the change in single-channel kinetics when systematic changes are performed in the pore lumen? Which electrostatic trap has a major effect on the rate constant of association? Which electrostatic trap has a major effect on the rate constant of dissociation? Numerous questions like these reveal a lack of knowledge about the basic mechanisms and the biophysical rules governing polypeptide translocation. Sometimes the answers are not trivial, because the polypeptide–pore system encompasses features of molecular complexity, such as the polypeptide's secondary structure, and the heterogeneity of the electrostatic, polar and hydrophobic groups.

In the mitochondrial translocation system, the transmembrane potential of the inner membrane is thought to electrophoretically drive the insertion of the targeting presequence into the pore lumen.[39] Our major hypothesis was that the electrostatic trap, either on the *trans* entrance of the pore (K131D, entry) or on the *cis* end of the β-barrel (K147D, exit) implies an additional binding site that corresponds to a minimum in the free energy landscape.[40] We envisioned

that there are two distinct possibilities: either this minimum and the transition states in the free energy landscape are far away from each other, in which case they have an independent contribution to the overall kinetics, or this minimum and one of the transition states overlap each other, so that the energetic barrier reduces, resulting in a faster translocation rate than that recorded with the WT-αHL pore (Fig. 3A).

In remarkable accord with our prediction, the electrostatic trap located on the *trans* entrance of the pore produced a substantial increase in the rate constant of association k_{on} (Fig. 3B), which is proportional to the frequency of the single-channel polypeptide-induced current blockades. This means that the transition state of the entry barrier (Fig. 1) overlaps with the minimum created by the electrostatic trap on the *trans* entrance of the pore, significantly decreasing the energetic height of the entry barrier. In contrast, the electrostatic trap on the *cis* end of the β-barrel produced a significant increase in the rate constant of dissociation k_{off}, which is the reciprocal of the dwell time of the single-channel polypeptide-induced current blockades, but no apparent modification of the rate constant of association k_{on} (Fig. 3C).[33] In this case, the transition state of the exit barrier (Fig. 1) overlaps with the minimum created by the electrostatic trap located on the *cis* end of the β-barrel, substantially altering the energetic height of the exit barrier.

For the double trap-containing αHL pore ($K131D_7/K147D_7$), there are two minima that correspond to the electrostatic traps located on the *trans* entrance of the pore and the *cis* end of the β-barrel (Fig. 3D). There is also a minimum that corresponds to the native binding site near the pore constriction.[13] The K131D trap modifies the rate constant of association k_{on}, and the K147D trap changes the rate constant of dissociation k_{off} (Fig. 3D). Overall, we find the effects produced by the electrostatic traps to be non-additive, because of the major alterations to the energetic heights of the entry and exit barriers.

One immediate issue is the discrimination between polypeptide translocation and the binding followed by the release of the polypeptide backwards to the aqueous phase from which it came. In principle, a single transient polypeptide-induced current blockade cannot be a diagnostic for the "true" translocation of the polypeptide from one side of the lipid bilayer to the other. Nevertheless, with careful calculations and voltage-dependence single-channel experiments, one can determine the fraction of single-channel events attributed to the "true" translocation.[13,33] In addition, one may ask how the hydrophobic content of the polypeptides alters the underlying kinetics. It is known that facilitated translocation of polypeptides through protein pores employs either one or more well-defined hydrophobic binding sites that interact with the hydrophobic regions of the passengers.[41,42] Indeed, we observed longer translocation times with more hydrophobic polypeptides,[33] confirming the complexity of the free energy landscape of the polypeptide–pore interaction that includes electrostatic, polar and hydrophobic contributions. The more hydrophobic polypeptides exhibited a substantial decrease in the rate constant of association to the pore lumen, having to cross a greater energetic height due to the hydrophilic feature of the pore lumen.[33]

4. Playing with a single temperature-responsive plug: hydrophobic collapse within a nanopore

Recently, we were able to engineer an unusual pore-based nanostructure with a temperature-responsive plug.[28] The plug was an elastin-like polypeptide (ELP) loop with varying length, between 25 and 100 residues, engineered in a single subunit of the αHL protein pore, and near the central region of the large vestibule. This approach complements previous methodology that has employed tethered movable polymers for stochastic sensing of proteins and DNA.[4,11,12,43,44] We anticipated that the ELP loop would produce transient current blockades, the nature of which is dependent on the features of the plug, including the amino acid sequence and the loop length. Single-channel electrical recordings with ELP loop-containing αHL protein pores with a single short plug (~36 residues) exhibited reduced unitary conductance decorated by highly frequent, short-lived transient spikes with durations in the range of tens of microseconds. In contrast, the measurements with ELP loop-containing αHL protein pores with a single medium-sized plug (~61 residues) showed not only a significantly smaller unitary conductance, but also long-lived transient current blockades in the range of several milliseconds (Fig. 4A).

Of course, one has to consider the sensitivity of the single-channel recordings not only with the length of the polypeptide plug, but also with its amino acid sequence. In contrast to the single-channel electrical traces obtained with a medium-sized ELP plug-containing αHL protein pore, we obtained a unique single-channel electrical signature decorated by highly frequent and very short-lived current spikes when a medium-sized Gly-Ser-rich loop-containing αHL protein pore was explored (Fig. 4B). The short duration of the current fluctuations was consistent with the high flexibility of the Gly-Ser-rich polypeptide due to the absence of the bulky side chains at Gly residues.

Since the transient current blockades observed with the medium-sized ELP plug-containing αHL pore were time-resolvable, it was straightforward to explore its single-channel conductance as a function of temperature, given the

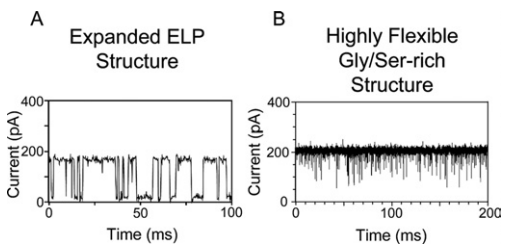

Fig. 4 Typical single-channel electrical traces of a polypeptide loop-containing αHL pore recorded at 40 °C: (A) an expanded elastin-like polypeptide (ELP) loop-containing αHL pore; (B) a Gly-Ser-rich polypeptide loop-containing αHL pore.[28] Reproduced with permission from *J. Am. Chem. Soc.* 2006, **128**, 15332–15340. ©2006 American Chemical Society.

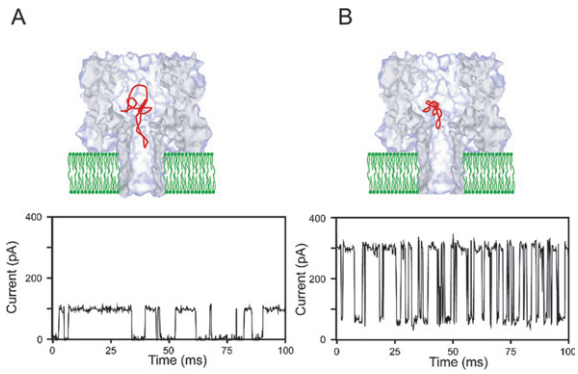

Fig. 5 An ELP loop engineered within the large vestibule of the αHL pore functions as a temperature-responsive plug.[28] This polypeptide loop gates the αHL pore similar to the highly flexible loops of the outer membranes' proteins in mitochondria and Gram-negative bacteria. The ELP loop undergoes an inverse temperature-dependent conformational transition (the upper panels): (A) typical single-channel trace of a medium-sized ELP loop-containing αHL pore recorded at 20 °C, (B) representative single-channel trace of a medium-sized ELP loop-containing αHL pore recorded at 60 °C.[28] Reproduced with permission from *J. Am. Chem. Soc.* 2006, **128**, 15332–15340. ©2006 American Chemical Society.

unusual inverse temperature transition of the ELPs.[45–53] At 20 °C, the 61-residue ELP plug produced full transient current blockades (Fig. 5A), but at elevated temperatures ~40 °C or above, we observed partial current blockades (Fig. 5B). We interpreted that at temperatures below the inverse transition temperature T_t, the ELP plug was fully hydrated and in an expanded conformation,[51,52] thus blocking the pore completely, but reversibly. In contrast, at temperatures above the transition temperature T_t, the ELP plug was dehydrated, and its structure was collapsed.[51,52] The excursions of a high-temperature collapsed ELP plug produced a partial current blockade. Therefore, this experiment indirectly confirmed the hydrophobic collapse of a polypeptide at single-molecule resolution.

This protein engineering can be expanded further by the redesign of other temperature-responsive protein pores, with features that can be finely tuned by changing the amino acid sequence of the repetitive unit of the ELP loop. Alternatively, the temperature-responsive polypeptide plug might be replaced by a light- or pH-responsive polypeptide loop. Needless to say, engineering a gating polypeptide plug within the pore lumen of a β-barrel protein is a reversal of the process of engineering loop deletions of the outer membrane proteins for a better understanding of the single-channel current fluctuations that they produce.[54] For example, various protein design avenues might employ engineered plugs that contain elements of secondary structure, such as sandwiches of β-sheets or α-helical bundles, like those in other outer membrane proteins.[55,56]

5. Unresolved questions and future prospects

Recent single-molecule studies of polypeptide transport though a single protein pore provide details of a ubiquitous, fundamental, but very complex process in biology.[16,33,41,57–61] The kinetics of polypeptide translocation are a result of an array of molecular factors, and electrostatic, driving, and entropic forces exerted on polypeptides through narrow protein pores.[62] In principle, the underlying kinetics of the protein–pore interactions should be tuned by protein design of either the protein pore or the translocating polypeptide. However, it is not yet clear how changes in the structural and stability features of translocating polypeptides are translated into alterations of the kinetics and thermodynamics of translocation. There are numerous outstanding questions pertinent to polypeptide translocation through a protein nanopore. For example, what is the balance of the energetic factors that must be overcome upon the conversion from a folded state to a "crossable" state in order to traverse the protein pore? What are the interactions between a protein passenger and the side chains of the transmembrane pore? What is the passage time of a polypeptide through a transmembrane protein pore? Why are the pore-mediated unfolding rates of the proteins a few orders of magnitude faster than the corresponding rates obtained from global or thermodynamic unfolding, which is induced by chemical denaturants or temperature? How do the hydrophobic fragments of the translocating polypeptides interact with the hydrophobic binding sites within the lumen of the pore? These numerous questions are endurie because polypeptide translocation has not yet been pursued extensively at single-molecule resolution. In addition, the molecular nature of polypeptide translocation is quite complex, encompassing the interplay between protein–pore interaction and pore–mediated unfolding.

With such complexity, obtaining a comprehensive understanding of polypeptide translocation through a transmembrane pore is indeed a problem for biophysicists, since the translocation and unfolding are intimately related as part of the same overall process, and the exploration of this process requires a quantitative approach of its individual steps. We showed in this review article that a deep understanding of the polypeptide–pore interaction can be accomplished by systematic changes in the pore lumen through functional binding sites.

It might be interesting to examine folded protein domains fused to a positively-charged targeting fragment interacting with a β-barrel pore that contains negatively-charged traps. This situation appears in the mitochondrial protein import system through the translocase of the outer membrane (TOM).[36,63,64] In the future, we plan to examine the pore-mediated unfolding of proteins with altered local stability by protein engineering of the passenger. Collectively, these efforts will help to obtain a new and comprehensive picture of how a protein traverses a narrow protein-conducting β-barrel pore.

It is likely that a coming breakthrough will be probing the local mechanical stability of a polypeptide[65,66] by direct force measurements in a protein pore or synthetic nanopore *via* optical tweezers. Specifically, the mechanisms by which

proteins are unfolded and transported through protein pores might be examined by a combination of single-channel recordings and an optical tweezer.[67,68] In this case, the N-terminal targeting fragment, which will eventually be attached to a polystyrene bead, would be pulled through the nanopore by an optical trap. Such an instrument would have the potential for probing the balance between driving and entropic forces at the single-molecule level in a sequential fashion from the N- to C-terminal, which is not achievable by mechanical unfolding *via* AFM or optical tweezer alone.[69] From a practical point of view, this type of mechanical unfolding of a protein domain through a nanopore is more relevant to a natural process such as protein degradation by ATP-dependent proteases and protein import into mitochondria and chloroplasts[63–65,70,71] than simple AFM measurements.[69]

For example, in mitochondrial transport the transmembrane potential ($\Delta\psi$) through the inner mitochondrial membrane catalyzes protein unfolding by unraveling the polypeptide sequentially from the positively-charged N-terminal to the C-terminal.[39,72,73] Mechanistically, the unfolding during protein import or degradation is fundamentally different from global or thermodynamic unfolding. The resistance of a protein to $\Delta\psi$-catalyzed pore-mediated unfolding is not determined by its stability against global unfolding. In contrast, the resistance to unfolding in protein translocation is mainly determined by the local structure of the passenger interacting with the pore.[74] In the case of mechanical unfolding *via* AFM pulling experiments, the polypeptide chain is stretched out from both ends, so the force is equally distributed along the chain.[75] Just as enzymes can alter the pathway of a reaction, the transmembrane potential $\Delta\psi$ can catalyze the unfolding pathway of the translocating protein. For instance, the small ribonuclease Barnase is a quite stable protein with $\Delta G^{\circ} \approx 10$ kcal mol^{-1} in dilute buffer at 25 °C, and its activation energy for unfolding is very high (>20 kcal mol^{-1}).[76] Yet, surprisingly, Barnase does not resist force well, and is mechanically unfolded by very low forces.[75] Therefore, the thermodynamic stability of the protein is not well correlated with its mechanical stability. This is one example in which the measurements on protein unfolding thorough a nanopore hold promise for revolutionizing our knowledge about complex protein unfolding pathways in critical biologically relevant processes, such as protein degradation and protein translocation. Therefore, the nanopore technique will represent a local force probe for unraveling unique unfolding pathways in proteins,[77] which is not possible using conventional optical tweezer techniques or simple AFM measurements.[68,69]

On the other hand, the transport of a single polypeptide through a protein pore is of fundamental importance in many arenas, so the results of future studies will have a profound and far-reaching impact on medical biotechnology areas, such as drug delivery, separation-based science, nanobiotechnology, proteomics, and stochastic biosensors. Certainly, single-molecule stochastic sensing of proteins employing a single protein nanopore will be one of the spin offs of future studies. However, the lipid bilayer-membrane protein system remains a fragile component for projected detectors. The feeling among scientists is that the solid-state nanopores hold promise for the next breakthroughs in fundamental and applicative biomedical sciences.[78] However, placing a single functional chemical group at a strategic position within the inner surface of a synthetic nanopore remains a distant dream.

Because the nanopore measurements have potential for parallel high-throughput screening arrays and nanoscale miniaturization, this technique can be further expanded for lab-on-a-chip technologies[23] and analytical assays for protein characterization at high temporal and spatial resolutions.[68] In the future, it might be possible to develop large array-based devices for accessing and analyzing individual proteins. These nanopore-based arrays will have potential for real-time, rapid and inexpensive screening in drug design, proteomics, cancer research, and other applicative fields in medical biotechnology. Of course, these high-throughput devices will need to overcome several technical challenges, such as integration of nanopores into nanofluidics,[23,79] amalgamation of protein pores with synthetic nanopores,[80,81] and chemical modification of the protein pores.[5,30,32]

Finally, we anticipate that the results of these studies will stimulate new theoretical and computational efforts in the area of full-atomistic molecular dynamics to examine polypeptide traffic through transmembrane protein pores.[16,40,66,74,82–84] Computational studies combined with structural analysis should lead to a qualitative understanding of how polypeptides undergo conformational fluctuations when they traverse a protein nanopore. For example, it is unclear how longer polypeptides traverse even a cylindrical β-barrel pore. Are they translocated in completely unfolded or misfolded conformation? It is likely, although challenging, that coupling single-molecule electrical recordings with fluorescence or other optical platforms will be able to concurrently provide information about kinetic, thermodynamic and structural alterations of the translocating polypeptide.

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References

1 H. Bayley and C. R. Martin, *Chem. Rev.*, 2000, **100**, 2575–2594.
2 S. M. Bezrukov, *J. Membr. Biol.*, 2000, **174**, 1–13.
3 J. Sanchez-Quesada, M. R. Ghadiri, H. Bayley and O. Braha, *J. Am. Chem. Soc.*, 2000, **122**, 11757–11766.
4 L. Movileanu, S. Howorka, O. Braha and H. Bayley, *Nat. Biotechnol.*, 2000, **18**, 1091–1095.
5 H. Bayley and P. S. Cremer, *Nature*, 2001, **413**, 226–230.
6 H. Xie, O. Braha, L. Q. Gu, S. Cheley and H. Bayley, *Chem. Biol.*, 2005, **12**, 109–120.
7 S. Cheley, H. Xie and H. Bayley, *ChemBioChem*, 2006, **7**, 1923–1927.

8 J. J. Kasianowicz, E. Brandin, D. Branton and D. W. Deamer, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 13770–13773.
9 M. Akeson, D. Branton, J. J. Kasianowicz, E. Brandin and D. W. Deamer, *Biophys. J.*, 1999, **77**, 3227–3233.
10 A. Meller, L. Nivon, E. Brandin, J. Golovchenko and D. Branton, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 1079–1084.
11 S. Howorka, L. Movileanu, O. Braha and H. Bayley, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 12996–13001.
12 S. Howorka, S. Cheley and H. Bayley, *Nat. Biotechnol.*, 2001, **19**, 636–639.
13 L. Movileanu, J. P. Schmittschmitt, J. M. Scholtz and H. Bayley, *Biophys. J.*, 2005, **89**, 1030–1045.
14 T. C. Sutherland, Y. T. Long, R. I. Stefureac, I. Bediako-Amoa, H. B. Kraatz and J. S. Lee, *Nano Lett.*, 2005, **4**, 1273–1277.
15 R. Stefureac, Y. T. Long, H. B. Kraatz, P. Howard and J. S. Lee, *Biochemistry*, 2006, **45**, 9172–9179.
16 C. P. Goodrich, S. Kirmizialtin, B. M. Huyghues-Despointes, A. P. Zhu, J. M. Scholtz, D. E. Makarov and L. Movileanu, *J. Phys. Chem. B*, 2007, **111**, 3332–3335.
17 G. Oukhaled, J. Mathe, A.-L. Biance, L. Bacri, J.-M. Betton, D. Lairez, J. Pelta and L. Auvray, *Phys. Rev. Lett.*, 2007, **98**, 158101.
18 R. Stefureac, L. Waldner, P. Howard and J. S. Lee, *Small*, 2008, **4**, 59–63.
19 Z. Siwy, L. Trofin, P. Kohli, L. A. Baker, C. Trautmann and C. R. Martin, *J. Am. Chem. Soc.*, 2005, **127**, 5000–5001.
20 A. Han, G. Schurmann, G. Monding, R. A. Bitterli, N. F. de Rooij and U. Staufer, *Appl. Phys. Lett.*, 2006, **88**.
21 C. Muro, S. M. Grigoriev, D. Pietkiewicz, K. W. Kinnally and M. L. Campo, *Biophys. J.*, 2003, **84**, 2981–2989.
22 S. D. Zakharov, V. Y. Erukova, T. I. Rokitskaya, M. V. Zhalnina, O. Sharma, P. J. Loll, H. I. Zgurskaya, Y. N. Antonenko and W. A. Cramer, *Biophys. J.*, 2004, **87**, 3901–3911.
23 H. Craighead, *Nature*, 2006, **442**, 387–393.
24 L. Z. Song, M. R. Hobaugh, C. Shustak, S. Cheley, H. Bayley and J. E. Gouaux, *Science*, 1996, **274**, 1859–1866.
25 M. P. Schwartz and A. Matouschek, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 13086–13090.
26 S. C. Hinnah, R. Wagner, N. Sveshnikova, R. Harrer and J. Soll, *Biophys. J.*, 2002, **83**, 899–911.
27 X. F. Kang, L. Q. Gu, S. Cheley and H. Bayley, *Angew. Chem., Int. Ed.*, 2005, **44**, 1495–1499.
28 Y. Jung, H. Bayley and L. Movileanu, *J. Am. Chem. Soc.*, 2006, **128**, 15332–15340.
29 H. Bayley, *Curr. Opin. Biotechnol.*, 1999, **10**, 94–103.
30 H. Bayley and L. Jayasinghe, *Mol. Membr. Biol.*, 2004, **21**, 209–220.
31 L. Movileanu, S. Cheley, S. Howorka, O. Braha and H. Bayley, *J. Gen. Physiol.*, 2001, **117**, 239–251.
32 H. Bayley, O. Braha, S. Cheley and L. Q. Gu, in *NanoBiotechnology*, ed. C. M. Niemeyer and C. A. Mirkin, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2004, pp. 93–112.
33 A. J. Wolfe, M. M. Mohammad, S. Cheley, H. Bayley and L. Movileanu, *J. Am. Chem. Soc.*, 2007, **129**, 14034–14041.
34 L. Movileanu, S. Cheley and H. Bayley, *Biophys. J.*, 2003, **85**, 897–910.
35 K. Hill, K. Model, M. T. Ryan, K. Dietmeier, F. Martin, R. Wagner and N. Pfanner, *Nature*, 1998, **395**, 516–521.
36 L. Becker, M. Bannwarth, C. Meisinger, K. Hill, K. Model, T. Krimmer, R. Casadio, K. N. Truscott, G. E. Schulz, N. Pfanner and R. Wagner, *J. Mol. Biol.*, 2005, **353**, 1011–1020.
37 M. Bohnert, N. Pfanner and M. van der Laan, *FEBS Lett.*, 2007, **581**, 2802–2810.
38 W. Wickner and R. Schekman, *Science*, 2005, **310**, 1452–1456.
39 N. Pfanner and K. N. Truscott, *Nat. Struct. Biol.*, 2002, **9**, 234–236.
40 D. E. Makarov, *Biophys. J.*, 2007, **92**, 4135–4136.
41 B. A. Krantz, R. A. Melnyk, S. Zhang, S. J. Juris, D. B. Lacy, Z. Wu, A. Finkelstein and R. J. Collier, *Science*, 2005, **309**, 777–781.
42 G von Heijne, *Science*, 2005, **309**, 709–710.
43 S. Howorka, L. Movileanu, X. F. Lu, M. Magnon, S. Cheley, O. Braha and H. Bayley, *J. Am. Chem. Soc.*, 2000, **122**, 2411–2416.
44 L. Movileanu and H. Bayley, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 10137–10141.
45 C. H. Luan, T. M. Parker, K. U. Prasad and D. W. Urry, *Biopolymers*, 1991, **31**, 465–475.
46 D. W. Urry, D. C. Gowda, T. M. Parker, C. H. Luan, M. C. Reid, C. M. Harris, A. Pattanaik and R. D. Harris, *Biopolymers*, 1992, **32**, 1243–1250.
47 D. W. Urry, T. Hugel, M. Seitz, H. E. Gaub, L. Sheiba, J. Dea, J. Xu and T. Parker, *Philos. Trans. R. Soc. London, Ser. B*, 2002, **357**, 169–184.
48 D. W. Urry, *Biopolymers*, 1998, **47**, 167–178.
49 D. W. Urry, *J. Phys. Chem. B*, 1997, **101**, 11007–11028.
50 J. Hyun, W. K. Lee, N. Nath, A. Chilkoti and S. Zauscher, *J. Am. Chem. Soc.*, 2004, **126**, 7330–7335.
51 C. Nicolini, R. Ravindra, B. Ludolph and R. Winter, *Biophys. J.*, 2004, **86**, 1385–1392.
52 E. Schreiner, C. Nicolini, B. Ludolph, R. Ravindra, N. Otte, A. Kohlmeyer, R. Rousseau, R. Winter and D. Marx, *Phys. Rev. Lett.*, 2004, **92**.
53 M. Baer, E. Schreiner, A. Kohlmeyer, R. Rousseau and D. Marx, *J. Phys. Chem. B: Condens. Matter Mater. Surf. Interfaces Biophys. Chem.*, 2006, **110**, 3576–3587.
54 S. Biswas, M. M. Mohammad, D. R. Patel, L. Movileanu and B. van den Berg, *Nat. Struct. Mol. Biol.*, 2007, **14**, 1108–1109.
55 K. P. Locher, B. Rees, R. Koebnik, A. Mitschler, L. Moulinier, J. P. Rosenbusch and D. Moras, *Cell*, 1998, **95**, 771–778.
56 M. S. Sansom, *Curr. Biol.*, 1999, **9**, R254–R257.
57 L. K. Koriazova and M. Montal, *Nat. Struct. Biol.*, 2003, **10**, 13–18.
58 S. Zhang, E. Udho, Z. Wu, R. J. Collier and A. Finkelstein, *Biophys. J.*, 2004, **87**, 3842–3849.
59 S. Zhang, A. Finkelstein and R. J. Collier, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 16756–16761.
60 B. A. Krantz, A. Finkelstein and R. J. Collier, *J. Mol. Biol.*, 2006, **355**, 968–979.
61 A. Fischer and M. Montal, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 10447–10452.
62 S. M. Simon, C. S. Peskin and G. F. Oster, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 3770–3774.
63 A. Matouschek, *Curr. Opin. Struct. Biol.*, 2003, **13**, 98–109.
64 S. Prakash and A. Matouschek, *Trends Biochem. Sci.*, 2004, **29**, 593–600.
65 A. J. Wilcox, J. Choy, C. Bustamante and A. Matouschek, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 15435–15440.
66 D. K. West, D. J. Brockwell and E. Paci, *Biophys. J.*, 2006, **91**, L51–L53.
67 U. F. Keyser, B. N. Koeleman, S. van Dorp, D. Krapf, R. M. M. Smeets, S. G. Lemay, N. H. Dekker and C. Dekker, *Nat. Phys.*, 2006, **2**, 473–477.
68 C. Dekker, *Nature Nanotechnology*, 2007, **2**, 209–215.
69 T. Sato, M. Esaki, J. M. Fernandez and T. Endo, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 17999–18004.
70 A. Matouschek and B. S. Glick, *Nat. Struct. Biol.*, 2001, **8**, 284–286.
71 K. Shariff, S. Ghosal and A. Matouschek, *Biophys. J.*, 2004, **86**, 3647–3652.
72 S. Huang, K. S. Ratliff, M. P. Schwartz, J. M. Spenner and A. Matouschek, *Nat. Struct. Biol.*, 1999, **6**, 1132–1138.
73 S. Huang, K. S. Ratliff and A. Matouschek, *Nat. Struct. Biol.*, 2002, **9**, 301–307.
74 L. Huang, S. Kirmizialtin and D. E. Makarov, *J. Chem. Phys.*, 2005, **123**, 124903.
75 R. B. Best, B. Li, A. Steward, V. Daggett and J. Clarke, *Biophys. J.*, 2001, **81**, 2344–2356.
76 A. Matouschek, A. Azem, K. Ratliff, B. S. Glick, K. Schmid and G. Schatz, *EMBO J.*, 1997, **16**, 6727–6736.
77 R. P. De Los, A. Ben Zvi, O. Slutsky, A. Azem and P. Goloubinoff, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 6166–6171.
78 J. Griffiths, *Anal. Chem.*, 2008, **80**, 23–27.
79 Y. Astier, H. Bayley and S. Howorka, *Curr. Opin. Chem. Biol.*, 2005, **9**, 576–584.
80 R. Hemmler, G. Bose, R. Wagner and R. Peters, *Biophys. J.*, 2005, **88**, 4000–4007.
81 R. Peters, *Traffic*, 2005, **6**, 199–204.
82 S. Kirmizialtin, V. Ganesan and D. E. Makarov, *J. Chem. Phys.*, 2004, **121**, 10268–10277.
83 P. Tian and I. Andricioaei, *J. Mol. Biol.*, 2005, **350**, 1017–1034.
84 S. Kirmizialtin, L. Huang and D. E. Makarov, *Phys. Status Solidi B*, 2006, **243**, 2038–2047.