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Multistep protein unfolding during nanopore translocation

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Abstract

Cells are divided into compartments and separated from the environment by lipid bilayer membranes. Essential molecules are transported back and forth across the membranes. We have investigated how folded proteins use narrow transmembrane pores to move between compartments. During this process, the proteins must unfold. To examine co-translocational unfolding of individual molecules, we tagged protein substrates with oligonucleotides to enable potential-driven unidirectional movement through a model protein nanopore, a process that differs fundamentally from extension during force spectroscopy measurements. Our findings support a four-step translocation mechanism for model thioredoxin substrates. First, the DNA tag is captured by the pore. Second, the oligonucleotide is pulled through the pore, causing local unfolding of the C terminus of the thioredoxin adjacent to the pore entrance. Third, the remainder of the protein unfolds spontaneously. Finally, the unfolded polypeptide diffuses through the pore into the recipient compartment. The unfolding pathway elucidated here differs from those revealed by denaturation experiments in solution, for which two-state mechanisms have been proposed.

In cells, essential molecules are often transported between compartments. In an important case, folded proteins are unfolded during translocation through narrow pores in membranes and then refolded in a recipient compartment. Examples include the import of certain proteins into mitochondria^{1–3} and the transfer of harmful subunits of bacterial toxins into target cells^{4,5}. Analogously, the proteasome pulls a folded protein against a constriction, so that the unfolded polypeptide enters the degradation chamber^{6,7}.

The energetics of co-translocational unfolding are poorly understood^{8,9}. As well as the free energy of protein unfolding, additional considerations must be taken into account. These include the free energy required to thread the end of the polypeptide into the pore and the free energy required to confine the flexible polymer within the pore lumen, both of which have substantial entropic contributions^{9,10}. Interactions between the polypeptide and the internal surface of the pore may also be appreciable^{9–11}.

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Author contributions

D.R.-L. and H.B. planned the research. D.R.-L. performed the experiments and data analysis. D.R.-L. and H.B. wrote the paper.

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Important advances have been made with various physiological systems. For example, the translocation of edema factor (EF) and lethal factor (LF) through the pore formed by the protective antigen (PA) of anthrax toxin have been investigated^{4,12,13,14}. In planar bilayers, the unfolding and translocation of these proteins occurs in a few seconds, and is initiated by a positively charged leader sequence^{14,15}. While the "S-shaped kinetics" suggest a multistep process, no unfolding intermediates have yet been observed in single-molecule experiments^{14,16}. The kinetics can be modeled as Brownian motion^{4,17,18} coupled to drift in the applied potential. However, in living cells, the driving force is more likely to be the pH across the endosome membrane^{4,12,19}, with contributions from the membrane potential (ψ) and binding to chaperonins upon entry into the cytoplasm⁴. Unfolding is assisted at the endosomal pH of ~ 5.5 , at which EF and LF are molten globules²⁰, and may be rate-limiting at low ψ and pH values¹⁴.

Brownian ratchets have been proposed as a general mechanism for polypeptide translocation¹⁷. However, directly driven co-translocational unfolding also occurs, e.g. during transport through the membranes of organelles or bacteria. For example, in mitochondria, the inner membrane ψ and the mtHsp70 motor are likely to operate directly for part of the translocation process^{3,21}. In this context, co-translocational unfolding has been examined in a computational study in which barnase with a positively charged leader sequence was pulled through a model pore⁸. The translocation intermediates differed from those in thermal and denaturant unfolding, and from those induced by pulling on both ends of the polypeptide (cf. force spectroscopy approaches).

Here, we have examined experimentally, at the single-molecule level, the kinetics of the co-translocational unfolding of a model protein of 108 amino acids, *E. coli* thioredoxin, through a model pore, staphylococcal α -hemolysin (α HL). Previous work with model systems has established the ability of nanopores, including the α HL pore, to distinguish between folded, partly unfolded and unfolded states^{22–27}. We take advantage of this capability to illuminate for the first time the dynamics of an intermediate generated during co-translocational unfolding. We reveal a 4-step mechanism, which includes two separate unfolding events with a detectable, partly unfolded intermediate, that differs from the two-state mechanism observed in solution^{28,29}.

Step 1→2: the oligonucleotide leader threads into the pore

We initiated the translocation of a model protein, thioredoxin, through the α -hemolysin (α HL) protein pore by linking an oligonucleotide (oligo) to its C terminus. Single-stranded nucleic acids are transported unidirectionally through the α HL pore in an electric field, and in a positive potential the negatively charged oligo was expected to i) facilitate the threading of the C-terminal end of the construct into the mouth of the pore; ii) provide a tunable driving force both for protein unfolding and the early stages of protein translocation, estimated at ~ 5 to 10 pN^{30–32}; and iii) prevent backward movement of the oligo-protein conjugate.

We used a thioredoxin variant with the catalytic disulfide removed (C32S/C35S), which mimicks the reduced form of the protein^{33,34}. Three stabilizing mutations were also

introduced (A22P/I23V/P68A)²⁹ and a C-terminal cysteine added (C109). We refer to this variant as V5-C109; all further mutations described in this work were made in the V5-C109 background. A 30-mer oligo(dC) oligonucleotide was linked to Cys-109 through a disulfide bond³⁵, and the conjugate, V5-C109-oligo(dC)₃₀, was purified by ion exchange chromatography (Supplementary Fig. S1).

To observe protein translocation, the ionic current passing through a single α HL pore in a planar lipid bilayer was monitored³⁶ (Fig. 1a). When purified V5-C109-oligo(dC)₃₀ was added to the cis chamber and a potential of +80 to +140 mV applied, we observed a series of transient current blockades (Fig. 1b,c), which in >90% of the cases showed a cyclic pattern with 4 current levels separated by 4 irreversible steps (Fig. 1d,e). The cycle begins with step 1→2, the blockade of the open pore (level 1, $I_{+140\text{mV}} = 290.5 \pm 2.0$ pA) to produce level 2, which is of lower conductance ($I_{\text{RES},+140\text{mV}} = 48.5 \pm 1.2$ pA; I_{RES} is the current flowing during a blockade). The rate of occurrence of this event increased both at higher applied potentials and with higher concentrations of the conjugate, the latter reflecting a bimolecular mechanism ($k_{12} = 4.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at +90 mV and $2.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ at +140 mV). In step 2→3, level 2 converts to the more highly conducting level 3 ($I_{\text{RES},+140\text{mV}} = 64.2 \pm 0.8$ pA). The rate constant for this step (k_{23}) has an exponential dependence on the applied potential, and ranges from $k_{23} = 1.45 \pm 0.3 \text{ s}^{-1}$ at +90 mV to $k_{23} = 69 \pm 21 \text{ s}^{-1}$ at +140 mV. In step 3→4, level 3 converts to the final, weakly conducting level 4 ($I_{\text{RES},+140\text{mV}} = 9.1 \pm 1.6$ pA), with a voltage- and concentration-independent rate constant, $k_{34} = 1.7 \pm 0.1 \text{ s}^{-1}$. A new cycle begins after step 4→1, when the pore reassumes its open state (level 1). Step 4→1 is also voltage- and concentration-independent, $k_{41} = 96 \pm 5 \text{ s}^{-1}$.

To further define step 1→2, the voltage dependence of the association rate constant for V5-C109-oligo(dC)₃₀, k_{12} , was compared to that of a 92-mer oligonucleotide³⁷ (Fig. 2a). Both the magnitudes of the association rate constants and their voltage dependences are similar, suggesting that an interaction with the oligonucleotide portion of the V5-C109-oligo(dC)₃₀ conjugate initiates the interaction with the α HL pore. To gain additional evidence for oligo threading, we used the ability of the α HL pore to discriminate between nucleobases³⁸. We attached the same thioredoxin variant to oligo(dA)₃₀, rather than oligo(dC)₃₀. The oligo(dA) was expected to give a greater current blockade by comparison with oligo(dC)₃₀³⁸. When the two oligo-protein conjugates were compared, this was the case for level 2, but not for levels 3 and 4, suggesting both that the oligonucleotide end indeed threads first (Fig. 3) and that only during level 2 is the oligonucleotide located within the pore.

Steps 2→3 and 3→4: the crossing of two unfolding barriers

As expected, the rate of step 2→3 (k_{23}) showed no dependence on the concentration of V5-C109-oligo(dC)₃₀ (Supplementary Fig. S2), but k_{23} had an exponential dependence on the applied potential (Fig. 2b). This is consistent with level 2 representing a state with the oligo threaded through the β barrel of the α HL pore but unable to proceed to the trans side because it is held back by the folded thioredoxin, i.e. the barrier presented by the unfolding of thioredoxin must be overcome for translocation to proceed. By contrast, the rate constants for steps 3→4 and 4→1 (k_{34} and k_{41}) are not voltage-dependent (Fig. 2c and 2d), suggesting that the oligo is no longer in the α HL pore during these transitions. This is supported by the

finding that V5-C109-oligo(dC)₃₀ and V5-C109-oligo(dA)₃₀ exhibit similar I_{RES} values for levels 3 and 4 (Fig. 3). The lack of voltage-dependence is explained by the low charge density of the polypeptide, and the fact that the side-chain charges are both positive and negative (Supplementary Fig. S3).

Because folded thioredoxin is too large to enter the α HL pore (largest diameter at the cis entrance = 33 Å, smallest diameter 26 Å), steps 2→3 and 3→4 must represent unfolding events. To confirm this and to examine unfolding in more detail, we examined the effect of urea on the translocation process. In these experiments, we took advantage of the high stability of the α HL pore, which remains folded and functional at high urea concentrations³⁹. Urea molecules bind the backbone of solvent-exposed polypeptide chains, thereby lowering the free energy of both the unfolded state and transition state for unfolding so that unfolding in urea proceeds at higher rates⁴⁰.

The rate constant (k_{12}) for association between V5-C109-oligo(dC)₃₀ and the α HL pore, step 1→2, decreased at high urea concentrations (data not shown) which might be attributed to the increase in the bulk viscosity³⁹. By contrast, the rate constants for steps 2→3 and 3→4 (k_{23} and k_{34}) increased at high urea concentrations by ~10-fold over the range 0 M to 4.3 M, suggesting that both steps involve unfolding of the protein (Fig. 4a,b). However, step 4→1 (k_{41}) has no significant dependence on urea, with the rate varying by less than 2-fold (Fig. 4c), consistent with a largely unfolded protein diffusing through the pore, which thereby returns to the open state, level 1.

A set of correlations has been obtained that relates the dependence of the free energy of unfolding on urea concentration (the m -value) and structural parameters associated with unfolding such as the change in exposed surface area or the number of amino acids unfolded⁴¹. Although these correlations are derived from equilibrium unfolding studies, they can be used to make a gross structural characterization of the transition state for unfolding⁴². By assuming that the structure of the transition state does not change significantly with the applied potential, kinetic m -values of 1.13 and 1.43 kJ mol⁻¹ M⁻¹ were obtained from $\ln k$ versus [urea] plots for steps 2→3 and 3→4 respectively, which correspond to changes in surface area of 2455 and 3112 Å², 36 and 43 residues respectively⁴¹. These values are each smaller than that obtained from urea unfolding experiments in bulk solution (~2.5 kJ mol⁻¹ M⁻¹, roughly 80 residues²⁸), and strongly suggest that the unfolding pathway mediated by the α HL pore proceeds through two sequential transition states. Furthermore, thioredoxin has been judged to be a two-state unfold in solution^{28,43}, while our results indicate an unfolding pathway where at least one intermediate is present. This finding is not necessarily surprising given that i) the pore presents a physical barrier at its entrance that might force the protein to take a different unfolding route and ii) single-molecule experiments are able to discern non-rate limiting steps that are hidden in bulk experiments^{8,44}. Indeed, our data suggest that at low potentials the mean duration of level 2 will become longer than that of level 3 (Fig. 2b, c), i.e. unfolding would appear to be two-state in the bulk, but a short-lived intermediate would be visible in a single-molecule experiment.

To gain further insight into the unfolding steps, we used thioredoxin mutants in the V5-C109 background, all tagged at the C terminus with oligo(dC)₃₀. We hypothesized that disruption

of a network of ionic interactions between the C-terminal α -helix and the rest of the thioredoxin structure (Fig. 5a) would facilitate unfolding of the C terminus when it was subjected to a pulling force.

We first removed the charged group from position 96 by mutating Lys-96 to Ala, eliminating the electrostatic interactions with positions 44 and 48, with the expectation that the C-terminal α -helix would be released from the protein core more easily. The mutation indeed increased the rate of step 2 $\rightarrow$ 3 by almost 20-fold (Fig. 5c), while the effect on the rates of steps 3 $\rightarrow$ 4 and 4 $\rightarrow$ 1 were less than 2-fold (Fig. 5d,e).

Next, we substituted three attractive interactions by repulsive interactions by mutating Lys-90 and Lys-96 to Asp residues (K90D/K96D), which introduces repulsive interactions between residues 90 and 101, 96 and 44, and 96 and 48. It was predicted that this would have an even greater effect on step 2 $\rightarrow$ 3. Indeed, step 2 $\rightarrow$ 3 was 10,000-fold faster with K90D/K96D-oligo(dC)₃₀ compared with V5-C109-oligo(dC)₃₀ and level 2 could only be observed at low potentials (Fig. 5f). The K90D/K96D mutation also caused an $\sim$ 4-fold increase in the rate of step 3 $\rightarrow$ 4 (Fig. 5g), while the change in the rate of step 4 $\rightarrow$ 1 was less than 2-fold (Fig. 5h).

We also examined the destabilization of the core of the protein with the double mutation P22A/V23I. These mutations are known to cause an approximately 10°C decrease in the T_m of thioredoxin²⁹. In this case, the rates of both step 2 $\rightarrow$ 3 and step 3 $\rightarrow$ 4 were increased (Fig. 5i, 5j). The 3-fold increase in k_{23} might be explained by a weakening of the Van der Waals interactions between residues 22 and 23 and the final portion of the C-terminal α helix. The rate of step 3 $\rightarrow$ 4 was increased 8-fold, suggesting that residues 22 and 23 are in their native configuration before step 3 $\rightarrow$ 4 proceeds. The effect of the P22A/V23I mutations on the rate of step 4 $\rightarrow$ 1 was less than 2-fold and we conclude that this step does not involve further unfolding of the protein.

Step 4 $\rightarrow$ 1 involves a completely unfolded protein

If the protein is completely unfolded after step 3 $\rightarrow$ 4, step 4 $\rightarrow$ 1 would represent the diffusion of the unfolded protein out of the pore into the trans compartment. We assume that this diffusion has overall directionality, as no complete backward translocations are observed, most likely because the oligonucleotide works as a ratchet preventing movement of the C terminus back to the cis side. Refolding on the trans side will contribute only at the very end of the translocation process, because a major fraction of the protein must be completely translocated to initiate refolding.

We confirmed the nature of step 4 $\rightarrow$ 1 by conducting experiments in which we examined an unfolded population with the α HL pore. We made use of the demonstrated ability of nanopores to detect the unfolded fraction in a protein solution at different urea concentrations from the event frequency^{22,26}. We used the K96D/K90D thioredoxin mutant, which has a denaturation midpoint in urea of $C_{1/2} = 5.3$ M, by comparison with a value of $C_{1/2} = 8.8$ M for V5-C109 (Supplementary Fig. S4). Two kinds of events were observed in the presence of high urea concentrations (Fig. 6), events with the series of blockades described previously (levels 1 to 4) and events that only showed one blockade level, with the

same I_{RES} as level 4, i.e. lacking levels 2 and 3. The fraction of events that lack steps 2→3 and 3→4 increased with the urea concentration, suggesting that they are caused by translocations that are initiated with unfolded thioredoxin. To confirm that these events are caused by unfolded proteins diffusing through the pore, we measured the fraction of unfolded protein in solution at different urea concentrations by circular dichroism. The nanopore approach and the circular dichroism measurements revealed dependences of the (presumed) unfolded population on urea concentration that could be fitted to a reversible two-state model with similar $C_{1/2}$ and $m_{1/2}$ values ($m_{1/2}$, the energetic destabilization of the native state caused by 1 M denaturant): nanopore, $C_{1/2} = 4.9 \pm 0.05$ M, $m_{1/2} = 3.6 \pm 0.25$ kJ mol⁻¹ M⁻¹; circular dichroism, $C_{1/2} = 5.3 \pm 0.06$ M, $m_{1/2} = 4.3 \pm 0.32$ kJ mol⁻¹ M⁻¹ (Figure 6). The small differences may be explained by the time resolution of the nanopore technique. At high urea concentrations, unfolding is accelerated. When steps 2→3 and 3→4 are shorter than 50 μ s, they cannot be resolved and therefore the unfolded fraction at high urea concentrations is overestimated.

Conclusions

Previous work with model systems has demonstrated the ability of nanopores, including the α HL pore^{22–27}, to distinguish between folded, partly unfolded and unfolded states of proteins at the single-molecule level. Here, we have taken advantage of this ability to measure the lifetime of a partly unfolded intermediate that is generated during translocation of a model protein, thioredoxin, through a model protein nanopore, α -hemolysin.

All told, we observe 4 steps in the translocation process and we have inferred a molecular description of each. In solution, thioredoxin has been considered to present a paradigm of two-state folding^{28,43}. Our nanopore experiments show two sequential unfolding events, the first depends on the applied potential (k_{23}), while the second is voltage-independent (k_{34}). Further, extrapolation to 0 M urea in bulk unfolding experiments and to 0 mV in the nanopore approach yields unfolding rates of 4.1×10^{-8} s⁻¹ and 1.4×10^{-3} s⁻¹ (an estimate of k_{23} at 0 mV), respectively, a difference of more than 4 orders of magnitude (Supplementary Fig. S5), suggesting that topologically different pathways are taken^{8,9}. We foresee further differences should unfolding kinetics obtained by single-molecule force spectrometry become available given the entropic restriction associated with the attachment of both ends of a protein to surfaces⁴⁵.

Our findings suggest a general mechanism for co-translocational protein unfolding, which is broadly similar to that proposed on the basis of ensemble measurements by Krantz and colleagues¹⁴. Single-molecule experiments can reveal additional detail including the existence of intermediates, whether or not they are rate limiting. For example, we have demonstrated a partly unfolded intermediate in the present work. In our mechanism, the translocated protein contains a charged oligopeptide terminus that is drawn into the pore. Pulling at this end then causes partial unfolding. In the case explored here, the unfolding rate has an exponential dependence on the applied potential, which has also been observed for the nanopore-mediated unzipping of DNA duplexes⁴⁶. The partial unfolding destabilizes the remainder of the folded structure, which then unfolds spontaneously and diffuses through the pore. Reversal is prevented by a ratchet mechanism that is effective for a period

(seconds) sufficient to drive forward translocation, and which in the case described here is provided by the covalently attached oligonucleotide^{17,47}. Such a ratchet mechanism is an attractive possibility for movement of EF and LF through the anthrax PA pore^{4,18}, and for mitochondrial import^{2,48}. In nature, such translocation is usually N terminus first (C terminus for the proteasome) and translocated proteins may have acquired low stability against one-end pulling. Except for the initial engagement with the pore, a Brownian ratchet mechanism is largely independent of polypeptide charge distribution¹⁷.

The model system we have established is amenable to further development to allow the investigation of additional components of translocation systems. Chaperone proteins might be added to either compartment, and enzymes that mediate posttranslational modification included on the trans side. The effects of refolding conditions in the trans compartment could also be explored. We might also explore model pores with a variety of geometries, and internal surfaces with different distributions of charge and hydrophobicity.

Methods

α HL pores

Wild-type α HL monomers were produced by expression in an Escherichia coli in vitro transcription/translation (IVTT) system and oligomerized into heptameric pores on rabbit red blood cell membranes by Ellina Mikhailova. The heptamers were purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)³⁷. A portion of the resulting protein solution (0.2 μ L) was added to the cis compartment of a bilayer apparatus (see below).

Thioredoxins and oligonucleotide-thioredoxin conjugates

The thioredoxin V5-C109 gene (TopGene) was obtained in the pET 30a+ plasmid. The mutants used in this work were made with the Quick Change II XL site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing. Protein overexpression was carried out with E. coli BL21(DE3) cells (Novagen). Oligonucleotides carrying a 5'-thiol modifier with a hexamethylene linker were obtained from Integrated DNA Technologies and activated with 2,2'-dipyridyl disulfide. The thioredoxins were reduced for 24 h in 1 mM dithiothreitol and then reacted with the 5'-S-thiopyridyl oligonucleotides for 16 h at room temperature as described³⁴. The desired oligo-thioredoxin conjugates were purified by ion exchange chromatography on a HiTrap Q FF column (GE Healthcare) eluted with 0 to 1 M KCl in 10 mM Tris.HCl, 1 mM EDTA, pH 8.0. The mass of each conjugate was verified by mass spectrometry (Supplementary Fig. 1c). The concentration of each oligo-thioredoxin conjugate was determined by using the calculated molar extinction coefficient of the oligonucleotide at 260 nm.

Electrical measurements and data analysis

Planar lipid bilayer recordings were carried out at 22.0 ± 1.5 °C. Briefly, and as described elsewhere³⁵, we used a bilayer of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL, USA) across an aperture 100 μ m in diameter in a Teflon film (Goodfellow) that separated the two compartments (1 mL each) of the apparatus, cis and

trans. The buffer was 10 mM HEPES, 2 M KCl, pH 7.2. After the insertion of a single α HL pore from the cis compartment, the solution was replaced with fresh buffer by manual pipeting. Ionic currents were measured by using Ag/AgCl electrodes with a patch-clamp amplifier (Axopatch 200B, Axon Instruments). The amplified signal was low-pass filtered at 5 kHz (or 10 kHz) and sampled at 20 kHz (or 50 kHz) with a Digidata 1440A digitizer (Axon Instruments). The signal was not further filtered unless otherwise stated. Initial data analysis was performed with pClamp software (Molecular Devices) by carrying out a threshold search, which gave the inter-event intervals at level 1. In this threshold search, blockades below the resolution time ($<50 \mu\text{s}$) and very long blockades (5 to 10 % of the events) were discarded. A second threshold search with three levels was carried out on the events obtained in the first search, which gave the dwell times at levels 2, 3 and 4. For each level, we constructed a cumulative unbinned histogram of the duration times, which is the probability distribution and which was fitted to a single exponential function (Igor Pro 6.12A, WaveMetrics) to yield the mean event duration. The errors given in the text represent the standard deviations between independent experiments.

Urea denaturation experiments and circular dichroism

A desired urea concentration was obtained by diluting a known volume of a freshly made 10 M urea solution into the buffer of both compartments for the electrical recordings or the optical cuvette for the circular dichroism measurements. The final urea concentrations were checked by measuring the refractive indices of the solutions. All measurements were carried out after an incubation time of 30 min to 3 h to let the sample equilibrate. Circular dichroism measurements were performed with a Chirascan instrument (Applied Photophysics) at 222 nm in the presence of 1 mM DTT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Interaction of V5-C109-oligo(dC)₃₀ with the αHL pore

a) The pore is inserted in a lipid bilayer from the cis compartment and a potential is applied causing an ionic current to flow through the pore. b) Current trace at +140 mV in 2 M KCl in the absence of V5-C109-oligo(dC)₃₀. c) Current trace at +140 mV in 2 M KCl with V5-C109-oligo(dC)₃₀ (0.4 μM, cis). d) and e) Level 1: V5-C109-oligo(dC)₃₀ is in solution and the pore is unoccupied. Level 2: the oligonucleotide threads into the pore and pulls on the protein. Level 3: the pulling force causes partial unfolding allowing the oligonucleotide to traverse the pore and the unfolded segment of the polypeptide to enter. Level 4: the

remainder of the polypeptide unfolds spontaneously, diffuses through the pore and leaves through the trans entrance.

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Figure 2. Voltage-dependences of the rate constants for transitions between current levels

a) (○), Voltage-dependence of the frequency of occurrence of interactions between the V5-C109-oligo(dC)₃₀ and the α HL pore (step 1→2, k_{12}). (□), Voltage-dependence of the frequency of occurrence of interactions between a 96-mer oligonucleotide and the same wild-type α HL pore used here³⁶. b) Voltage-dependence of the rate constant for step 2→3 in the translocation of V5-C109-oligo(dC)₃₀ (k_{23}). c) Voltage-dependence of the rate constant for step 3→4 (k_{34}). d) Voltage-dependence of the rate constant for step 4→1 (k_{41}). Error bars represent the standard deviations between independent experiments ($n = 6$).

Figure 3. Oligonucleotide insertion in step 1→2

a) Histogram of the current blockade levels observed with V5-C109-oligo(dC)₃₀. b) Histogram of the current blockade levels in the presence of equal concentrations of V5-C109-oligo(dC)₃₀ and V5-C109-oligo(dA)₃₀. $I_{RES}\% = (I_{RES}/I_O) \times 100$, where I_{RES} is the current flowing during a blockade and I_O is the current through the unblocked pore.

Figure 4. Voltage-dependences of the rate constants for transitions between current levels in the presence of urea

a) The effect of urea on step 2→3 (k_{23}). b) The effect of urea on step 3→4 (k_{34}). c) The effect of urea on step 4→1 (k_{41}). Error bars represent the standard deviations between independent experiments ($n = 3$).

Figure 5. The effect of mutations on the rate constants for transitions between current levels
 a) The thioredoxin structure with arrows to highlight the network of ionic interactions (green residues) that link the C-terminal α -helix (center) with the rest of the structure. Pro-22 and Val-23 (red) make Van der Waals interactions both with the C-terminal α -helix and the core of the protein. Stars, residues mutated in this work. b) Secondary structure of thioredoxin mapped onto the primary sequence. Arrow, direction of translocation (C to N). c), d), e) Voltage dependencies of k_{23} , k_{34} and k_{41} for K96A-oligo(dC)₃₀ (□) and V5-C109-oligo(dC)₃₀ (○). f), g), h) Voltage dependencies of k_{23} , k_{34} and k_{41} for K96D/K90D-oligo(dC)₃₀ (△) and V5-C109-oligo(dC)₃₀ (○). The y-axis in 'f' is on a logarithmic scale. i), j), k) Voltage dependencies of k_{23} , k_{34} and k_{41} for P22A/V23I-oligo(dC)₃₀ (◇) and V5-C109-oligo(dC)₃₀ (○). Error bars represent the standard deviations between independent experiments (n = 4).

Figure 6. Detection of events from the unfolded population in urea solution

Left. The two types of events that are detected by electrical recording (events with level 4 only, and events with levels 2, 3 and 4) and the variation of the percentage of each type with the urea concentration. The events that lack distinct current steps (level 4 only) represent the translocation of unfolded thioredoxin. *Right.* The data are fitted to a two-state reversible process (black) assuming that the protein is completely unfolded in 12 M urea. Data from CD measurements in bulk solution are also shown (red).