Published in final edited form as:
Protein Pept Lett. 2014 March ; 21(3): 256–265.

Characterization of Protein Unfolding with Solid-state Nanopores

Jiali Li[*], **Daniel Fologea**, **Ryan Rollings**, and **Brad Ledden**
Department of Physics, University of Arkansas, Fayetteville, AR 72701

Abstract

In this work, we review the process of protein unfolding characterized by a solid-state nanopore based device. The occupied or excluded volume of a protein molecule in a nanopore depends on the protein's conformation or shape. A folded protein has a larger excluded volume in a nanopore thus it blocks more ionic current flow than its unfolded form and produces a greater current blockage amplitude. The time duration a protein stays in a pore also depends on the protein's folding state. We use Bovine Serum Albumin (BSA) as a model protein to discuss this current blockage amplitude and the time duration associated with the protein unfolding process. BSA molecules were measured in folded, partially unfolded, and completely unfolded conformations in solid-state nanopores. We discuss experimental results, data analysis, and theoretical considerations of BSA protein unfolding measured with silicon nitride nanopores. We show this nanopore method is capable of characterizing a protein's unfolding process at single molecule level. Problems and future studies in characterization of protein unfolding using a solid-state nanopore device will also be discussed.

Keywords

Bovine Serum Albumin (BSA); current blockage; excluded volume; protein unfolding; solid-state nanopore; translocation time

1. Introduction

Protein molecules are made up of long chains of amino acids. The native (functional) state of most protein molecules is in a tightly folded three-dimensional conformation. Within cells, unfolding and refolding of proteins occurs on a continuous basis. Fluctuations between native protein structure and partially or fully unfolded states affect key biological processes such as binding, translocation across membranes, secretion, or degradation [1]. To be able to characterize the folding-unfolding process of proteins is of fundamental importance not only for basic science but also in biotechnological applications. Owing to the physiological implications, many bulk based experimental methods as well as theoretical approaches have been employed for analyzing the conformational changes accompanying the folded-unfolded process: Fluorescence [2], NMR [3], and molecular dynamics simulations [4] are a few such

[*]Corresponding Author: Jiali Li, Department of Physics, Room 226, University of Arkansas, 825 w Dickson Street, Fayetteville, AR 72701, jialili@uark.edu, Phone: (479) 575-7593. Fax: (479) 575-4580.

techniques. In recent years, it has been demonstrated that ion channels (protein pores) suspended in lipid membranes [5–8] and later solid-state nanopores [9] are capable of characterizing protein unfolding at single molecule level [10].

Polypeptides and proteins measured by protein pores

Protein pores or ion channels suspended in lipid membranes were first shown to be capable of sensing single polypeptide molecules [11, 12] or proteins [5–8, 13–15]. Protein pores such as αhemolysin have reproducible geometry, well-defined structure and dimensions. However, due to their small fixed pore diameter, ~1.5 nm, only polypeptides or denatured proteins are able to pass through, which limits protein pores being used in measuring the process of protein unfolding. In addition, protein pores become unstable under protein denaturing conditions.

Protein unfolding measured by solid-state nanopores

Solid-state nanopores fabricated with desired geometry and dimensions, have been demonstrated to be capable of detecting protein molecules both in their native (folded) [16–25] and in their denatured (unfolded) states [9, 26, 27]. Motivated to develop a solid-state nanopore based device to identify proteins by measuring them both in their folded and unfolded states, we have studied the process of protein unfolding with sold-state nanopores. The protein molecules we have studied are: β-lactoglobulin (βLGa) and Hpr [9], Laminin [10], Bovine Serum Albumin (BSA), and two sets of protein markers (NativeMark from Invitrogen, Protein Marker from NBL) in their native and denaturing conditions. Because the complexity of protein unfolding process and the sophistication of current signals observed for each protein, in this review we will mainly focus on one protein, BSA unfolding, as an example to describe the experiments, results, data analysis, and problems of using solid-state nanopores to characterize the process of protein unfolding.

2. Principles of measuring protein unfolding by a solid-state nanopore

The center part of a solid-state nanopore device is a single nanopore fabricated in an insulating membrane (Fig. 1a and 1b) which separates two PDMS chambers filled with salt solution: the *cis* chamber in which protein samples is added, and the *trans* chamber where protein molecules move into after they translocate through the nanopore. A pair of silver chloride electrodes is embedded in the chambers. The electrodes are used to apply a constant voltage Ψ across the membrane and to measure the ionic current flow through the nanopore. The *cis* chamber is often electrically grounded and the *trans* is positively or negatively biased depending on the charge of the protein to be measured. The nanopores used for protein unfolding experiments reported so far are fabricated by a Focused Ion Beam (FIB) [26] or by a combination of FIB and low energy noble gas ion beam [28–30]. The nanopores are often 10–30 nm in thickness and 2–25 nm in diameter depending on the size of proteins.

2.1. Parameters to be measured: current drop amplitude ΔI_b and time duration t_d

A protein molecule of ~10^2 amino acids in a nanopore will partially block the flow of ions in the pore, increase the pore resistance, and cause a transient current drop event from the open pore current I_0 as shown in Fig. 1c. Each current drop event in Fig. 1c represents a protein

molecule interacting with or translocating through the pore. The current drop magnitude caused by a translocating protein is a function of time, $\Delta I_b(t)$. Analysis of current drop events caused by protein molecules has been and is still a challenging task. For initial event sorting, we often calculate the mean current drop amplitude ΔI_b and the dwell time t_d (Fig. 1c). The dwell time t_d of an event is defined as the time at half height of ΔI_b. Below we describe how these two parameters are connected to the protein unfolding process.

$\Delta I_b(t)$ is proportional to the excluded volume Λ (t) of a protein molecule in a nanopore—Approximately, the mean current drop amplitude ΔI_b is determined by the pore geometry, conformation or shape of a protein molecule, and the solution conductivity. If we idealize a nanopore as a cylinder with diameter D_p and length H_{eff}, assuming a protein molecule occupies a volume $\Lambda(t)$ in the pore at time t (the instantaneous excluded volume), and further we assume that a translocating protein molecule in a nanopore driven by an applied voltage Ψ obeys Ohm's law, the relationship between $\Delta I_b(t)$ and $\Lambda(t)$ can be written as [31–34]:

$$\Delta I_b(t) = -I_0 \frac{\Lambda(t)}{H_{eff} A_p}[1+f(d_m/D_p, l_m/H_{eff})] \approx -I_0 \frac{\Lambda(t)}{V_p} \quad (1)$$

Where $I_o=\Psi/R_0$ is the open pore current, $R_0= H_{eff}\,\sigma/A_p$ is the pore resistance, σ is the solution conductivity, H_{eff} is the effective thickness of the pore taking into account the access resistance region on both sides of the nanopore [35–37], A_p is the area of the pore with an average diameter D_p, $V_p = H_{eff}A_p$ the volume of the pore, and d_m and l_m are the diameter and length of the protein molecule. $f(d_m/D_p, l_m/H_{eff})$ is a correction factor for the relative size and shape of the pore and the protein molecule and is often ignored for simplicity. For a folded protein molecule that can fit entirely inside a nanopore, f is less than one and contributes little to the current drop [32]. For a peptide chain that is much longer than the pore, $f(d_m/D_p, l_m/H_{eff}) \approx 0$, therefore $\Delta I_b \approx I_0\Lambda/V_p$, or $\Lambda \approx (\Delta I_b/I_0)V_p$. This relates the relative current drop amplitude, $\Delta I_b(t)/I_0$, to the excluded volume of the protein molecule $\Lambda(t)$.

The simple model described in Eq. (1) shows that if a nanopore's volume V_p is kept a constant, the relative current drop magnitude, $\Delta I_b(t)/I_0$, is directly proportional to a protein's excluded volume, Λ, in the nanopore. For a completely unfolded protein going through a nanopore as a linear peptide chain with $\ell_m > H_{eff}$, only a segment of the protein is in the pore at any time, the excluded volume of the local segment is expected to be smaller than the whole protein at its 3D form, $\Lambda_{localSeg} < \Lambda_{folded}$, therefore a smaller $\Delta I_b(t)/I_0$ is expected to be measured for the unfolded protein than its 3D folded conformation [9]. Below we use BSA as an example to illustrate this point more quantitatively.

BSA excluded volume Λ in a nanopore and its conformation state—BSA belongs to the most abundant (with a typical concentration of ~77 mM) serum albumin protein family which is essential to maintain many important body functions in mammals. The BSA (66,430 D, Sigma) used in this work has 583 amino acid residues. One of the possible shapes and dimensions of a native state BSA shown in Fig. 1d is a prolate ellipsoid [38]. BSA is water soluble, monomeric, globular shape, and negatively charged at pH 7.4.

Its isoelectric point at 25 °C is around 5.0 depending on the solution and salt concentration. BSA has 17 disulphide (covalent) bonds that make it as one of the most stable proteins. BSA is one of the most widely studied proteins and is used in numerous biochemical applications due to its stability.

The conformation of a BSA molecule can be a folded (Fig. 1d), partially (Fig. 1e), and fully unfolded (Fig. 1f) states. By adding the atomic volume of all 583 amino acids [39, 40], the total atomic volume of a BSA molecule is estimated to be $\Lambda = 87.0\ nm^3$. The estimated contour length of a completely unfolded BSA is about $\ell_m \approx 200$ nm. When a BSA molecule translocates a nanopore ($H_{eff} < \ell_m$) as a linear peptide chain, only a segment of the amino acid chain will be inside the nanopore. As illustrated in Figure 2a, for a nanopore with H_{eff} = 20 nm, the maximum excluded volume of a BSA molecule in its folded form is Λ_{fold}=87.0 nm^3, as a linear peptide chain, $\Lambda_{unfold} \approx 9.0\ nm^3$. For a H_{eff} = 10 nm pore, the maximum values are Λ_{fold}=62.2 nm^3 and $\Lambda_{unfold} \approx 4.5\ nm^3$ (Fig. 2a, dotted lines). The ratio of Λ_{unfold} : Λ_{fold} = 1: 9.7, this suggests that the $\Delta I_b(t)/I_0$ measured for a BSA molecule in a nanopore is expected to be 10 times smaller for a linear unfolded BSA chain comparing to a globular shaped folded BSA molecule.

The excluded volume calculated by adding the atomic volumes of all amino acids in a nanopore provides an easy way to estimate the relative ratio of $\Lambda_{unfold}/\Lambda_{fold}$. However, this estimation has ignored complicated issues such as the space inside a folded protein, hydration layer and territory bound water and ions on protein surface, the real excluded volume of a protein molecule in a nanopore is expected to be larger. For example, here we calculate Λ_{fold}=87.0 nm^3 for a folded BSA, this is smaller than the value reported by Freedman et al. [25] calculated by formula $\Lambda=(4/3)\pi a^2 b$ =117 nm^3 using the dimensions shown in Fig. 1d, and it is also smaller than experimentally measured value166±29 nm^3.

In conclusion, if the same nanopore is used, a BSA molecule's excluded volume is expected to be Λ (folded) > Λ (partially unfolded) > Λ (unfolded). During a nanopore experiment, the measured relative current drop magnitudes are expected to have: $\Delta I_b/I_0$(folded) > $\Delta I_b/I_0$ (partially unfolded) > $\Delta I_b/I_0$ (unfolded). Thus a solid-state nanopore device can be used to characterize the process of protein unfolding.

Protein unfolding states and the time duration t_d—The time duration of a current blockage event, t_d, is also related to a protein's conformation state. When a BSA molecule in a nanopore is folded, it is expected to behave like a particle with a total charge Q= −18e at pH7 as shown in Fig. 2b. Under the electrical field strength of $E=\Psi/H_{eff}$, the electric force exerted on a BSA molecule is $F_e=QE=Q\Psi/H_{eff}$, which is opposed by a viscous drag force $F_{drag}=\eta C_f \upsilon$ assuming a BSA molecule is moving with a terminal velocity $\upsilon=H_{eff}/t_d$, then t_d can be written as

$$t_d = C_f \frac{\eta H_{eff}^2}{Q\Psi} \quad (2)$$

Here ηC_f is the friction coefficient [41], η is the solution viscosity, and C_f is a constant. In this case, the time duration t_d is expected to be inversely proportional to the applied voltage

Ψ, and the time distribution histogram for t_d is expected to be a Gaussian [9, 10, 41]. This equation also works for a uniformly charged amino acid chain, which is expected to behave like a DNA molecule with $\upsilon=(l_m+H_{\text{eff}})/t_{\text{d}}$.

When a protein is completely unfolded and going through a nanopore as a linear amino acid chain, the time distribution is expected to depend on the protein's charge sequence [9, 10]. As shown in Fig. 2b for an unfolded BSA, the net charge inside a nanopore is a function of translocation position of the amino acid chain; and the net charge could be positive, neutral, and negative. The net electrical driving force would change direction when the net charge of the local segment changes sign, which could drive the protein back and forth near the electrically neutral charged region. The polypeptide chain could be transiently trapped in the nanopore due to a protein's heterogeneous charge sequence, the translocation process could be thermally activated and the time duration distribution is expected to be multiple exponentials [9, 10] instead of a Gaussian for a folded BSA molecule.

Pore-protein interactions affect the dynamics of protein translocation—The above discussions on ΔI_{b} and t_{d} have ignored the interactions between a nanopore surface with a passing protein molecule. Reports on protein translocation in solid-state nanopores have shown that if the size of a protein and the diameter of the pore are close, the pore-protein interactions could dominate a protein's translocation dynamics [9, 19]. The pore-protein interactions could be controlled by functionalizing a nanopore's surface to adjust its charge, surface hydrophobicity/hydrophilicity, and by performing a nanopore experiment at different salt concentrations. Although these studies are critically important, understanding and controlling pore-protein interactions are still challenging tasks today. To reduce the pore-protein interactions, nanopores several nanometers larger in diameter than a protein is preferred.

2.2 Methods to unfold proteins for solid-state nanopore analysis

Most native-state proteins have a well-defined 3D structure, which can be disrupted by heat, denaturants, and extreme pH [42]. The task to completely unfold a native state protein to a linear amino acid chain and measure it with a solid-state nanopore has been a challenge. Here we briefly summarize several denaturing agents used in our lab: Guanidine HCl, urea, and SDS plus DTT.

Guanidine hydrochloride

CH_6ClN_3 or $[CH_6N_3]^+Cl^-$ in solution, is one of the strongest denaturants used in studies of protein unfolding. Since it contains Cl^- ions, it can be used with or without additional Cl^- containing salt that is required for a nanopore experiment using AgCl electrodes. In 6M guanidine chloride solution, all proteins lose their well-ordered structures, and most of them become randomly coiled.

Urea

$CO(NH_2)_2$, has two NH_2 groups joined by a carbonyl (C=O) functional group. It is highly soluble in water and non-toxic. In 8M urea, most proteins are denatured. However, since urea is electrically neutral, adding 8M urea decreases solution conductivity significantly [9].

Sodium dodecyl sulfate

(SDS) +DTT, $CH_3(CH_2)_{11}OSO_3Na$, is an anionic surfactant and is a common component used in cleaning products. SDS can denature a protein and coat a layer of negative charge on a peptide chain. This would be ideal because such a negatively charged peptide chain would go through a nanopore like a uniformly charged DNA molecule. However, attempts at adding SDS directly to the *cis* and *trans* chambers had not been successful in our lab because SDS can form bubbles easily. **Dithiothreitol (DTT), $C_4H_{10}O_2S_2$**, is an unusually strong reducing agent and is often used to break disulfide bonds.

3. Measuring BSA unfolding with solid-state nanopores

3.1. BSA in SDS + DTT + temperature

The experiment—Below we use BSA as an example to demonstrate how a solid-state nanopore can be used to characterize the unfolding process of a protein with disulfide bonds. To compare $\Delta I_b/I_0$ signal of folded BSA with partially and completely unfolded, the protein needs to be measured at least in 3 conformations, folded, partially denatured, and completely unfolded. This requires a nanopore that is large enough to be occupied by a native state globular shaped protein. One model of a BSA molecule has a dimension of d_m=4 nm and l_m=14 nm (Fig. 1d), hence a pore larger than 16 nm is preferred. The nanopore used for this BSA unfolding experiment was about ~16 nm (Fig. 1b). The electrolyte solution contains 1M KCl with 10 mM Tris and 1mM EDTA at pH 7. BSA protein (Sigma-Aldrich) was first dissolved to make a stock solution of 1 mg/ml (~ 15 μM) in ~150 mM KCl TE buffer. The *trans* chamber was positively biased to drive the negatively charged BSA (pH 7) to pass through the nanopore. The ionic current signal was recorded using an Axopatch 200B (Molecular Devices) in event driven and voltage-clamp mode. The low pass Bessel filter in the Axopatch 200B was set to 10 kHz or 100 kHz as indicated.

Results—After the BSA sample was added to the *cis* chamber to a final concentration of ~12 nM, current blockage events were observed. Several examples of these events are shown in Fig. 3a. After calculating the average current drop amplitude ΔI_b and the dwell time t_d for each event, we plotted the ΔI_b vs t_d in Fig. 3b. Fig. 3b shows the event distribution of BSA measured in 1M KCl at room temperature ~22°C without denaturants.

To denature the BSA, 1.4 mg SDS (3.9 mM) with 2 mM DTT was added to the stock BSA (1mg BSA in 1ml) solution. This mixture was heated at 45° for 5 minutes, then immediately cooled down in a water bath at room temperature. We then added this BSA treated with SDS +DTT and heated at 45° to the *cis* chamber. The resulting event distribution is shown in Fig. 3c. The same experiment was repeated for the BSA heated at higher temperatures are shown in Fig. 3d (preheated at 60° C) and Fig. 3e (preheated at 90° C).

For native BSA at room temperature, the current drop ΔI_b histogram on the right axis of Fig. 3b shows approximately two clusters of events, consistent with our earlier observations [16]. One cluster has a smaller current drop peak at ΔI_b =60 ± 10 pA, the other cluster has a broader ΔI_b = 100–300 pA (Fig. 3b). For the BSA protein preheated at 45° C, the plot of ΔI_b vs t_d in Fig. 3c shows features similar to the native state BSA data displayed in Fig. 3b, except more events have larger ΔI_b and longer t_d. Preheated at 60°C, the ΔI_b vs t_d plot only

shows one peak (Fig. 3d) at ΔI_b =60 ± 10 pA. Preheated at 90 °C, the same one peak at ΔI_b =60 ± 10 pA (Fig. 3e) was observed.

Discussion—The disappearance of the broader peak at ΔI_b = 100–300 pA observed for BSA in Figure 3b and 3c suggests that under the conditions of SDS + DTT preheated above 60 °C (Fig. 3d and 3e), the BSA molecules were unfolded and all BSA protein molecules have lost their 3D structure. Note that the process of protein folding-unfolding is dynamic and the time scale is in the order of milliseconds. The observed one peak at ΔI_b =60 ± 10 pA suggests that the BSA preheated above 60 °C remained denatured measured by the nanopore, possibly the heating treatment had permanently unfolded the protein. This also suggests that the small current blockage peak observed at $\Delta I_b \approx 60$ pA at all temperatures likely belongs to the denatured BSA translocation events. This conclusion is also consistent with our earlier studies on other protein unfolding measured with solid-state nanopores [9] and proteins and peptides measured with protein nanopores [5, 11, 12]. In the work by Oukhaled at el. and Merstorf et al., the authors concluded that the small ΔI_b and short t_d blockages were due to the passage of completely unfolded proteins, since their frequency increased as the concentration of the denaturing agent increased. This frequency dependency has also been observed with an increase of the temperature [8]. However, from this experiment, we can't exclusively conclude that all the events in the small ΔI_b = 60 pA peak represented unfolded BSA translocation, some short events could be produced by the collision of BSA molecules with the nanopore without translocation.

The broader peak with greater ΔI_b =100–300 pA in Fig. 3b implies larger excluded volume of protein molecules in the pore, possibly produced by folded BSA. The same larger ΔI_b =100–300 pA peak in Fig. 3c indicates that most BSA molecules were still having larger excluded volumes (folded state), but could have some degree of denaturation and aggregation. Since some events have larger ΔI_b (400–600pA) and very longer t_d, an increased pore-protein interactions at this condition was also possible. It is puzzling why the native state BSA in a nanopore produced such a broad peak in ΔI_b, implying a broad distribution of the excluded volume Λ and translocation configurations. Broad range of ΔI_b distributions were also observed by previous reports studying BSA and other protein translocation in solid-state nanopores [9, 16–19, 21, 25–27], suggesting some protein molecules going through solid-state nanopores with multiple conformations, some could be partially denatured.

Fig. 3f shows that the relative current drop amplitude $\Delta I_b/I_0$ is close to 2% for foplded BSA and about 0.5% for unfolded. Assuming V_p doesn't change, the ratio of $\Delta I_b/I_0$ for unfolded/folded measured in this experiment is about 1:4, not the ratio Λ_{unfold}: $\Lambda_{fold} \approx$ 1:10 predicted in Fig. 2a. Two possibilities could explain this smaller ratio: 1) if the single peak in Fig. 3d represents the completely unfolded BSA peptide chain in the pore, then events in the broader ΔI_b peak were produced by partially denatured BSA under the experimental condition; 2) if the broader peak at ΔI_b = 100–300 pA represents the intact folded BSA protein translocation in the nanopore, then the peak at ΔI_b = 60 pA represents unfolded but looped translocation configuration, not the linear amino acid chain imagined in Fig. 2a.

The time duration histograms in Fig. 3g show that the peak values of t_d were similar for BSA in its native state and for BSA pretreated at 45°C. The t_ds had increased and the distributions were broader for the unfolded BSA pretreated 5 minutes at 60 °C and 90 °C. It is not clear if the negatively charged SDS had affected the t_d measured in this experiment.

This example demonstrates that by measuring the relative current drop magnitude $\Delta I_b/I_0$, a solid-state nanopore device can characterize a protein's unfolding process. Next, we show two more examples: 1) BSA in 6M GuHCl that BSA can only be partially denatured; 2) BSA voltage dependence without chemical denaturants to see if a native state BSA behaves like a rigid charged particle.

3.2. BSA in 6M Guanidine Chloride

Proteins can be completely or partially (if disulphide bonds exist) denatured in 6M *Guanidine Chloride* (GuHCl). The conductivity of 6M Guanidine hydrochloride solution was approximately σ=179.3 ms/cm at room temperature (22 °C) at pH=7.0 (σ=114 for 1M KCl). Using a pore with D_p ~18 nm (TEM image shown in Fig. 4) in 6M GuHCl solution at Ψ=120 mV, an open pore current I_0=12.9 nA was measured. BSA protein was first dissolved in 6M GuHCl, and then added into the cis chamber. The BSA concentration in the *cis* chamber was ~100 nM. The *trans* chamber was positively biased.

Examples of current blockage events produced by the BSA sample are shown for Ψ=120 mV (Fig. 4a) and for Ψ=60 mV (Fig. 4b). Comparing with the completely denatured BSA which only showed one cluster of events in Figure 3c and 3d, the plot of ΔI_b vs t_d (Fig. 4c) shows two clusters of events at both voltages, ~80% of the events are in the cluster of longer t_d with smaller ΔI_b, indicating not all BSA molecules are completely denatured in 6M GuHCl. Figure 4d shows that the peak values of current drop amplitudes are: ΔI_b = 34±6 pA and the relative blockage $\Delta I_b/I_0$=0.54% at Ψ=60 mV, I_b = 54±8 pA and $\Delta I_b/I_0$=0.42% at Ψ=120 mV. The ratio of $\Delta I_b/I_0$ is decreased at Ψ=120 mV compared to Ψ=60 mV. To see the behavior of the two clusters of events more clearly, the time duration histograms are shown separately in Figure 4e at both voltages. Fitting with a Gaussian to the events of larger ΔI_b and short t_d, the peak values are: t_d^p = 33.6 ± 0.7 μs for Ψ=60 mV and t_d^p = 34.7 ± 1.4 μs for Ψ=120 mV. Two exponential fittings to the events of smaller ΔI_b and longer t_d, the exponential decal constants are tau1 =26.7 ± 5 μs and tau2 = 125 ± 37 μs for 60mV, tau1 =26.1 ± 4 μs and tau2 = 130 ± 40 μs for 120 mV. The histograms of the waiting time between events at both of these voltages in Figure 4f shows that the inter-event times are longer at Ψ=60 mV than Ψ=120 mV.

Discussion—This measurement shows that BSA molecules in 6M GuHCl were not completely unfolded, which is consistent with that guanidine is not capable of breaking the disulphide bonds in a protein. The ratio of $\Delta I_b/I_0$ decreased from 0.54% at Ψ=60 mV to 0.42% at Ψ=120 mV, this suggests a BSA molecule might become more stretched at 120 mV so it blocked less amount of current flow, consistent with recent observations reported by Cressiot et al on studying a maltose binding protein [27]. The same ratio of $\Delta I_b/I_0 \approx$ 0.5% was observed here suggesting that the peak at ΔI_b = 60 pA in Figure 3 is most likely from looped BSA translocation configuration. The events in the slow cluster with short

tau1≈26 μs could be produced by collision of BSA molecules with the pore, the longer t_d were most likely produced by partially unfolded BSA. With tau2 = 125 μs for 60mV and tau2 = 130 μs for 120 mV, the dwell times of these events were longer at Ψ=120 mV, suggesting that these events with longer t_ds could be produced by BSA linear translocation as shown in Figure 2B. The translocation process could be thermally activated. Another possibility is that BSA molecules become more stretched at higher voltage that it takes longer time for them to translocate a nanopore. However, without a reference molecule like a DNA that doesn't change its shape in a pore, the BSA excluded volume in the pore can't be estimated precisely, therefore we can't determine if the BSA molecules were in linear or looped configuration in a pore. The inter-event histogram (Fig. 4f) shows that the current drop frequency increased as the applied voltage increased from 60 mV to 120 mV, consistent with earlier report by Cressiot et al. [27].

3.3. Voltage dependence of BSA in 1M KCl without denaturant

Recent measurements with solid-state nanopores have shown that the electric field in a nanopore can change protein structure: folded protein molecules could be partially denatured and partially folded proteins could be further stretched. Using β-lactoglobulin, our previous experiment demonstrated that a fraction of β-lactoglobulin seemed partially denatured without any denaturing agent [9]. Freedman et al. had shown that the electric field in a nanopore did denature folded BSA [25]. Oukhaled et al. [26] and Cressiot et al. [27] had demonstrated that unfolded protein molecules could be further stretched in nanopores with increased applied voltage. Here we show an example of the electric field effects to folded BSA and double stranded DNA molecules by nanopore experiment and computer simulation.

Results—To probe if the strong electric field in a nanopore, $E \approx \Psi / H_{eff} = 10^5$V/cm, will change the conformation of a very stable protein like BSA in folded state, we measured voltage dependence of BSA in 1M KCl solution without any denaturing agent. Figure 5a shows that when the applied voltage Ψ was increased from, 60 mV, to 120 mV and 240 mV, the time duration t_d was slightly increased (Fig. 5b, open diamond ◇). The same trend was also observed when a set of native proteins (NativeMark from Invitrogen, contains IgM, Apoferritin, Lactate Dehydrogenase, BSA, etc.) were measured with the same nanopore (data not shown). If the BSA molecules were rigid charged particles as shown in Figure 1d, the translocation time t_d would be expected to follow Eq. 2, therefore t_d would be shorter as increased. To aid visualization, by assuming BSA molecules are rigid charged particles (with position profile shown in Fig. 2) driven through a nanopore by voltage Ψ, but simultaneously moves randomly under thermal motion, we have simulated BSA translocation times (Fig. 5b, open squares □). The simulated BSA distributions (Fig. 5c) show that the particle like BSA translocation times decreased as voltage Ψ increased. To further validate the computer simulation program (see details in Appendix), a rigid uniformly charged 7k dsDNA translocation time was also simulated and the results are compared with the data measured in a nanopore (Fig. 5 def). For both folded BSA and uniformly charged DNA molecules, as voltage Ψ increases, the time durations simulated decrease and the time distributions are Gaussians (Fig. 5d and 5f).

Discussion—The study in Figure 5 suggests that the highly charged native state BSA molecules can't be treated as rigid particles during a nanopore translocation experiment. Highly charged protein molecules could be partially denatured upon entering a nanopore at the voltages used, consistent with previous observation using β-lactoglobulin [9] and more recent report using BSA [25]. This assumption is reasonable considering that some proteins unfold at pulling forces larger than ~5 pN [43, 44]. The electrical field strength in a nanopore is $E \approx \psi / H_{eff} = 10^5 V/cm$. The electric force experienced per charge is estimated to be $eE \approx 1.6$ pN/*e*. A BSA molecule has about 100 negative and positive residues each at pH 7.0. When a BSA molecule is entering a nanopore, opposite charges in the protein will be driven in opposite directions by the electric field (see Fig. 2b.) and this electric force is estimated to be much larger than the strength of the hydrogen bonds and will reduce the attractive electric force between these charges that hold a protein in a folded shape, thus a BSA could be partially denatured. In addition, the heat produced by current flow in a pore may also contribute to protein denaturing. This electric field effect is not an advantage for the purpose of protein identification; it could cause a previous unfolded protein entering a nanopore with a charged region, not one end of a peptide chain, increase the chance of looped protein translocation configuration.

Conclusion

From a very limited number of reports on studying protein unfolding with solid-state nanopores, we conclude that a solid-state nanopore device is capable of characterizing the process of protein unfolding at single molecule level by measuring a protein's relative current drop amplitude that is directly proportional to the protein's excluded volume in a nanopore. A protein in a folded state occupies a larger excluded volume in a nanopore therefore blocks more ionic current flow than the protein in unfolded or random coil state. The technique of solid-state nanopore is not only sensitive enough to distinguish the folding state of a protein; it also gives the distribution of possible states.

The disadvantage of this method is that the electric field used to drive a protein molecule through a nanopore and to produce an ionic current signal can change protein structure, partially unfold a native state protein or stretch an unfolded peptide chain. When an unfolded protein molecule enters a nanopore, due to its heterogeneous charge distribution, a negatively or positively charged region may enter first depending on the voltage polarity, which could cause looped or multiple peptide chain translocation configurations. This looped translocation configuration will not affect the determination of a protein's folding state, however, this will be a key problem for future applications such as identifying proteins by their linear amino acid chain translocation signal in a nanopore.

Perspective of studying protein unfolding by solid-state nanopores—Characterization of protein unfolding using solid-state nanopores is a new research field that only started several years ago and is still at its initial developing stage. To progress further in this field, experimental and theoretical studies are needed to understand how the translocation dynamics and configurations of unfolded protein molecules in a nanopore are related to protein structure, nanopore geometry, and pore-protein interactions.

Acknowledgments

The authors thank the funding support of this research provided by NIH R21HG003290, NIH R21HG004776, and ABI0710. The authors also thank Professor Jene Golovchenko and the Harvard nanopore group for their assistance on nanopore fabrication.

References

1. Dobson CM. Protein folding and misfolding. Nature. 2003; 426:884–890. [PubMed: 14685248]
2. Eftink MR. The Use of Fluorescence Methods to Monitor Unfolding Transitions in Proteins. Biophys. J. 1994; 66:482–501. [PubMed: 8161701]
3. Meinhold DW, Wright PE. Measurement of protein unfolding/refolding kinetics and structural characterization of hidden intermediates by NMR realaxation dispersion. PNAS. 2011 May 11.
4. Daggett V, Levitt M. Protein Unfolding Pathways Explored through Molecular Dynamics Simulations. J. Mol. Biol. 1993; (232):600–619. [PubMed: 7688428]
5. Oukhaled G, Mathe J, Biance A-L, Bacri L, Betton J-M, Lairez D, Pelta J, Auvray L. Unfolding of Proteins and Long Transient Conformations Detected by Single Nanopore Recording. Phys. Rev. Lett. 2007; 98
6. Stefureac R, Waldner L, Howard P, Lee JS. Nanopore Analysis of a Small 86-Residue Protein. Small. 2008; 4(1):59–63. [PubMed: 18058890]
7. Merstorf C, Cressiot B, Pastoriza-Gallego M, Oukhaled A, Betton JM, Auvray L, Pelta J. Wild Type, Mutant Protein Unfolding and Phase Transition Detected by Single-Nanopore Recording. ACS Chem. Biol. 2012; 7(4):652–658. [PubMed: 22260417]
8. Payet L, Martinho M, Pastoriza-Gallego M, Betton J-M, Auvray L, Pelta J, Mathé J. Thermal Unfolding of Proteins Probed at the Single Molecule Level Using Nanopores. Anal. Chem. 2012; 84(9):4071–4076. [PubMed: 22486207]
9. Talaga D, Li J. Single-molecule protein unfolding in solid state nanopores. J. Am. Chem. Soc. 2009; 131:9287–9297. [PubMed: 19530678]
10. Ledden, B.; Fologea, D.; Talaga, DS.; Li, J. Sensing Single Protein Molecules with Solid-state Nanopores, in Nanopores: Sensing and Fundamental Biological Interactions. In: Iqbal, SM.; Bashir, R., editors. New York: Springer; 2011. p. 129-150.
11. Sutherland TC, Long Y-T, Stefureac R-I, Bediako-Amoa I, Kraatz H-B, Lee JS. Structure of Peptides Investigated by Nanopore Analysis. Nano Lett. 2004; 4(7):1273–1277.
12. Movileanu L, Schmittschmitt JP, Scholtz JM, Bayley H. Interactions of peptides with a protein pore. Biophys. J. 2005 Aug.89:1030–1045. [PubMed: 15923222]
13. Stefureac R, Long Y, Kraatz H, Howard P, Lee J. Transport of alpha-helical peptides through alpha-hemolysin and aerolysin pores. Biochemistry. 2006; 45(30):9172–9179. [PubMed: 16866363]
14. Pastoriza-Gallego GGM, Thiebot B, Betton J-M, Pelta J. Polyelectrolyte and unfolded protein pore entrance depends on the pore geometry. Biochimica et Biophysica Acta - Biomembranes. 2009; 1788:1377–1386.
15. Pastoriza-Gallego M, Rabah L, Gibrat G, Thiebot B, Goot FGvd, Auvray L, Betton J-M, Pelta J. 1.Dynamics of Unfolded Protein Transport through an Aerolysin Pore. J. AM. CHEM. SOC. 2011; 133(9):2923–2931. [PubMed: 21319816]
16. Fologea D, Ledden B, McNabb DS, Li J. Electrical Characterization of Protein Molecules in a Solid-State Nanopore. Appl. Phys. Lett. 2007; 91
17. Han A, Creus M, Schurmann G, Linder V, Ward TR, Rooij NFd, Staufer U. Label-Free Detection of Single Protein Molecules and Protein-Protein Interactions Using Synthetic Nanopores. Anal. Chem. 2008; 80:4651–4658. [PubMed: 18470996]
18. Han A, Schurmann G, Mondin G, Bitterli RA, Hegelbach NG, de Rooij NF, Staufer U. Sensing protein molecules using nanofabricated pores. Appl. Phys. Lett. 2006; 88:093901–093903.
19. Firnkes M, Pedone D, Knezevic J, Döblinger M, Rant U. Electrically Facilitated Translocations of Proteins through Silicon Nitride Nanopores: Conjoint and Competitive Action of Diffusion, Electrophoresis, and Electroosmosis. Nano Lett. 2010; 10(6):2162–2167. [PubMed: 20438117]

20. Stefureac RI, Trivedi D, Marziali A, Lee JS. Evidence that small proteins translocate through silicon nitride pores in a folded conformation. j. Phys. Condens. Matter. 2010; 17(22):454133. [PubMed: 21339619]
21. Niedzwiecki DJ, Grazul J, Movileanu L. Single-Molecule Observation of Protein Adsorption onto an Inorganic Surface. Journal of the American Chemical Society. 2010; 132(31):10816–10822. [PubMed: 20681715]
22. Yusko EC, Johnson JM, Majd S, Prangkio P, Rollings RC, Li J, Yang J, Mayer M. Controlling the translocation of proteins through nanopores with bioinspired fluid walls. Nature Nanotechnology. 2011
23. Yusko EC, Prangkio P, Sept D, Rollings RC, Li J, Mayer M. Single-Particle Characterization of Aβ Oligomers in Solution. ACS nano. 2012; 6(7):5909–5919. [PubMed: 22686709]
24. Sexton LT, Mukaibo H, Katira P, Hess H, Sherrill SA, Horne LP, Martin CR. An Adsorption-Based Model for Pulse Duration in Resistive-Pulse Protein Sensing. J Am Chem Soc. 2010; 132:6755–6763. [PubMed: 20411939]
25. Freedman KJ, Jürgens M, Prabhu A, Ahn CW, Jemth P, Edel JB, Kim MJ. Chemical, Thermal, and Electric Field Induced Unfolding of Single Protein Molecules Studied Using Nanopores. Anal. Chem. 2011; 83:5137–5144. [PubMed: 21598904]
26. Oukhaled A, Cressiot B, Bacri L, Pastoriza-Gallego M, Betton J-M, Bourhis E, Jede R, Gierak J, Auvray L, Pelta J. Dynamics of Completely Unfolded and Native Proteins through Solid-State Nanopores as a Function of Electric Driving Force. Acs nano. 2011; 5:3628–3638. [PubMed: 21476590]
27. Cressiot B, Oukhaled A, Patriarche G, Pastoriza-Gallego M, Betton J, Auvray L, Muthukumar M, Bacri L, Pelta J. Protein transport through a narrow solid-state nanopore at high voltage: experiments and theory. Acs nano. 2012; 6(7):6236–6243. [PubMed: 22670559]
28. Cai Q, Ledden B, Krueger E, Golovchenko JA, Li J. Nanopore Sculpting with Noble Gas Ions. J. Appl. Phys. 2006; 100
29. Li J, Stein D, McMullan C, Branton D, Aziz MJ, Golovchenko JA. Ion-beam sculpting at nanometre length scales. Nature. 2001 Jul; 412(12):166–169. [PubMed: 11449268]
30. Stein D, Li J, Golovchenko JA. Ion-Beam Sculpting Time Scales. Phys. Rev. Lett. 2002; 89(27): 276106-1-4. [PubMed: 12513225]
31. Bezrukov SM. Ion Channels as Molecular Coulter Counters to Probe Metabolite Transport. J. Membr. Biol. 2000; 174:1–13. [PubMed: 10741427]
32. DeBlois RW, Bean CP. Counting and Sizing of Submicron Particles by the Resistive Pulse Technique. Rev. Sci. Instrum. 1970; 41(7):909–916.
33. Gregg EC, Steidley KD. Electrical Counting and Sizing of Mammalian Cells in Suspension. Biophys. J. 1965; 5:393–405. [PubMed: 5861698]
34. Henriquez RR, Ito T, Sun L, Crooks RM. The Resurgence of Coulter Counting as a Nanoscale Analytical Method. Analyst. 2004; 129:478–482. [PubMed: 15222315]
35. Hall JE. Access Resistance of a Small Circular Pore. J.Gen. Physiol. 1975; 66:531–532. [PubMed: 1181379]
36. King GM, Golovchenko JA. Probing Nanotube-Nanopore Interactions. Phys. Rev. Lett. 2005; 95(21)
37. Hyun C, Rollings R, Li J. Probing Access Resistance of Solid-State Nanopores with a Scanning-Probe Microscope Tip. small. 2012; 8(3):385–392. [PubMed: 22393313]
38. Peters TJ. Serum Albumin. Adv. Protein Chem. 1985; 37:161–245. [PubMed: 3904348]
39. Sirimulla S, Lerma M, Herndon WC. Prediction of Partial Molar Volumes of Amino Acids and Small Peptides: Counting Atoms versus Topological Indices. J. Chem. Inf. Model. 2010; 50:194–204. [PubMed: 20058884]
40. Zamyatnin AA. AMINO ACID, PEPTIDE, AND PROTEIN VOLUME IN SOLUTION. Ann. Rev. Biophys. Bioeng. 1984; 13:145–165. [PubMed: 6378067]
41. Li J, Talaga DS. The distribution of DNA translocation times in solid-state nanopores. J. Phys. Condens. Matter. 2010; 22 p. 454129 (8pp).

42. Rocco AG, Mollica L, Ricchiuto P, Baptista AM, Gianazza E, Eberini I. Characterization of the Protein Unfolding Process Induced by Urea and Temperature. Biophys. J. 2008; 94:2241–2251. [PubMed: 18065481]

43. Lellermayer MSZ, Smith SB, Granzer HL, Bustamante C. Folding-Unfolding Transitions in Single Titin Molecules Characterized with Laser Tweezers. Science. 1997; 276:1112. [PubMed: 9148805]

44. Bechtluft P, Leeuwen RGHv, Tyeman M, Tomkiewicz D, Nouwen N, Tepper HL, Driessen AJM, Tans SJ. Direct Observation of Chaperone-Induced Changes in a Protein Folding Pathway. Science. 2007 Nov; 318(30):1458–1461. [PubMed: 18048690]

Appendix

Translocation time simulation

To better understand protein translocation time distribution, we have simulated t_d in figure 5. Below is a brief description of the computer simulation. We model a BSA molecule as rigid with charge distribution shown as in Figure 1d. When this BSA molecule is pulled through a nanopore by the electric field in a nanopore, the BSA molecule also moves randomly due to Brownian motion along the centerline of the pore. The motion of the BSA molecule can be modeled by a 1-D Langevin equation:

$$m\frac{dv}{dt}=F_e(x) - fv+\kappa W(t)$$

Where ν is the speed of the molecule, $F_e(x)=Eq(x)$ is the electric force, $q(x)=\sum Q_{in}$ is the total charge of a BSA in the pore, f is the drag coefficient, related to the diffusion coefficient by $f= k_bT/D$, κ is defined by the fluctuation-dissipation theorem, and W(t) is a 'noise term' or Wiener process with zero mean that represents the random thermal forces on the molecule. The variable × is the position of the first part of the molecule that enters the pore.

To calculate time durations t_d, we simulated ensembles of hundreds of molecules travelling through the pore using the Euler-Murayama numerical methods to solve the one dimensional Langevin equation, which includes the Wiener process term, ΔW, which adds a normally distributed random number with mean zero for each timestep Δt. The change in speed is calculated by

$$v(i+1)=v(i)+\left(\frac{q(x_i)E}{m}-\frac{\alpha}{m}v(i)\right)\Delta t+\frac{\sqrt{2\alpha k_BT}}{m}\Delta t\Delta W$$

And the change in position is calculated by

$$\mathrm{x(i+1)=x(i)}+v(\mathrm{i})\Delta t$$

The translocation time t_d is calculated by adding all the timesteps it takes for a BSA or DNA molecule to pass through the nanopore.

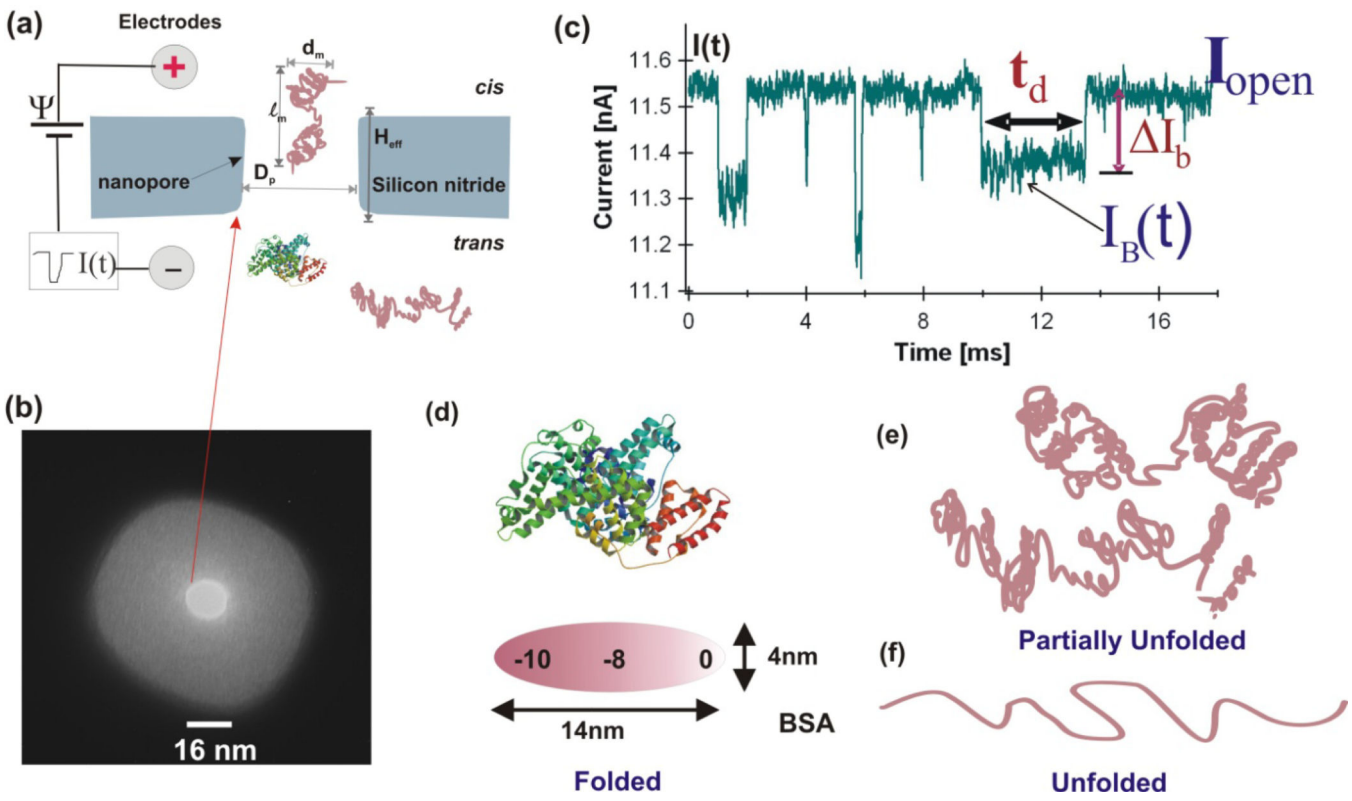

Figure 1.

(a) Schematic diagram of a nanopore experiment setup. (b) A TEM image of a ~16 nm pore used for BSA measurement. (c) Several recorded BSA current blockage events in partially denatured condition (SDS+DTT+45 °C at pH 7 and 1M KCl measured with the nanopore shown in (b). (d) Illustration of one of the possible conformations of a BSA protein at native state (PDB, 3v03). (e) Possible partially denatured form of BSA. (f) completely unfolded form of a BSA molecule.

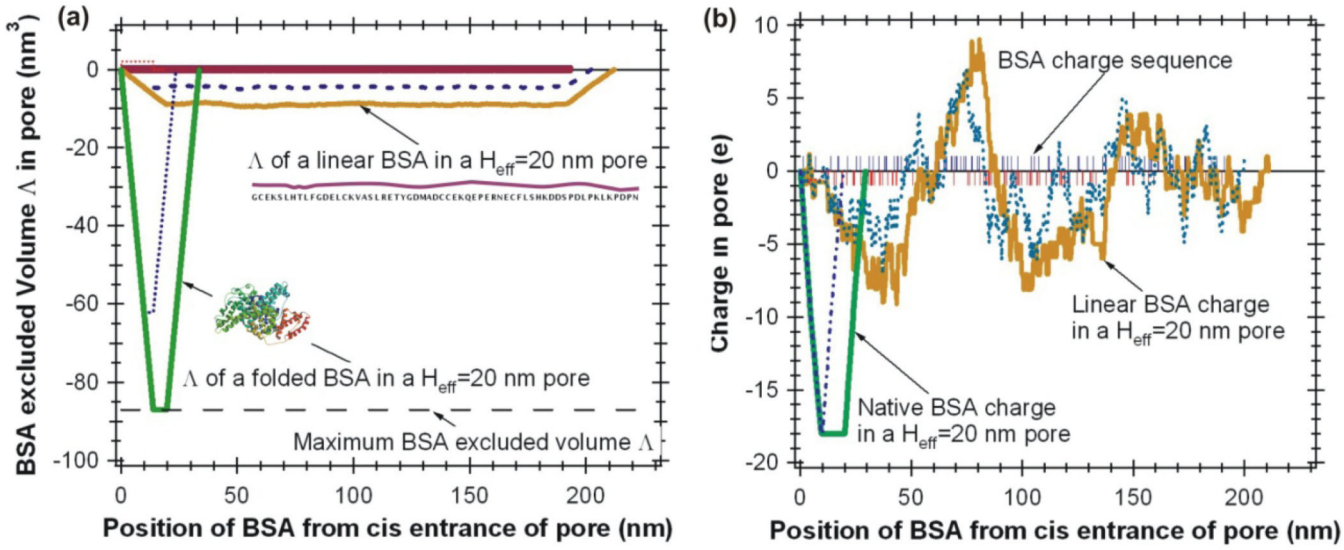

Figure 2.

(a) Predicted excluded volume of BSA in a nanopore at folded globular state and unfolded linear shape in a H_{eff}=20 nm pore (solid lines) and H_{eff}=10 nm pore (dotted lines). (b) Predicted electrical charge of a BSA in a nanopore at folded globular state and unfolded linear shape (without SDS) in a H_{eff}=20 nm pore (solid lines) and H_{eff}=10 nm pore (dotted lines). The BSA protein sequence was obtained from: http://www.rcsb.org/pdb/explore/remediatedSequence.do?structureId=3V03.

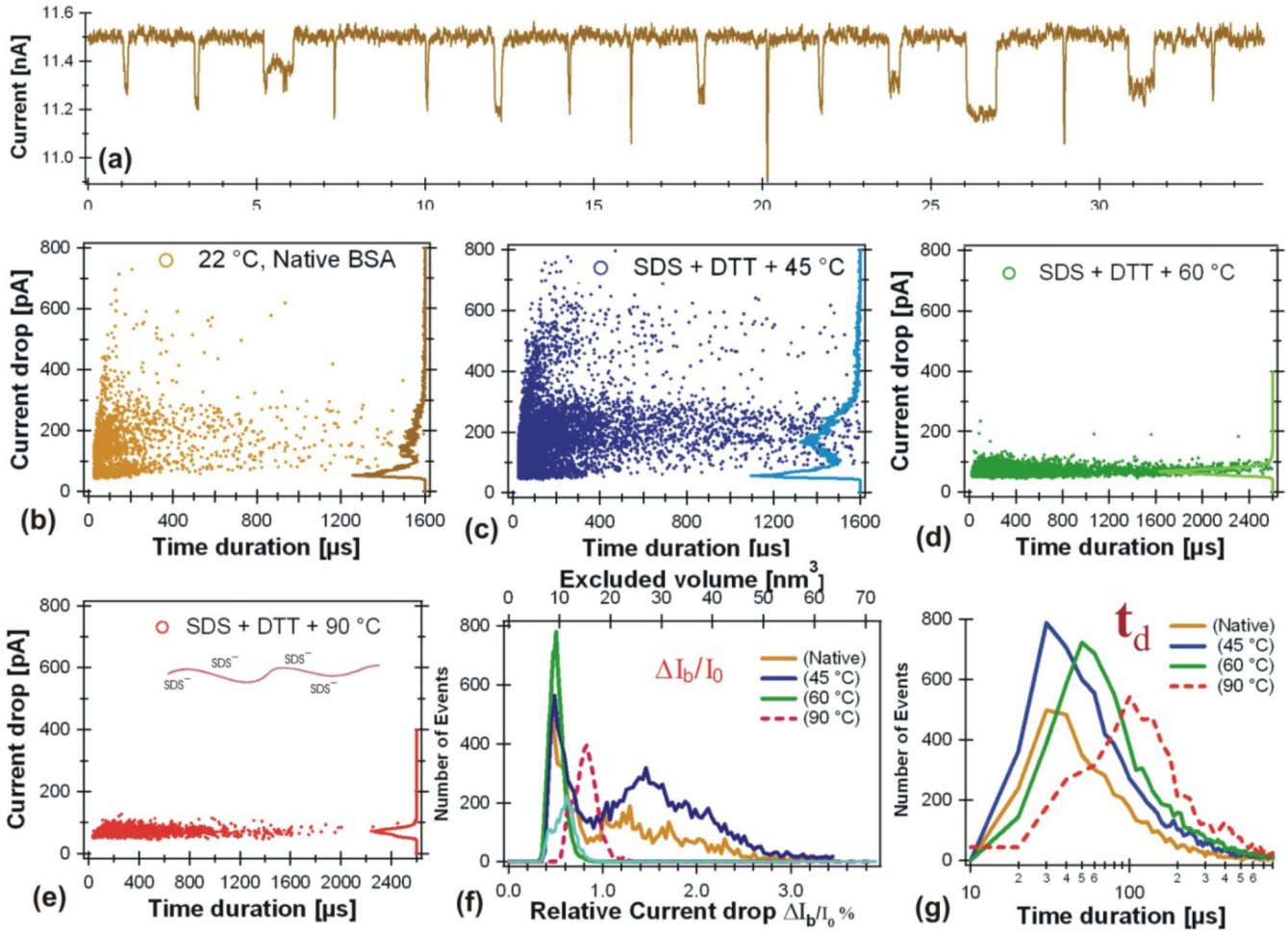

Figure 3.

BSA unfolding states measured in a ~16 nm pore (Fig. 1b) in 1M KCl at pH 7. (a) Current drop events observed for BSA in 1M KCl at pH 7 with no denaturant. The stock BSA sample contains 1.4 mg SDS (4.85 mM) and 2mM DTT with 1 mg BSA (15μM) in 1ml 1M KCl solution were kept for 5 minutes at the higher temperature indicated in the figures, than immediately cooled down in a water bath kept at room temperature. The BSA protein was then added to the cis chamber. The bias voltage was Ψ=120 mV. The open pore current was ΔI_0=11.5 (b), 11.5 (c), 13.4 (d), and 8.7 (e) nA respectively. (f) Number of events distributions vs $\Delta I_b/I_0$ for data shown in panels b, c, e, and d. (g) Number of events distributions vs t_d for data shown in panels b, c, e, and d. The final concentration of the BSA protein in the cis chamber was about 12 nM for all the measurements. The final concentrations of SDS and DTT were 3.9 μM and 1.6 μM respectively.

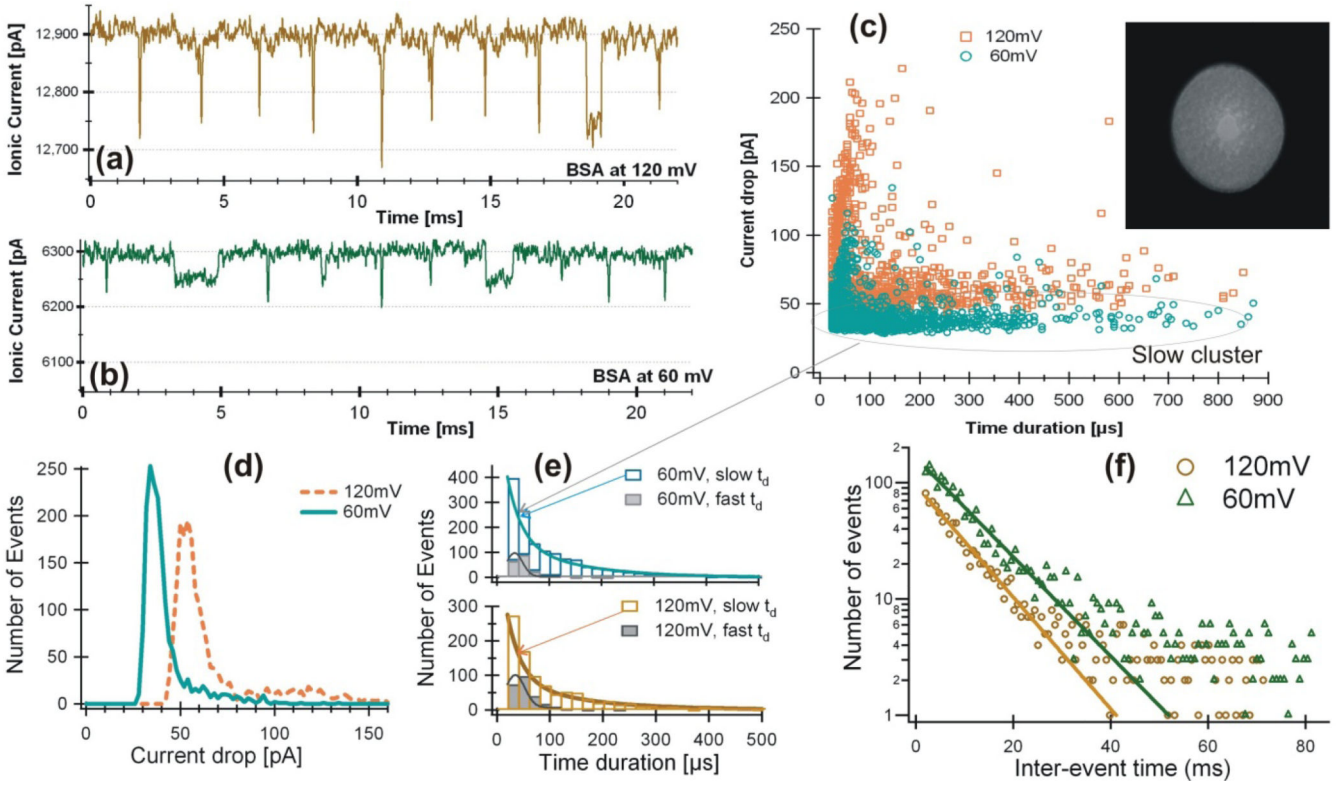

Figure 4.

BSA protein in 6M Guanidine Hydrochloride solution measured by a pore of D_p=18±2 nm. Examples of current blockage events at 120 mV (a, I_0=12.9 nA), 60 mV (b, I_0=6.3 nA), and ΔI_b vs I_0 (c). The nanopore used for this experiment is shown in the insert of (c). Histogram distributions are shown for current blockage ΔI_b (d), time duration t_d (e), and the interval between events (f).

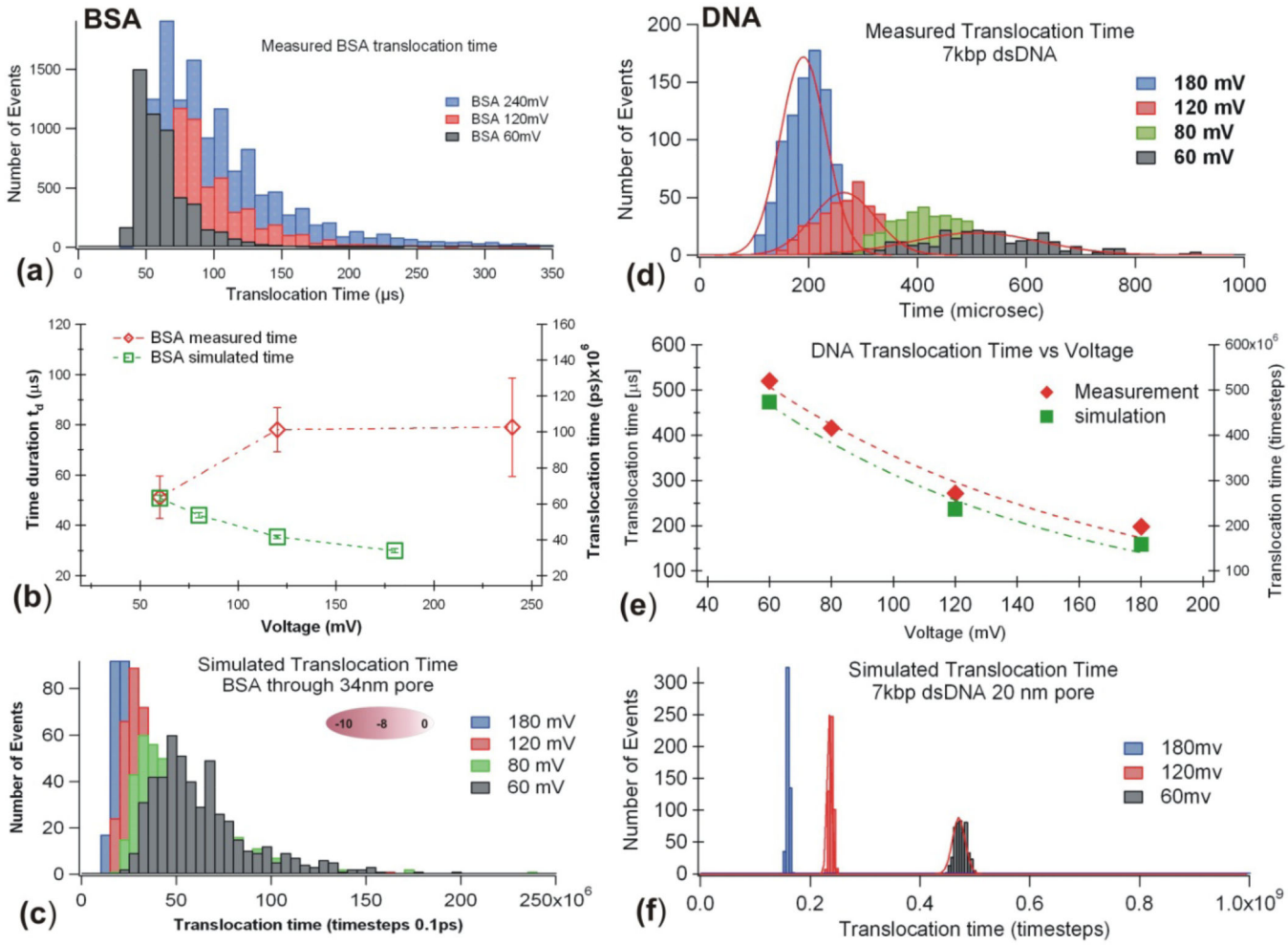

Figure 5.

For the native state BSA, I_0=13.2 nA in 1M KCl at 120 mV during the measurement. Voltage dependence of the time duration distributions measured by the nanopore (a), the most probable values of t_d as a function of voltages (b, ◇), Computer simulated time histograms vs voltage (c). For the 7k dsDNA, I_0=11.5±1.5 nA in 1M KCl at 120 mV during the experiment. Voltage dependence of the time duration distributions measured by the nanopore (d), the most probable values of t_d as a function of voltages (e), Computer simulated time durations vs voltage (f). Error bars were the standard deviation from Gaussian fit to the data.