Unfolding of Proteins and Long Transient Conformations Detected by Single Nanopore Recording

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(Received 7 September 2006; published 9 April 2007)

We study the electrophoretic blockades due to entries of partially unfolded proteins into a nanopore as a function of the concentration of the denaturing agent. Short and long pore blockades are observed by electrical detection. Short blockades are due to the passage of completely unfolded proteins, their frequency increases as the concentration of the denaturing agent increases, following a sigmoidal denaturation curve. Long blockades reveal partially folded conformations. Their duration increases as the proteins are more folded. The observation of a Vogel-Fulcher law suggests a glassy behavior.

DOI: 10.1103/PhysRevLett.98.158101 PACS numbers: 87.15.Cc, 82.37.−j, 87.14.Ee

The folding of an amino-acid chain into a functioning protein is a crucial process in biology [1]. The understanding of this mechanism remains a challenge. In most biological systems, protein folding takes place as nascent protein is expelled progressively out of the channel of ribosome. Several years ago, Simon and Blobel [2] demonstrated the existence of transmembrane protein conducting channels by electrophysiological methods. Later, the transport of small peptides has also been directly observed through the mitochondrial cationic channel [3]. These experiments suggested to us the idea of studying the folding of proteins through passive pores of nanometer size and, in a first step, the artificial translocation of partially folded proteins in the presence of a denaturing agent. We use as a passive pore, the α-hemolysin [4] pore of *Staphylococcus aureus*, a very convenient protein widely used in studies of polymer and DNA translocation [5–13]. It offers the advantage of being resistant to denaturation [14] and the diameter of its internal channel (equal to 2 nm) is large enough to allow the passage of unfolded proteins, while forbidding the translocation of a folded one. Two experiments probing the transport of different oligopeptides through α-hemolysin have been published recently [15,16]. The first one performed on collagenlike peptides shows that the transport is sensitive to association in double or triple helix, the second one shows strong interaction effects between cationic α-helical peptides and the pore. Probing the dynamics of peptides through small channels is also interesting because the studies of the kinetics of translocation at realistic time scales is still out of reach of numerical simulations although a first attempt has been made recently for the archaeal Sec YEβ translocon [17].

The recent renewal of interest for translocation of macromolecular chains through protein pores [5,6] is based on the observation of complete passage of individual single-stranded DNA molecules through a single protein channel in a planer lipid bilayer performed in 1996 [7]. Several teams in the world explored the numerous applications of this technique [8–10,16,18]. Recent developments include the transformation of nanopores into manipulation tools by techniques of active control [11–13] and search for artificial channels [19–21].

Many efforts are devoted to the understanding of translocation and folding of proteins [22–26]. Most of the protein folding experiments have been performed in bulk and can only probe the average behavior of an ensemble of molecules. Recently developments in single molecule methods, fluorescence spectroscopy and force spectroscopy, allow observing individual molecules along their folding pathway [24,25]. Simulations of the unfolding of short proteins in a nanopore have been recently reported in [26], showing that the unfolding by nanopore differs from the unfolding by pulling probed in optical tweezers experiments [25]. *In vivo* the mechanisms of translocation and folding are active and regulated. Here we restrict ourselves to a much simpler and particular problem, the passive transport of a protein under the conditions of denaturation and strong confinement. We choose the recombinant maltose binding protein [27] of *Escherichia coli* (MBP) because this protein has been extensively used as a model of protein folding and protein translocation [28], and does not contain disulfide bonds. This protein is a large two domain periplasmic protein with 370 residues ($M_r = 40\,707$ Da). Its structure is ellipsoidal with overall dimensions of $30 \times 40 \times 65$ Å^3 [29]. The protein is negatively charged ($Z = -8$) at physiological pH. The denaturation of MBP by guanidium chloride (Gdm-HCl) has been studied in bulk [27,30]. The transition takes place for [Gdm-HCl] = $0.8M$.

MBP denaturation experiments have been performed by varying [Gdm-HCl] between 0 and $1.35M$. We checked the integrity of α-hemolysin pore between 0 and

$2M$ Gdm-HCl by observing the blockades due to short poly(ethyleneglycol) chains ($M_w = 3400$ Da). Blockades duration and frequency were identical whatever [Gdm-HCl]. Experiments were carried at room temperature by incubating 0.35 μM of recombinant MBP [27] in appropriate final concentrations of Gdm-HCl (Sigma) in buffer (5 mM HEPES, pH = 7.5) with 1M KCl. Bilayers lipid membranes are made by using the following method [7]: a film of a 1% solution of diphytanoylphosphatidylcholine-Lecithine (Avanti) in decane is spread across a 150 μm wide hole drilled in a polysulfone wall separating the two compartments of a chamber. Each compartment contains 1 ml of 1M KCl, 5 mM HEPES (at pH = 7.5), 0 to 1.35M Gdm-HCl. After thinning of the decane film and formation of a planar bilayer, the channels are inserted by adding 0.15 nanomole of monomeric α-hemolysin (Sigma) from a mother solution in one compartment. The ionic current through a α-hemolysin channel is measured with a Axopatch 200B amplifier. Data are filtered at 10 kHz and acquired at 10 μs intervals with the DigiData 1322A digitizer coupled with the Clampex software (Axon Instruments, Union City, U.S.A.). Measurements of events frequency and pulse duration are based on the statistical analysis of the current traces. Data were systematically checked for reproducibility.

Native MBP proteins do not enter the α-hemolysin pore [Fig. 1(a)]. No events are observed during the time of the experiments varying between 10 mn and 1 h. On the contrary many current blockades are visible when the same experiment is performed in presence of Gdm-HCl at 1.35M concentration [Fig. 1(b)]. At this Gdm-HCl concentration, MBP is completely unfolded [27,30]. We checked that the frequency of events was proportional to the protein concentration in the range 0.1–1.5 μM [Fig. 1(f)]. The mean dwelling time τ is independent of protein concentration, $\tau = 0.2 \pm 0.1$ ms at 100 mV. We observed by varying the applied voltage V that the events frequency f varies exponentially as a function of V, $f \propto \exp(V/V_0)$ with $V_0 = 42 \pm 2$ mV [Fig. 1(g)]. No blockade is observed below 50 mV as reported for polynucleotides in Refs. [9,31]. Theoretically V_0 is equal to k_BT/q, q being the charge of the portion of the protein in the pore and k_BT the thermal energy. The experimental value of q is 0.6 electronic charge. This corresponds on average to the net charge of 28 amino acids.

We studied the pore electrical activity due to MBP as a function of the concentration of denaturing agent. At $V =$ 100 mV, blockade events appear when [Gdm-HCl] reaches 0.8M [Fig. 2(a)]. Blockades are very rare at 0.8M but their frequency increases very rapidly as [Gdm-HCl] increases slightly [Fig. 2(b) and 2(c)]. In this zone of transition, long blockades alternate with series of short one. This intermittent behavior disappears when [Gdm-HCl] is higher than 1M [Fig. 2(d)]. The variation of the mean frequency of short blockades as a function of concentration of denaturing agent is a sigmoid curve quite similar to a classical denaturation curve obtained by measuring circular dichroism or tryptophane fluorescence of bulk solutions [27,30] [Fig. 3(a)]. By observing the current traces and analysing the statistical distribution of the blockades duration [Fig. 3(b)] we identify at least two characteristic times, a short one of the same order of magnitude as the transit time of one fully denatured chain (0.2 ms) and a long one that depends strongly on the concentration of denaturing agent (100 ± 25 s at [Gdm-HCl] = 0.85M, 0.5 ± 0.1 s at [Gdm-HCl] = 0.875M, 40 ± 10 ms at [Gdm-HCl] = 0.9M, 0.5 ± 0.1 ms at [Gdm-HCl] = 1M. A few very long time blockades (greater than 15 min) were also observed at [Gdm-HCl] = 0.825M but could not be measured reproducibly.

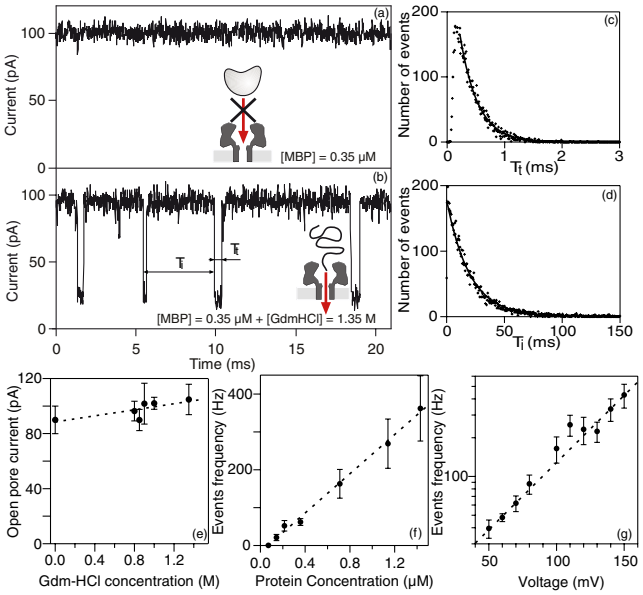

FIG. 1 (color online). Parts of current traces through α-hemolysin (at 100 mV) in the presence of MBP, examples of statistical analysis, open pore conductance *vs* [Gdm-HCl], and events frequency vs MBP and applied voltage. Left (a), without Gdm-HCl the current is constant at 100 pA, MBP cannot enter to the pore; (b) with [Gdm-HCl] = 1.35M well above the unfolding transition, the current trace decreases down to 20 pA when a MBP molecule is in the pore. The rms noise of the open pore current does not change at this [Gdm-HCl] value. Right, (c) distribution of blockades time T_f; (d) distribution of time intervals T_i between two successive pulses of current (computed over $\simeq$ 1500 events). Continuous lines are exponential fits, time constants define the average blockade duration (considering only the exponential tail of the distribution) and average intervals between events. In (a),(b),(c) and (d), [MBP] = 0.35μM. Bottom, (e) open pore current vs [Gdm-HCl]. The dashed line is a guide to the eye to highlight the slight conductance increase with [Gdm-HCl]. (f) Frequency of events vs [MBP]. The dashed line is a linear fit with a slope of 260 ± 10 Hz μM^{-1}. (g) Frequency of events *vs* applied voltage on a semilog scale. The dashed line is an exponential fit. The characteristic voltage is $V_0 = 42 \pm 2$ mV.

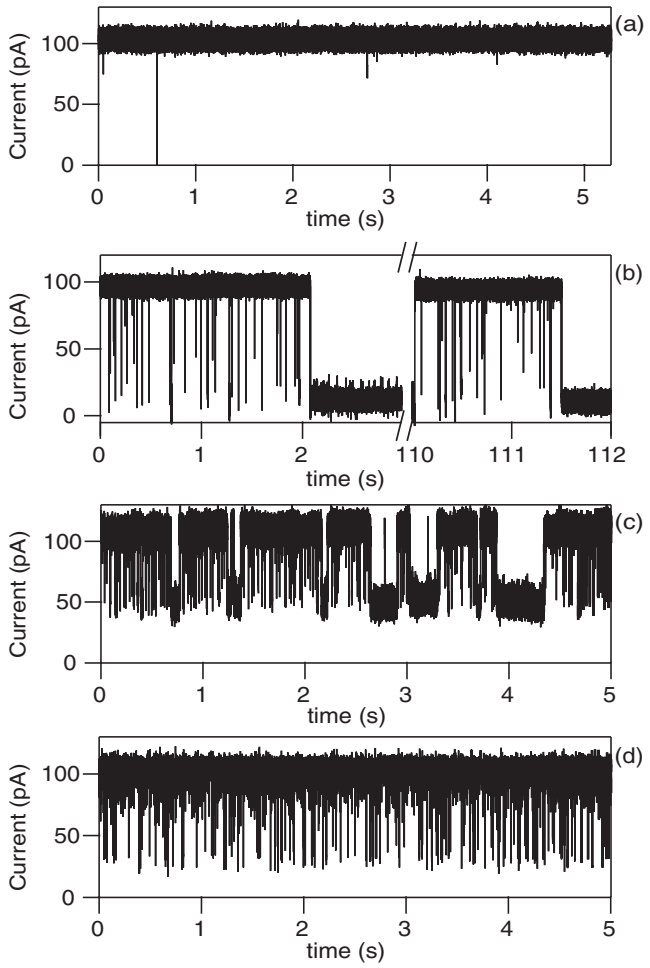

FIG. 2. Part of current traces through α-hemolysin pore *vs* [Gdm-HCl] at [MBP] = 0.35 μM. (a) at [Gdm-HCl] = 0.8M, very few short blockades are observed, their average duration is 0.2 ms. (b) at [Gdm-HCl] = 0.85M, long blockades of 100 s in average alternate with a series of short ones of 0.2 ms in average. (c) At [Gdm-HCl] = 0.9M, the frequency of the short blockades increases (of average duration 0.2 ms). The frequency of the long blockades also increases, their distribution is wide, their mean duration decreases, its value based on the statistical analysis of all events is 40 ms. (d) At [Gdm-HCl] = 1M, only short blockades (of average duration 0.5 ms) are observed, MBP molecules are almost completely unfolded. The frequency of short events does not further increase.

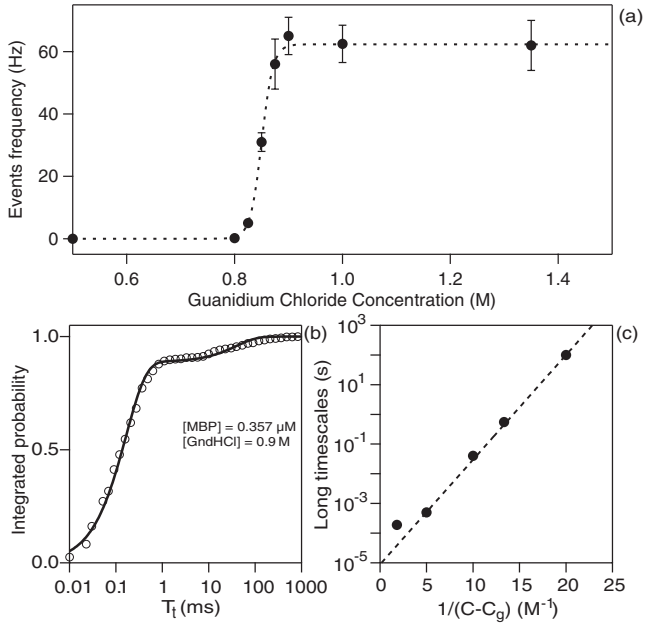

FIG. 3. The nanopore technique detects the unfolding of proteins and the existence of long transient glassy conformations. Here [MBP] = 0.35 μM. (a) Frequency of short blockades vs [Gdm-HCl]. The dashed line is a fit using a Fermi-Dirac sigmoid function. No blockade is observed below [Gdm-HCl] = 0.8M. At the unfolding transition, events frequency increases sharply with [Gdm-HCl]. (b) Integrated distribution of the blockade duration, i.e., number of blockade events with duration less than a given time t at [Gdm-HCl] = 0.9M. This time distribution is not monoexponential. The line is a fit using a two exponentials distribution. The mean duration of long time blockades is determined from this analysis when statistics is large enough (case of [Gdm-HCl] = 0.9 and 1M) or directly by averaging durations of long blockades when events are sparse (case of [Gdm-HCl] = 0.85 and 0.875M). (c) Vogel-Tammann-Fulcher representation of long blockades duration τ vs $1/(C - C_g)$, with C = [Gdm-HCl] and C_g = 0.8M. The continuous line is the best fit of the form $\tau = \tau_0 \exp(A/(C - C_g))$. The point at τ = 0.2 ms corresponds to the fully denatured state at [Gdm-HCl] = 1.35M.

These results rule out a two-state description of MBP unfolding deduced from macroscopic measurements [30]. Since native folded MBPs do not enter the pore whereas unfolded ones do, we should not observe different types of events. Because the duration of short time events is independent of [Gdm-HCl] and is equal to the dwell time of fully unfolded proteins we propose to impute the short time events to the translocation of unfolded chains. In this interpretation the frequency curve of Fig. 3(a) is a denaturation curve measured at the nanometer scale, i.e., a representation of the fraction of unfolded protein as a function of concentration of denaturing agent.

We do not observe in Fig. 2 any noticeable change of blockade levels between the short and the long pulses. We therefore deduce that the level of the current in the long time blockades is only due to the dwelling of an unfolded segment of the protein inside the pore and that a folded part outside the pore blocks the passage of the whole chain. In order to probe this hypothesis, we varied the applied potential during long events. There are two effects. The blockades stopped at low enough voltage, of order 15 mV, and at high voltage above 150 mV. This is consistent with an exit of the pore by backward diffusion at low voltage and unzipping at high voltage. The pulse duration of the long events at 100 mV is either the natural lifetime of the folded blocking part or the time needed for unzipping the folded part by the applied force.

A first explanation of long time blockades could be that unfolded parts of the proteins stick to α-hemolysin by

hydrophobic or other attractive interactions. This is, however, contradictory with the observation: blockades duration decreases as larger parts of MBP are unfolded (the shortest duration corresponds to completely unfolded MBP) (Fig. 2). This suggests that the size or strength of folded parts of proteins decreases. Protein folding is often described theoretically in terms of glass transition [32] associated to anomalous kinetics of folding and to anomalous distribution of lifetimes of metastable partially folded states. Here, being performed at thermal equilibrium, experiments are not sensitive to folding kinetics, but rather to the duration of intermediate conformations of proteins as they are submitted to external electrical and confinement forces. In several models where the variable is temperature, the distribution of lifetimes is a stretched exponential and the averaged lifetime is given by the Vogel-Fulcher law. In our case the variable is the concentration of the denaturing agent. Thus, we have compared the variations of the long blockades duration τ as a function of the denaturant concentration with the Vogel-Tamman-Fulcher law: $\tau = \tau_0 \exp(A/(C - C_g))$ with C_g the denaturant concentration at the folding transition. This law accounts for the data in the transition region over 5 orders of magnitude in time. A best fit yields to $C_g = 0.8 \pm 0.1M$; $\tau_0 = 10 \pm 1$ μs; $A = 0.8 \pm 0.1M$. This is a convincing experimental argument favoring the glassy state of folded proteins.

Nanopore recording appears to be a very suitable technique to study conformations of partially folded proteins with a good sensitivity and at a controlled spatial scale provided that the size of the pore can be varied in the future. Our experiments open the way to new studies and manipulations of proteins. In particular, it becomes possible to observe biomimetic renaturation of proteins in different biochemical environments associated with the passage from a denaturing towards a renaturing compartment. The active control of the translocation process should be used to measure interactions between proteins and chaperones or between proteins and DNA.

This work is supported by a program "ACI Nano sciences 2003" (French Ministry of Research), projects "Nanotubes and protein translocation" and "Molecular extrusion." We also thank H. Orland and T. Garel for useful discussions.

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