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Protein Unfolding Through Nanopores

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Abstract: In this mini-review we introduce and discuss a new method, at single molecule level, to study the protein folding and protein stability, with a nanopore coupled to an electric detection. Proteins unfolded or partially folded passing through one channel submitted to an electric field, in the presence of salt solution, induce different detectable blockades of ionic current. Their duration depends on protein conformation. For different studies proteins through nanopores, completely unfolded proteins induce only short current blockades. Their frequency increases as the concentration of denaturing agent or temperature increases, following a sigmoidal denaturation curve. The geometry or the net charge of the nanopores does not alter the unfolding transition, sigmoidal unfolding curve and half denaturing concentration or half temperature denaturation. A destabilized protein induces a shift of the unfolding curve towards the lower values of the denaturant agent compared to the wild type protein.Partially folded proteins exhibit very long blockades in nanopores. The blockade duration decreases when the concentration of denaturing agent increases. The variation of these blockades could be associated to a possible glassy behaviour.

Keywords: Aerolysin, α-hemolysin, first order transition, nanopore, protein denaturation, protein folding, single molecule experiment.

INTRODUCTION

Since the pioneer experiment of Kasianowicz [1] which consists in forcing the passage of ssDNA through a nanopore by an electric field, and of the direct proof of molecule translocation performed by PCR, a lot of studies have been performed with the motivation of ultra-fast DNA sequencing [2]. This year, the Oxford Nanopore Technologies Company announcement for the first result of viral genome sequencing by nanopore. This is a possible reason that some of groups working on DNA move to protein field in order to understand fundamental problems to develop future biotechnology applications.

At the beginning of the story of proteins viewed by nanopores, several groups studied the structure of peptides [3-8], showed electrical characterization of native proteins by large solid-state nanopores [9, 10], probed protein-pore interactions [11, 12] or antigen-antibody complexes formation [13, 14]. The next step will be to understand and to control the dynamics of unfolded proteins through nanopores. The protein translocation is an essential mechanism in living cells for proteins synthesis, export, degradation, during which molecules are usually transported through a protein channel in an unfolded state [15-17]. This motivation led some groups to work in this field, different kind of experiments are performed: a modulation of applied voltages [18-21], the nanopore charges surface modifications [22-28]. Theoretical and simulation studies have been also made in order to understand the physical process of protein entry and transport through nanopores [29-33]. Recently, other motivation and challenge are to understand protein folding using nanopore as biosensor [19, 34-40]. The idea is to study the transport of proteins through a single nanopore separating two different isolated chambers where the unfolding conditions will be controlled by the denaturing agent, guanidium or urea concentration, or temperature variation. We expect different translocation times depending on protein conformations, unfolded, partially folded chain. In 2007, our group showed for the first time that current traces, due to the channel blockade in the presence of protein, change as a function of unfolding level by varying guanidium concentrations [34]. The evolution of event frequency of unfolded proteins, current blockades, allows obtaining a denaturation curve at the single molecule level. Up to now few experiments have been performed by other groups to study protein unfolding. It was only shown the existence of different conformations, unfolded or partially folded proteins, depending on the concentration of chaotropic agent [36, 37]. Interestingly, the high sensitivity of the nanopore allows to discriminate the unfolding curve of the destabilized protein from the wild type protein [38]. Furthermore, the dynamics of partially folded proteins, leading to very long blockades in nanopores, could be associated to a glassy behavior [34, 38]. Recently, it was shown that the thermal denaturation curves performed at the single molecule level using nanopores and in bulk by circular dichroism CD experiments, are similar [39].

In this mini-review we present a new method of protein unfolding based on nanopore recording. First, we describe

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the concept of the experiment and some technical approaches of data processing and analysis. In the second step we treat the protein folding and stability through different nanopores and under chemical and physical denaturation. Finally we discuss recent results, the great interest for future applications, and also the potential of this emerging technique.

CONCEPT OF THE EXPERIMENT NANOPORE RECORDING

We introduce briefly the background necessary to obtain information from electric current traces (Fig. **1**). We apply a potential difference between both sides of a lipid bilayer using two Ag/AgCl electrodes (Fig. **1a**). This bilayer separates two compartments filled with an electrolyte buffer and behaves as an insulator. The insertion of one channel leads to an ionic current I0 of the empty pore (cf. base current of trace in Fig. **1b**). After the addition of proteins, we observe several current blockades (Fig. **1a**, **b**). It is important to distinguish the relevant blockades from all the fluctuations due to the noise. To detect each blockade; we use the "two-thresholds" method [41, 42]. This method allows to exclude the noise from the blockades of interest due to the proteins. Each blockade is characterized by its current (Ib) or its current variation Di=I0-Ib, by its duration Tt, called "dwell time" and by the inter-event time Ti between two successive events. If there is no correlation between two successive blockades, the inter-event time is fitted by an exponential function exp(-Ti/Tc) where Tc is a characteristic time (cf. Fig. **1d**). The event frequency f is defined as f=1/Tc. In Fig. **1d**, we show the example of the unfolded protein MalEwt. To distinguish the dwell time of the chains from other types of events, we represent each event in a scatter plot of the current variation of each event as a function of the corresponding dwell time (cf. Fig. **1e**). Several regions of interest are discriminated. The first one, characterized by low current variations (12 +/- 2.5 pA) and short dwell times (τs=45+/-20 μs), is due to the collision of the proteins to the pore entrance, which are approaching the nanopore without to be transported into it (red circle in Fig. **1e**). The second one with higher current variation (21 +/- 9 pA) and longer blockades (τl=351 +/- 20 μs) is attributed to the dwelling of the protein inside the nanopore (blue circle in Fig. **1e**). The dwell time distribution is exponential and its fit yields to a temporal decay constant τ. We discriminate both types of events described above: each is characterized by its time constant (Fig. **1c**) and its current variation (Fig. **1f**).

PROTEINS UNFOLDING USING NANOPORES

-Chemical Denaturation

We have reported in 2007 [34] the first experimental study of the MalEwt protein unfolding through a protein channel, α-hemolysin as a function of the concentration of denaturing agent, guanidium (Fig. **2**). The native protein size is larger than the pore diameter, thus the native protein does not enter inside the channel. In absence of protein, we do not observe any current blockade. In the presence of native proteins without denaturing agent, we observe very few spikes due to protein collision to the entrance of the channel. In the presence of low guanidium concentrations and proteins, the trace of the ionic current exhibits short blockades. When the guanidium concentration increases, both short and long blockades are observed and the frequency of only the short blockades increases. When the concentration of the denaturing agent is increased further, the long blockades disappear and the frequency of short events increases until a constant value. How are interpreted both types of spikes? As the mean duration of short blockades is independent of the guanidium concentration (Fig. **2c**), these blockades are due to the passage of completely unfolded proteins (Fig. **2b**). However, the duration of long blockades decreases as the concentration of denaturing agent increases (Fig. **2d**). The long blockades are associated to the existence of partially folded conformations, which block the passage of the proteins. How is interpreted the long current blockades evolution? We have shown the coexistence of partially and completely unfolded proteins for a fixed guanidium concentration. At the beginning of the denaturation process, near the native protein state, the dynamics of the long blockades is very slow. These observations drive us to suggest a possible glassy dynamics during protein unfolding transition [43-45]. Since a long time the theory of spin glasses was used to study the protein folding [43, 44, 46]. At low temperatures the glassy state exhibits the mechanical properties of a solid, but shows microscopic structural disorder. The protein can be viewed as a frustrated heterepolymer and protein folding is described in term of energy landscape theory. During the folding process, the protein explores multitudes of structures toward the low energy native structure or folded state. A nonexponential relaxation process is observed in the dynamics of protein folding [45]. The Vogel-Tamman-Fulcher law, t =t0 exp [A/(T-Tg), describes the distribution of lifetimes as a function of temperature during glass transition. At the transition temperature, the lifetime diverges. This temperature is called the Vogel-Fulcher temperature Tg. Here, we use a Vogel-Fulcher representation by using as a variable the denaturant concentration (C), guanidium, instead of temperature; t =t0 exp [A/(C-Cg)], t are the long dwell times of current blockade associated to partially folded proteins. These times are the times needed to unfold the protein structures at the pore entrance under the electric driving force. The effect of denaturant on Hydrogen bonds and solvent quality was the same as the one obtained by increasing the temperature. The Vogel-Fulcher law gives the best fit of our data. This is an experimental argument for a possible glassy behavior [43-46]. The variation of the long times as a function of the denaturing agent are well fitted by a Vogel-Tamman-Fulcher law strengthening our assumption (Fig. **2e**). These results rule out a two-state description of protein unfolding, which could have been deduced from macroscopic measurements. This protein folding is associated to a first-order transition linked to a possible glassy behavior. In order to confirm this possible behavior, it is necessary to perform other experiments with a different channel and protein, and study the distribution of unfolding rates.

In order to prove that protein unfolding transition is independent of the nanopore used, a new study has been performed with the same MalEwt protein with a different channel, aerolysin [38]. This channel does not have a vestibule domain [47]. In comparison with α-hemolysin one [48]. The aerolysin pore is essentially negatively charged (net charge of -52e) and α-hemolysin has a slightly positively charged

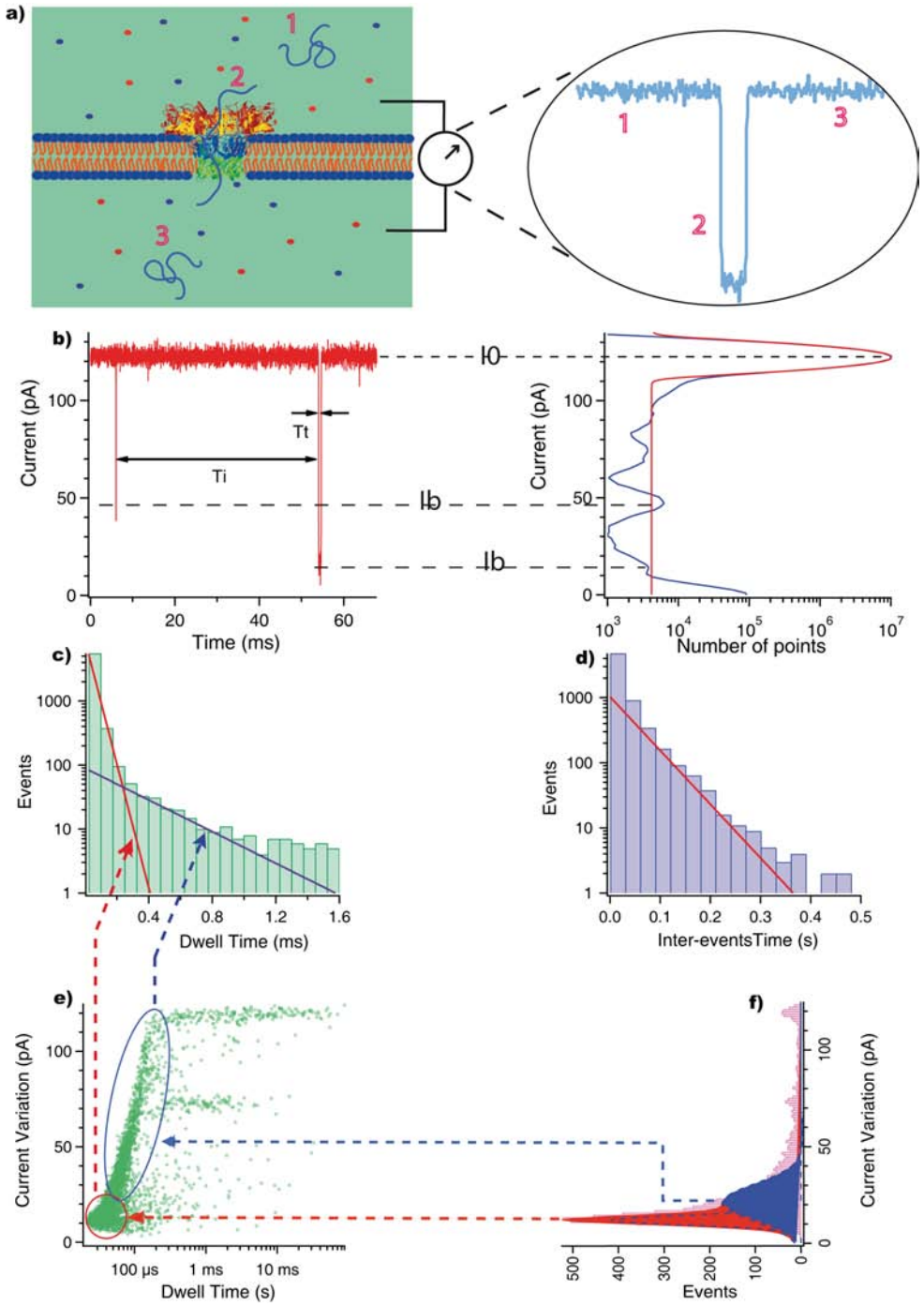

Figure 1. Relevant information from single protein dwelling: **a**) (left) Experimental set-up. One single aerolysin channel, inserted in a lipid bilayer, is submitted to an applied voltage (100mV), which induces an open pore ionic current I0. The buffer is composed by 1M KCl (K^+ ions depicted by red dots, Cl^- ions by blue dots), 5mM HEPES (pH=7.5). The protein is unfolded in the presence of guanidium chloride (1.5M) (experimental data issued from [18]). (right) The transport of an unfolded protein from one side of the membrane to the other side is represented by a three steps process: 1) the protein is located in the vicinity of the nanopore. We measure a steady ionic open pore current through the nanopore. 2) When it is dwelling through the channel, we observe a blockade of the ionic current. 3) The macromolecule is now transported into the opposite side of the membrane. The current has reached again its previously steady value. **b**) (left) Part of the ionic current trace. Ib is the current value of each blockade. The dwell time Tt is the duration of each blockade. The inter-event time Ti is the duration between two successive blockades. (right) Histogram of the current from the trace. **c**) Distribution of the dwell time of each blockade. The red and blue lines are single exponential fits corresponding to the short and long events respectively and allowing the calculation of both dwell time constants τc=45+/-20 µs and τl=351 +/- 20 µs. **d**) Histogram of the inter-events time. The red line is an exponential fit allowing the frequency calculation f= 19+/- 2 Hz. **e**) Scatter plot of the current variation of each event as a function of its duration. The red and blue circles are corresponding to the short and long events respectively. f) Distribution of current deviation of each blockade. The Gaussian fits in red and blue correspond to the short (12 +/- 2.5 pA) and long (21 +/- 9 pA) events respectively. (The color version of the figure is available in the electronic copy of the article).

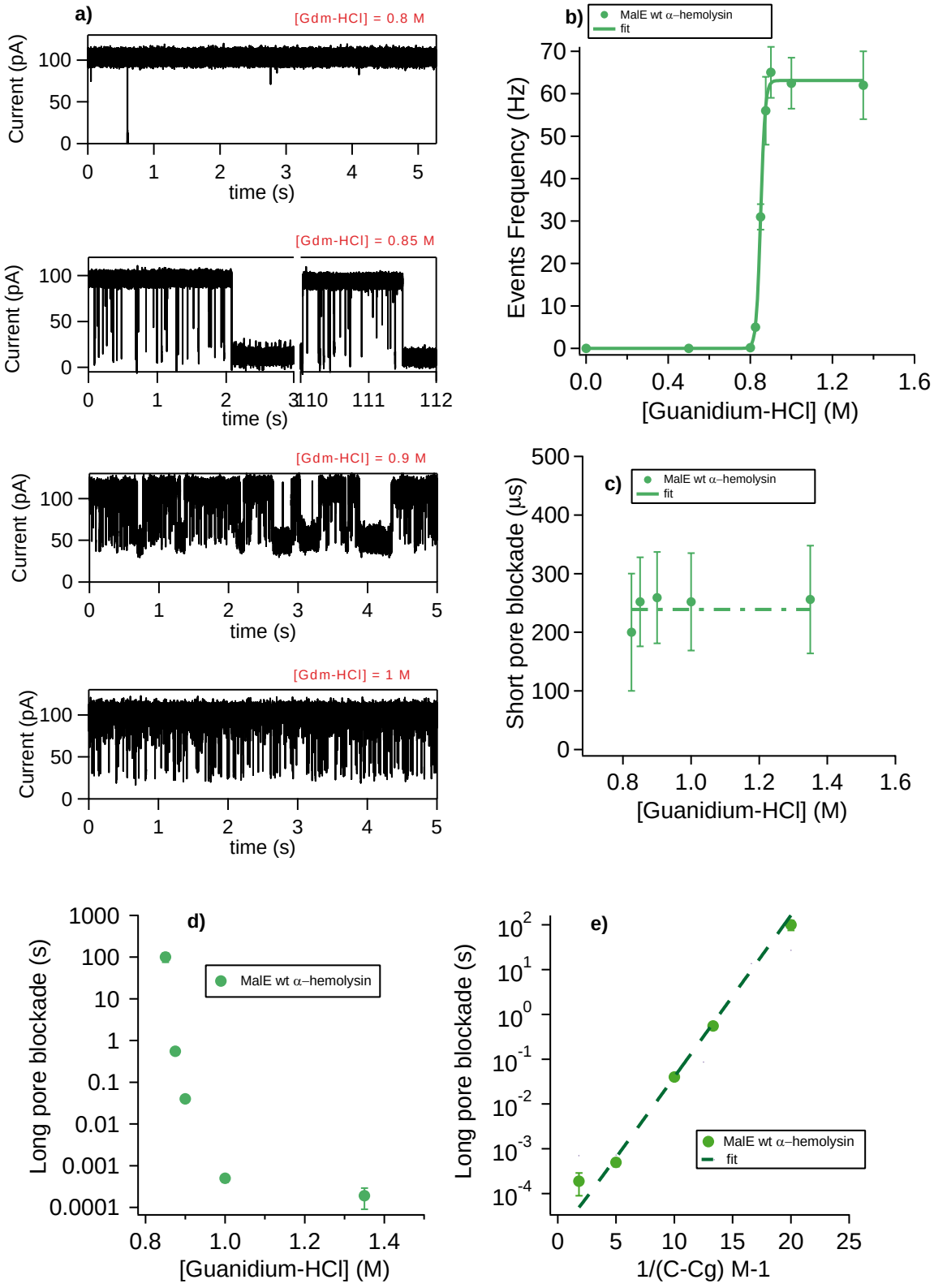

Figure 2. Chemical denaturation of the protein MalEwt through one α-hemolysin channel. (**a**) Current traces through α-hemolysin pore as a function of the concentration of Gdm-HCl in the presence of MalEwt. (**b**) The evolution of events frequency, short blockades, as a function of the concentration of Guanidium Chloride. The line is a sigmoidal fit of experimental curve. (**c**) The short (unfolded proteins) or (d) long (partially folded) pore blockade durations are plotted as a function of the concentration of denaturing agent. We obtain <tshort> = 239 ± 53 μs (e) Vogel-Tammann-Fulsher representation of long pore blockades versus 1 / (C – Cg), with C = [Gdm-HCl] and Cg = 0.8M. The dotted line is the best fit to the function t = t0 exp (A / (C – Cg)). The protein concentration is constant at 0.35 μM and the applied voltage is 100 mV, [NaCl] = 1M, pH = 7.4 (adapted from [34]). Here the proteins are added in the cis compartment of the chamber.

(net charge of+7e). The length of both channels is similar, but the aerolysin pore diameter is smaller than the α-hemolysin one. We find the same behaviour for MalEwt unfolding transition with aerolysin channel. The native protein does not enter the channel. In the presence of guanidium and proteins we observe the existence of long current blockades and short spikes (Fig. **3a**). The mean blockade currents are the same for the long and the short blockades. Thus, we can deduce that the unfolded part of the partially unfolded protein is inside the pore and the folded part one is outside the pore. The short blockades duration remains constant as a function of guanidium concentration (Fig. **3c**). Then the frequency of unfolded proteins events increases as the concentration of denaturing agent increases, we obtain a sigmoid denaturation curve similar to the one obtained with α-hemolysin pore. The long blockades duration decreases as a function of denaturant concentration up to 1M (Fig. **3d**). Finally, the variation of the long blockades duration as a function of guanidium concentration through aerolysin pore is also well described by a Vogel-Tamman-Fulcher law (Fig. **3e**).

In order to compare all data obtained with MalEwt in different experimental conditions, applied voltage, protein concentration, aerolysin or α-hemolysin pore, we have normalized the maximum event frequency of unfolded proteins to 100% of unfolded proteins. We obtain the same unfolded curves at the single molecule level (Fig. **4**). The denaturing concentrations at the midpoint of the unfolding transition have the same magnitude taking into account the standard deviation, $C_{half\text{-}aerolysin}$ = 0.87 ± 0.01 Mand $C_{half\text{-}hemolysin}$ =0.85 ± 0.01 M. We have shown that the nanopore structure, geometry, and the net charge do not change the folding transition. But, we have observed that both dynamics of unfolded protein entry and transport depend on the nanopore nature [18, 34, 38].

Jiali Li's group studied protein unfolding through solid-state nanopore [36] for different urea concentrations. Three different states are observed, folded, partially unfolded, and fully unfolded, according to three concentrations of denaturing agent. These conformations can be distinguished by the depth and duration of the measured current blockades. But the evolution of the event frequency of fully unfolded proteins as a function of urea is not shown as well as the dynamics of partially unfolded conformation.

-Physical Denaturation

As exposed above, it is possible to follow the denaturation of a protein upon addition of chemical denaturing agent. The same concept can be applied to the thermal unfolding of a protein keeping in mind that the transport dynamics will be faster as we increase the temperature. The temperature can easily be combined with the nanopore technique: the whole chamber is set to the desired temperature by a Peltier module. In this study we use either aerolysin or α-hemolysin as a nanopore and we measure the thermal denaturation curve of MalE219 which is known to have a destabilized denaturation transition compared to the wild type MalE [49]. The recordings of the current traces in Aerolysin are displayed in (Fig. **5a**). Without MalE219 the base line of the current is stable and no peak is observed. After the addition of the protein, we observe shallow peaks due to collisions of the native protein to the pore. By further increasing the temperature, we observe more and more deep peaks characteristic of the unfolded protein inserted in the pore. We clearly see that the frequency of event increase with temperature. This increase could be attributed to the enhanced thermal motion due to the temperature increase. But as shown in (Fig. **5b**), from 20 to 50°C, the event frequency (normalized to the maximum frequency) displays a sharp transition as about 45°C which cannot be explained by the simple enhancement of the thermal motion (for which the event frequency should follow an Arrhenius law). This clearly indicates that the proteins (too large to enter the pore in their native state) are unfolded at these temperatures. After 50°C, the frequency drops again. This can be understood from the event duration measurements (represented as a function of temperature in Fig. **5c**). Again as the thermal motion is enhanced at higher temperature, the velocity of the protein transport through the pore is increased. Thus the duration decreases with temperature down to a plateau starting at about 60°C, where the time scale reaches the limit of resolution of the system. This means that we do not observe all the events when their characteristic time is too small to be resolved [50]. Therefore, the event frequency above 50°C does not reflect the proportion of denatured proteins but rather the proportion of denatured proteins slow enough to be observed.

We measure as well the thermal denaturation curve of MalE219 using Circular Dichroism. We obtain, after baseline correction, the curve in (Fig. **5d**). This curve as well as the normalized frequency in (Fig. **5b**) for both pore, are fitted to a sigmoid. The transition temperatures are respectively 45.5±0.5°C and 44±1°C (equivalent values for both pores).

CONCLUSION, DISCUSSION

The understanding of protein folding remains a great challenge. The nanopore coupled with an electric detection is a recent method at single-molecule level to probe protein denaturation and protein stability as a function of different denaturing agents. Single-nanopore recording is highly sensitive to discriminate between partially folded and completely unfolded proteins. From only unfolded molecules event frequencies, we can obtain denaturation or unfolding curves. These curves depend on the protein stability or protein mutations but not on the structure, geometry, or electric charges of nanopores. At this stage of the discussion this method is similar to other single molecule methods to obtain denaturation curves, by fluorescence spectroscopy [51], force spectroscopy AFM [52] and tweezers [53]. The main advantages with the nanopore method are the ability to probe experimentally the whole conformational space, to detect rare events, to study the partially folded proteins dynamics and phase transition between folded to unfolded state.

What are the perspectives of this technique in the protein folding field? Up to now, the protein folding studies are mainly limited by the nanopores used. The protein channels could be unfolded by high concentrations of denaturing agent [54] and then only relatively small proteins could be study with this technique. We cannot perform experiments with multidomains proteins nor large size proteins. Today, solid-state nanopores do not allow to follow the unfolding

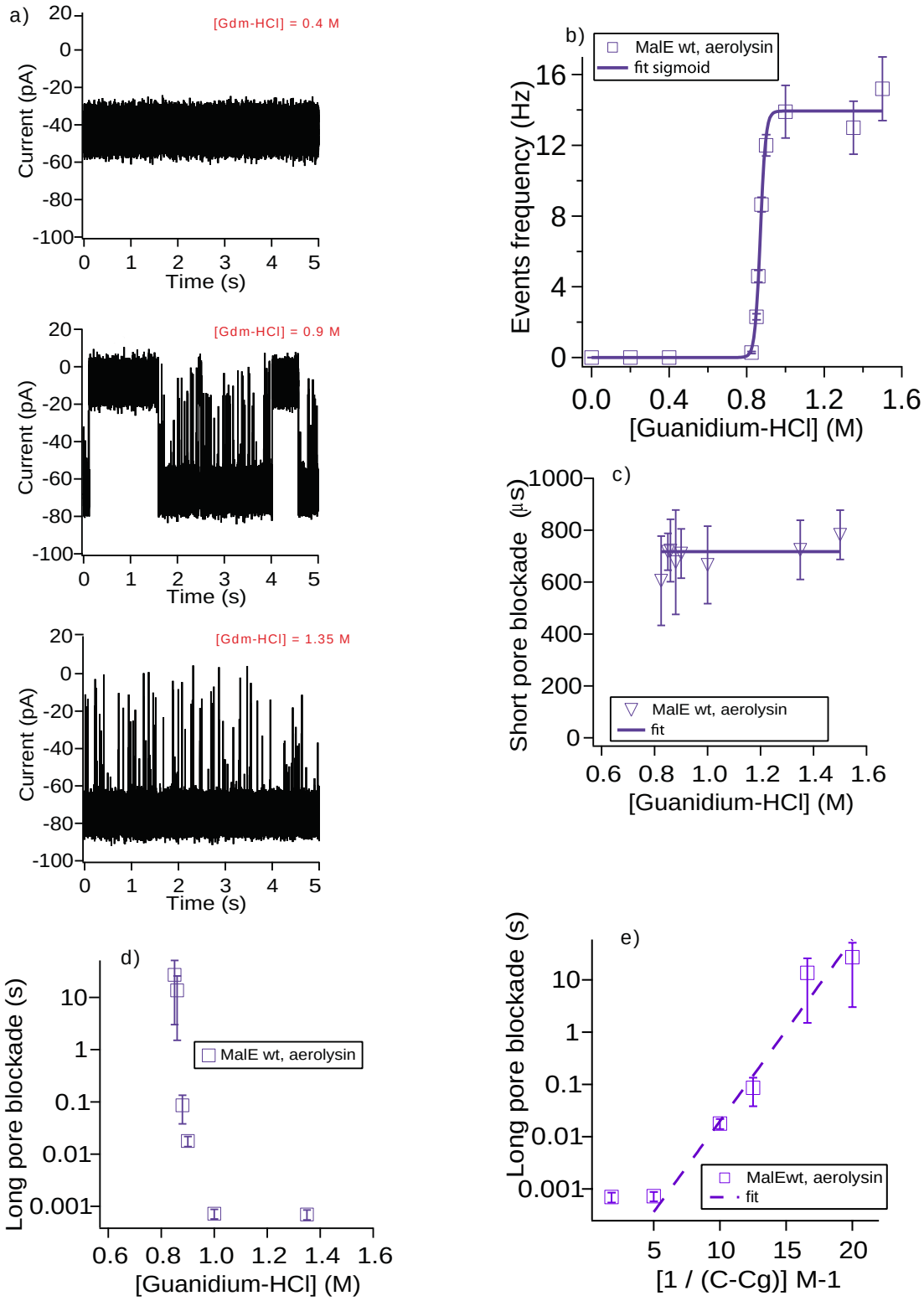

Figure 3. Chemical denaturation of the protein MalEwt through one aerolysin channel. (**a**) Current traces through aerolysin pore as a function of the concentration of Gdm-HCl in the presence of proteins. (**b**) Events frequency of short blockades as a function of guanidium chloride concentration. The line is a fit to a sigmoid function. (**c**) Short, unfolded proteins, or long, partially folded, (**d**) pore blockade duration as a function of guanidium chloride concentration. We obtain <tshort> = 717 ± 39 µs. (e) Vogel-Tammann-Fulcher representation of long pore blockade duration versus 1/(C-Cg) with C = [Gdm-HCl] and Cg = 0.8 M. Dotted line is the best fit to the function t = t0 exp (A/(C-Cg)). The applied voltage is V = -70 mV. Here the proteins (3µM) are added in the *trans* compartment of the chamber, [KCl] = 1M, pH = 7.5. (Adapted from [38]).

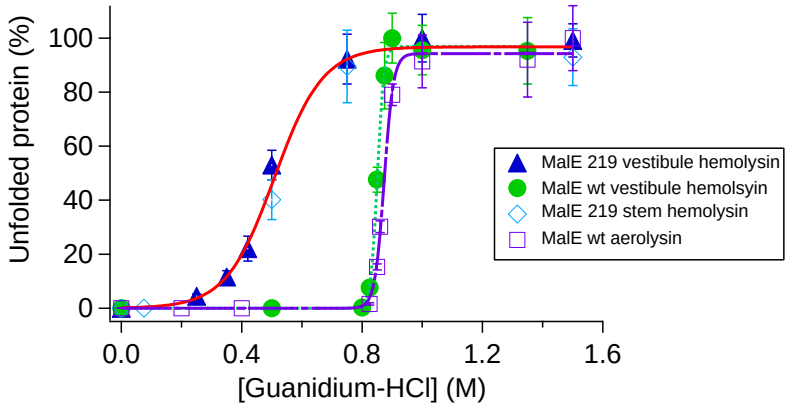

Figure 4. Unfolding curves at single molecule level using α-hemolysin or aerolysin nanopores and wild type, MalEwt, and instable mutant, MalE219, proteins. The events frequency are normalized to obtain this curve using as 100 % the maximum frequency of these short current blockades events. MalE219 is unfolded through α-hemolysin pore when the molecules enter by the two side of the channel. MalEwt is unfolded through aerolysin pore and with α-hemolysin pore. The data are adapted from [34] and [38].

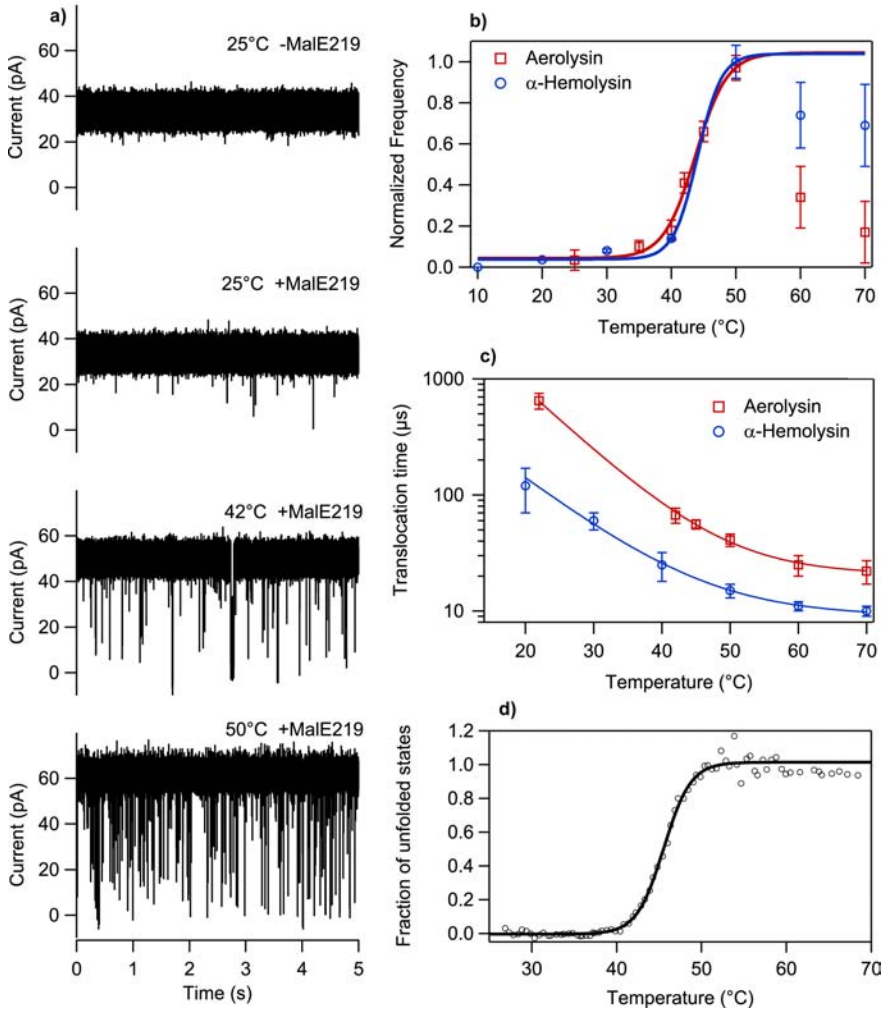

Figure 5. Thermal denaturation of the protein MalE219 probed by nanopore experiments. (**a**) Current traces recorded using aerolysin, without MalE219 (top) at 25°C, and with Male219 at 25,42 and 50°C (from top to bottom). (**b**) Frequency of events normalized to the maximum frequency as a function of Temperature, using aerolysin or α-hemolysin. The data are fitted to a sigmoid with a transition temperature of 44 ± 1 °C for both pores. (**c**) Event duration as a function of temperature. The lines are guides to the eyes. By increasing the temperature, the duration decreases down to a saturation value due to the limit of resolution of the system. This saturation explains the drop of the event frequency observed at 60 and 70°C. (**d**) Thermal unfolding transition curve of MalE219 obtained by Circular dichroism (ellipticity at 222 nm). The curve represents the fraction of unfolded MalE219 as a function of the temperature corrected by a baseline substraction. It is fitted to a sigmoid yielding a melting temperature of 45.5 ± 0.5°C. Adapted from [39].

transition. Indeed with solid-state nanopores, it is really difficult to control precisely the pore diameter, geometry and hydrophilic properties. These properties could change during the duration of the experiments. The new ways are to enhance surface modification with natural lipid [23] as well as chemical functionalization [24] to control the charge surface, the protein translocation velocity and to increase the protein selectivity. In the future, several applications will be connected to a control of proteins unfolding, like ultrafast peptides and proteins sequencing, and also recombinant proteins refolding at the pore exit.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Declared none.

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