Temperature and Chemical Denaturant Dependence of Forced Unfolding of Titin I27

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Single-molecule force measurement opens a new door for investigating detailed biomolecular interactions and their thermodynamic properties by pulling molecules apart while monitoring the force exerted on them. Recent advances in the nonequilibrium work theorem allows one to determine the free-energy landscapes of these events. Such information is valuable for understanding processes such as protein and RNA folding and receptor−ligand binding. Here, we used force as a physical parameter under the traditional chemical and temperature denaturing environment to alter the protein folding energy landscape and compared the change in the unfolding free-energy barrier of the I27 domain of human cardiac titin. We found that the trends in protein unfolding free-energy barriers are consistent for single-molecule force measurements and bulk chemical and temperature studies. The results suggest that the information from single-molecule pulling experiments are meaningful and useful for understanding the mechanism of folding of titin I27.

Introduction

Single-molecule manipulation and force measurement allows us to investigate interaction on a molecular basis. The development of this manipulation technique has made a quantum leap on the events that can be examined in detail.[1−11] For example, mechanical properties of molecules can be studied with a stretch−release cycle at the single-molecule level,[12−14] and the reaction rates of receptor−ligand binding[15,16] and protein folding[17] are increased to allow one to observe the event at laboratory time scales. Protein misfolding, in particular, has been linked to diseases such as Alzheimer's disease and Parkinson's disease.[18,19] The technique made it possible for us not only to observe the beginning and the end states but also to follow the entire trajectory, which provides much needed information about the detailed mechanisms of these complex biomolecular processes.

A detailed study by Cao and Li on protein mechanical unfolding in solutions containing chemical denaturants has demonstrated that combining chemical and mechanical unfolding provides important additional information about the folding/unfolding process.[20] They have observed a softening effect of the chemical denaturant on the resistance to mechanical unfolding of a small protein GB1, and the denaturant does not change the distance between the folded and transition states. The rich information contained in chemical, thermal, and mechanical unfolding has just begun to be explored, and the interplay among these different chemical and physical effects on protein folding offers great opportunity to explore the complex protein folding landscape[21−23] from a different viewpoint. One question is how to compare the results from different studies. The measured values in single-molecule manipulation techniques are usually expressed as force versus distance, which has obvious meanings in systems such as molecular motors and muscle proteins. However, the end-to-end distance is not a typical order parameter for reactions such as protein folding.[24,25] Free-energy surfaces as a function of end-to-end distance of molecules have been reconstructed from force measurements.[7,26−31] However, the lowest free-energy pathway, usually determined by chemical or temperature perturbations,[32−35] is simply defined using the reactant and the product in the reaction coordinate. Here, we investigate the mechanical unfolding of the I27 domain from human cardiac titin under thermal and chemical denaturing conditions. We compare the results from mechanical to chemical and thermal unfolding and find that similar trends exist. Such evidence suggests that mechanical and chemical unfolding of titin I27 follow a similar reaction coordinate.

Experimental Section

Titin I27 Sample Preparation. We used engineered recombinant protein chains of eight serial repeats of I27 domains of human cardiac titin (Athena ES, Baltimore, MD). A solution of 10 μL of protein at a concentration of 50−100 μg/ml was deposited on a fresh gold surface and incubated at room temperature for 10−20 min. The protein sample was rinsed with 1−2 mL of phosphate buffered saline (PBS). For temperature-dependent measurements, the protein sample was incubated for 1 h at a given temperature between 25 and 37 °C. For measurements dependent on denaturant concentration, 100 μL of guanidinium hydrochloride (GdmCl) solution at concentrations ranging from 0.2 to 2.0 M was added to the protein sample.

Force Measurements. Protein stretching measurements were done using a Multimode atomic force microscope (AFM) with PicoForce option (Veeco Instruments, Santa Barbara, CA). The spring constant for MLCT cantilevers (Veeco Probes, Camarillo, CA), determined using the equipartition theorem, was 0.05 N/m. Individual molecules were attached to the cantilever tip via a nonspecific binding interaction and stretched as the piezoelectric stage moved at constant velocity. Force−extension measurements were performed in solution at pulling velocities of 0.30 and 1.00 μm/s. For temperature-dependent measurements, the protein solution temperature was maintained within ±0.1 °C using a temperature controller (Veeco Instruments, Santa Barbara, CA). For measurements dependent on denaturant

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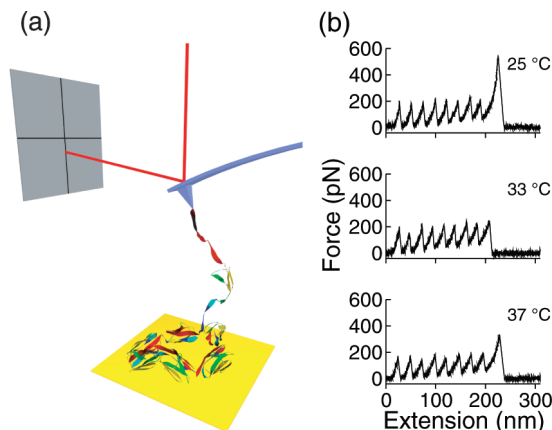**Figure 1.** AFM experimental setup and force−extension curves. (a) An illustration of the AFM experimental setup. One end of the molecule is attached to the gold surface and the other end to the silicon nitride cantilever. The force on the molecule is measured by the laser displacement, which is caused by the bending of the cantilever. (b) Representative force−extension curves of eight serially linked titin I27, obtained at different temperatures. Each peak represents an unfolding event of an I27 domain, with the last peak resulting from detachment of the molecule from the cantilever. The force peak decreases with increasing temperature without altering its characteristics.

concentration, molecules were stretched at room temperature and a pH of 7.4 ± 0.1.

Reconstructing Free-Energy Landscapes Using Jarzynski's Equality. Force−extension measurements were analyzed using Jarzynski's equality and previously described methods[7] to determine equilibrium free-energy surfaces for the unfolding of titin I27. We used an exact relationship derived by Hummer and Szabo[27] that determines the free energy as a function of the molecular end-to-end distance $G(z)$ from force−time data. The unfolding free-energy barrier, $\Delta G^{\ddagger}$, was calculated using the reconstructed free-energy landscape at the transition state and an assumption based on Bell's model.[36]

Results and Discussion

To measure the force-induced unfolding of single molecules, we used AFM to stretch titin molecules containing eight identical I27 domains, as illustrated in Figure 1a. Protein molecules were pulled under solutions at various temperatures and guanidinium hydrochloride (GdmCl) concentrations. Typical force−extension curves taken at various temperatures are shown in Figure 1b.

We determined the statistics of the peak force distribution as a function of temperature and chemical denaturant concentrations, as shown in Figure 2a. The distribution shifted to lower forces as the temperature increased, which is consistent with the expectation that the free-energy barrier of protein unfolding is lower with increasing temperature.[37] The most probable unfolding force as a function of temperature, obtained at 0.3 and 1 μm/s pulling velocities, is displayed in Figure 2b. Both data sets show that the force peaks decrease with temperature linearly, which agrees with the trend predicted by simulation[38] and observed experimentally in other proteins.[39] We have observed a similar trend when adding chemical denaturant (GdmCl); the unfolding force decreases with increasing concentration linearly, which has been observed in another protein.[20]

To compare the results with chemical unfolding quantitatively, we determined the free-energy surface of unfolding as a function of molecular extension using Jarzynski's equality. The free-energy barrier $\Delta G^{\ddagger}$ of unfolding was obtained from the G curve.[7,36] Figure 3 shows $\Delta G^{\ddagger}$ as a function of denaturant
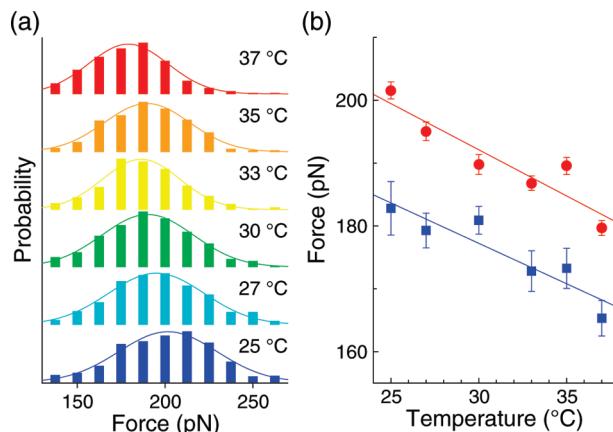

Figure 2. Unfolding force as a function of temperature. (a) Unfolding force distribution of titin I27 at different temperatures. The peak of the distribution shifted from 200 pN at 25 °C to 180 pN at 37 °C. (b) The most probable unfolding force, determined by fitting the distribution in (a) with the Gaussian distribution, is temperature-dependent. The two lines represent the experiments done at two different pulling velocities. Circle: 1 μm/s; square: 0.3 μm/s. The error bars represent one standard error.
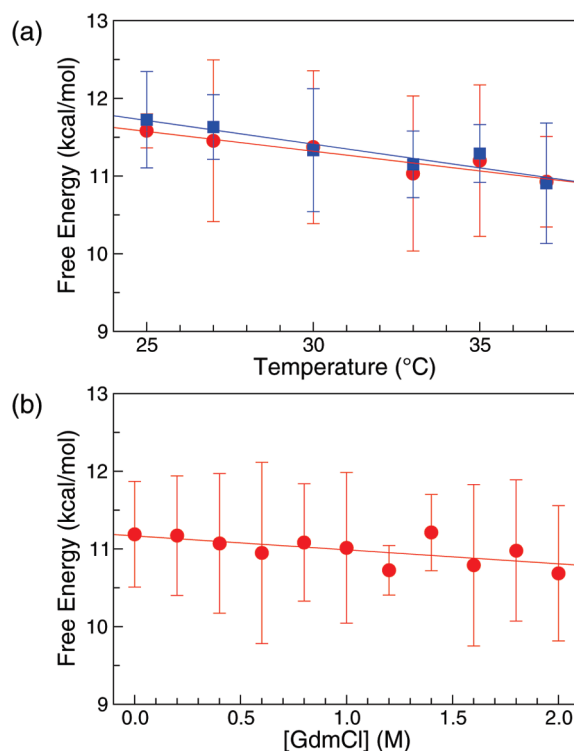

Figure 3. Change in free energies as a function of temperature and denaturant concentration. (a) Change in $\Delta G^{\ddagger}$ as a function of temperature. The two sets of data were obtained at two different pulling velocities. Circle: 1.0 μm/s; square: 0.3 μm/s. (b) Change in $\Delta G^{\ddagger}$ as a function of denaturant concentration. The uncertainty in the data, calculated using the bootstrap method, is 0.7 kcal/mol.

concentration and temperature. Note that due to the nonequilibrium nature of the technique, the value of the force peak depends on the pulling velocity, while the equilibrium free energy determined from Jarzynski's equality does not. This further verifies the method used and the consistency in trends that resulted from temperature-dependent unfolding characteristics. Similar temperature dependence in kinetic rate has been observed in chemical unfolding kinetic studies.[37] Using $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and the entropy change from folded to transition states, we obtained

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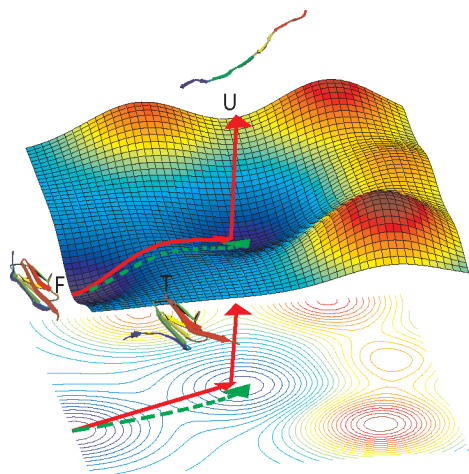*J. Phys. Chem. B, Vol. 113, No. 31, 2009* **10847**

Figure 4. Schematic representation of the free-energy landscape of titin I27. The solid line represents the free-energy pathway probed by single-molecule force measurement, and the dashed line indicates the reaction coordinate followed by the chemical denaturant. The chemical method results in unfolding through the lowest free-energy pathway but only probes the folded (F), transition (T), and compact unfolded states, while the force measurement probes the entire pathway, including that from the compact unfolded to stretched states (U). The pathway probed by force measurement may be slightly different from that of the chemical denaturant because the constraint of the molecular end-to-end distance, but it is not expected to differ significantly because the change in the free-energy landscape shows similar trends for both methods.

$\Delta H^{\ddagger} = 29$ kcal/mol and $\Delta S^{\ddagger} = 0.06$ kcal/mol·T. These values are in general agreement with the free-energy estimates derived from bulk kinetic rate studies.[37] Figure 3b shows $\Delta G^{\ddagger}$ at GdmCl concentrations less than 2 M, where most of the initial states are folded. Using $\Delta G^{\ddagger} = \Delta G^{\ddagger}(H_2O) - m[M]$, we determined the m value from single-molecule pulling experiments to be 0.17 ± 0.07 kcal/mol·M. This compares favorably with $m = 0.15$ kcal/mol·M from ensemble measurements.[40] In addition, using the unfolding rate k_u determined from chemical denaturant studies and the free energy $\Delta G^{\ddagger}$ from current single-molecule force measurement, we found that the prefactor k_0 in $k_u = k_0 e^{\Delta G^{\ddagger}/k_B T}$ is on the order of 10^5 s^{-1}, which is roughly constant with respect to temperature and chemical denaturant concentrations within the range studied. The prefactor is in good agreement with simulations[41] (10^5 s^{-1}) and falls within the range predicted by Kramers theory[32] (10^4–10^7 s^{-1}). Note that the numbers presented above may not be taken at their absolute values since the uncertainty in the estimated distance between folded and transition states carries directly to that in the free-energy estimate. However, assuming that the chemical denaturants do not alter the distance,[20] the change in the resulting m value will be linearly proportional to the change in distance but not the direction, that is, the sign of the slope, and we will focus on the general trends of the unfolding free-energy barrier as the denaturant and temperature increase.

The consistency in the trends from both the temperature-and denaturant-dependent studies indicate that the unfolding transition state from single-molecule force measurement is in the vicinity of that from chemical studies in the free-energy landscape. Note that both techniques probe equilibrium properties, with force measurements determining the free energy $\Delta G^{\ddagger}$ at equilibrium, that is, zero force, and chemical denaturant extrapolating the kinetic rate constant k_u to zero denaturant concentration. Assuming that the assumption for extrapolation is correct, the only difference is the reaction coordinate, where

force measurement has the molecular end-to-end distance as a constraint. The similarity in trends from both temperature (seen in $\Delta S^{\ddagger}$) and chemical (seen in the m value) studies suggests that external perturbation tilted the free-energy landscape by similar magnitude for both force and chemical reaction coordinates. Hence, the pathway for forced unfolding is expected to be close to that of the chemical unfolding, as illustrated in Figure 4. This is understandable since the size of the molecule, that is, the radius of gyration, is often used to define the folded and unfolded state, and the size as well as the molecular end-to-end distance of the molecule generally increases monotonically from the folded, to the transition, to the unfolded state. As illustrated in Figure 4, these two processes likely pass over similar free-energy surfaces through the transition state to the unfolded state.

Conclusion

In conclusion, we presented studies of the temperature and chemical denaturant dependence of mechanical unfolding of titin I27. The unfolding free-energy barrier decreases linearly with increasing temperature or chemical denaturant concentrations. This suggests that, at least for titin I27, the molecular end-to-end distance is a good reaction coordinate. Instead of being a passive observer who only sees the initial and final states of an individual event, single-molecule manipulation allows us to follow the entire trajectory and quantify the observables of the process. The values of the free energies determined will allow theories and models to be tested. In addition, single-molecule manipulation enables one to determine the region of the free-energy landscape that is not probed with bulk studies; hence, it allows one to map the entire protein folding landscape, including the highly stretched form, which resembles the conformation of the molecules when first synthesized.

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