TEMPERATURE-COUPLED FORCED UNFOLDING OF SINGLE PROTEINS

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ABSTRACT

Protein unfolding has been widely studied with denaturants, temperature, and most recently by single molecule extension using AFM. Pathways can in principle be distinct, with denaturants or temperature exerting a less directed influence than forced extension. Since spectrin family proteins are known to unfold with single repeat events as well as cooperative tandem repeat events, we sought to determine the coupled effect of temperature on forced unfolding of these widely expressed proteins that share a triple-helical repeat structure. Bimodal distributions of unfolding length were seen at all temperatures with the major peak corresponding to unfolding a single repeat and the minor peak at twice the length corresponding to unfolding a tandem repeat. Temperature has no significant effect on the peak-to-peak unfolding lengths, ruling out any influence on partial unfolding processes. Increased temperature does lead to a decrease in tandem repeat unfolding events, however. In addition, mean unfolding forces decrease steeply with temperature to about 50% of their value at 10 ^{o}C. Circular dichroism (CD) studies of the same protein in solution show a similar decrease in percent helix lost over the same temperature range. Cooling the protein from 42 to 23 ^{o}C, however, showed only a slight reversibility (<10%) in percentage of initial helix by CD in comparison to the large (>90%) recoveries in force and tandem events in the AFM studies. Comparing the free energy changes of thermal unfolding (CD) to thermomechanical unfolding (AFM) generally corroborates unfolding pathways. Increased temperature thus appears to soften both the helical linker between repeats and the triple-helical repeats themselves.

INTRODUCTION

Spectrin superfamily proteins play pivotal and ubiquitous roles in cell organization, membrane resilience, and even adhesion. In binding actin filaments and other components, these proteins function in both monomeric and associated forms and participate as extensible linkers. In the red blood cell, αI- and βI spectrin chains interact in an antiparallel fashion, with repeats characterized by coiled coils of anti-parallel triple-helices and connected by trans-domain helical linkers [Kreis and Vale, 1999]. β-spectrin's N-terminal actin binding domain leads to crosslinking and a crucial role in membrane network integrity.

Recent AFM studies on βI-spectrin have also demonstrated mechanical unfolding, including cooperativity in tandem repeat unfolding events which occur as frequently as single repeat events [Law et al., 2003]. Additionally, thermodynamic studies with urea and thermal unfolding in solution by McDonald and Pozharski. [2001] on various one and two repeat spectrin constructs have shown greater stability for tandem compared to single repeats. To further examine the intramolecular forces and the stability effect of tandem repeats on unfolding, we have applied the AFM technique of single molecule extension to four-repeat β-spectrin constructs at temperatures of 10, 23, 37, and 42 $^{\circ}$C.

METHODS AND MATERIALS

Protein preparation: The four N-terminal repeats of β-spectrin were expressed recombinantly, purified by gel permeation chromatography in phosphate-buffered saline (PBS), and kept on ice for AFM studies. Purified β-spectrin constructs exist only as monomers in solution. Immediately before use, any protein aggregates were removed by centrifugation at 166,000g at 2^{o}C for 1 hour; dynamic light scattering was used to verify monodispersity prior to experiment.

Dynamic force spectroscopy: An AFM experiment was begun by adsorbing 0.03–0.1 mg/ml protein for 15 min at room temperature onto either freshly cleaved mica or amino-silanized glass coverslips. The surface was then lightly rinsed with PBS and placed without drying, under the head of the AFM. Experiments were typically done at imposed displacement rates of 1 nm/msec as well as at 5 nm/msec. High temperature experiments were first done at 23 ^{o}C, raised to 37 ^{o}C, and then to 42 ^{o}C. The sample was then allowed to cool back down from 42 ^{o}C to 23 ^{o}C. Low temperature experiments (10 ^{o}C) were done inside a cold room. Temperatures were controlled and monitored using a Nanoscope Heater Controller by Digital Instruments. For any one temperature, thousands of surface to tip contacts were generally collected and later analyzed with a semi-automated, visual analysis

program. For a many hour experiment, initial results compared very favorably with results obtained near the end of the experiment.

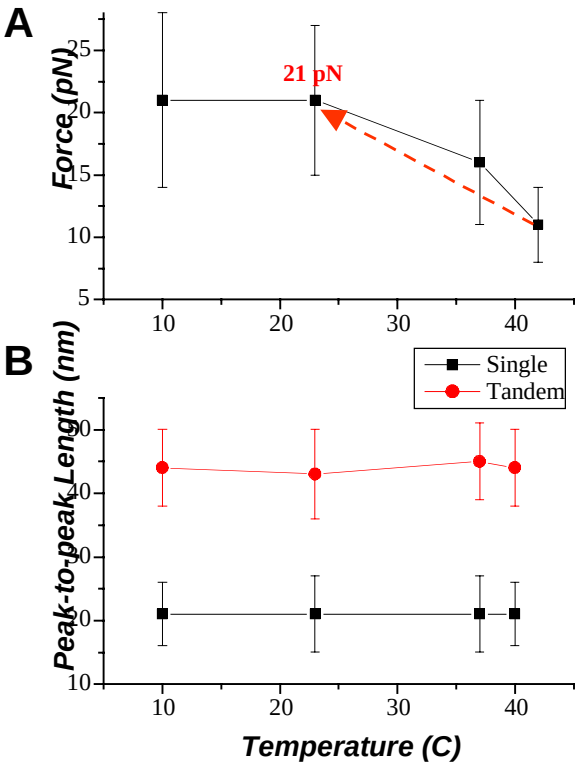

Fig. 1. Temperature versus unfolding force and length. (A) Unfolding forces of single spectrin molecules at various temperatures. The forces showed no change from 10°C to 23°C (21 pN) but decrease nonlinearly from 23°C to 37°C (16 pN) and 42°C (11 pN). The dotted arrow indicates that when the sample was brought back to 23°C from 42°C, the unfolding force returns back to 21 pN. (B) Peak-to-peak unfolding lengths at various temperatures for single repeat (square) and tandem repeat (circle). Temperature appears to have no effect on the peak-to-peak lengths: the peak-to-peak lengths are 24-26 nm for single repeats and 43-45 nm for tandem repeats.

RESULTS

The force and length data for 23 °C compare well with prior results [Law et al.; Lenne et al., 2000, Reif et al., 1999]. Both the length and force distributions appear bimodal with gaussian means differing by a factor of 2 for all the various temperatures studied. Average forces for 23°C and 10°C are nearly equal with the major gaussian means at about 21 pN. The gaussian means decrease to 16 pN and 11 pN for 37°C and 42°C, respectively. This non-linear relationship between unfolding forces and temperature is shown in Fig. 1A. When the protein was cooled back down to 23°C from 42°C, the major force gaussian mean goes back up to 21 pN (illustrated by the red dotted line in Fig. 1A). Average lengths for all temperatures appear equal with the major gaussian means ranging from 24 nm to 26 nm and are summarized in Fig. 1B. From a length versus force scatterplot analysis, single repeat and tandem unfolding events were identified [Law et al], and the percentage of each is summarized in Fig. 2. As with the force, cooling the protein back to 23°C from 42°C, leads to a very similar percentage of tandem repeat events.Temperature has no noticeable effect on the peak-to-peak unfolding lengths but has a dramatic effect on unfolding forces in the high temperature range. The constancy of both single and tandem repeat peak-to-peak lengths suggests that spectrin stays intact throughout the various temperatures studied even though the unfolding force changes significantly. The non-linear temperature effects on the unfolding forces agree very well with CD results of ours (data not shown) as well as thermal unfolding seen by others near 40°C [Minetti et al., 1986; MacDonald and Pozharski, 2001]. Fig. 2 clearly indicates that tandem repeat unfolding events occur less frequently at high temperatures. Since tandem repeat unfolding is likely to be facilitated by a contiguous helix between repeats, the lower force indicates less structure and the connecting helix seems likely to be the first helix lost. The resilience of spectrin indicated by the unfolding forces and tandem repeat unfolding events when cooled from 42°C to 23°C agrees well with irreversible unfolding at 48°C [Minetti et al., 1986].

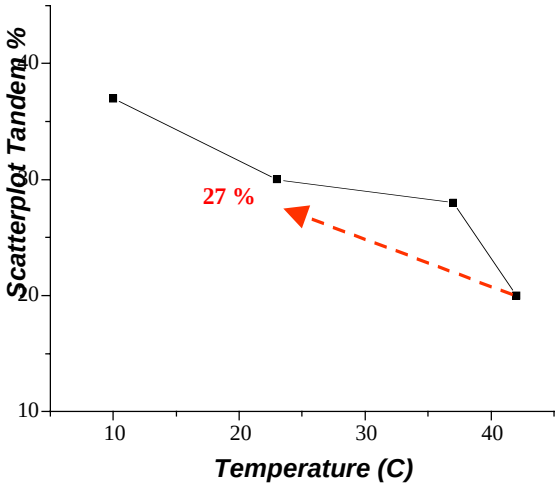

Fig. 2. Temperature versus scatterplot tandem events %. Tandem repeat unfolding events decrease as a function of temperature and appears more dramatic at high temperature range. The dotted arrow indicates that cooling back down to 23°C from 42°C is essentially reversible for AFM.

REFERENCES

P.-F. Lenne, A.J. Raae, S.M. Altmann, M. Saraste, and J.K.H. Horber, "States and transitions during forced unfolding of a single spectrin repeat," *FEBS Letters*, 476, pp.124-128, 2000.

T. Kreis, and R. Vale, *Guidebook to the Cytoskeletal and motor Proteins*, Oxford, 1999.

R. Law, P. Carl, S. Harper, P. Dalhaimer, D. Speicher, D. Discher, "Cooperativity in forced unfolding of tandem spectrin repeats," *Biophysical Journal* (to appear, Jan. 2003)

R. MacDonald and E. Pozharski, "Free energies of urea and of thermal unfolding show that two tandem repeats of spectrin are thermodynamically more stable than a single repeat," *Biochemistry*, vol. 40, 3974-3984, 2001.

M. Minetti, M. Ceccarini, A. Maria, D. Stasi, T. Petrucci, and V. Marchesi, "Spectrin involvement in a 40 °C structural transition of the red blood cell membrane," *J. Cell. Biochem.*, 30, 361-370, 1986.

M. Rief, J. Pascual,, M. Saraste, and H.E. Gaub, "Single Molecule Force Spectroscopy of Spectrin Repeats: Low unfolding forces in helix bundles," *J. Mol. Biol.*, vol. 286, pp.553-561, 1999.