Stepwise Unfolding of Ankyrin Repeats in a Single Protein Revealed by Atomic Force Microscopy

Lewyn Li,* Svava Wetzel,† Andreas Plückthun,† and Julio M. Fernandez*
*Department of Biological Sciences, Columbia University, New York, New York 10027; and †Biochemisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland

ABSTRACT Using single-molecule atomic force microscopy, we find that a protein consisting of six identical ankyrin repeat units flanked by N- and C-terminal modules (N6C) unfolds in a stepwise, unit-by-unit fashion under a mechanical force. Stretching a N6C molecule results in a sawtooth pattern fingerprint, with as many as six peaks separated by ~10 nm and an average unfolding force of 50 ± 20 pN. Our results demonstrate that a stretching force can unfold multiple repeat units individually in a single protein molecule, despite extensive hydrophobic interactions between adjacent units.

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Address reprint requests and inquiries to Julio M. Fernandez, Tel.: 212-854-9141; Fax: 212-854-4619; E-mail: jfernandez@columbia.edu.

Proteins containing repeats of the same structural motif are common in nature. The ankyrin (ANK) repeat is a 33-residue L-shaped motif, consisting of two antiparallel α-helices linked by a short loop (1). A β-turn forms the base of the L and connects two consecutive repeats (Fig. 1 *a*). Adjacent ANK repeats are stabilized by extensive hydrophobic and hydrogen bonding interactions (1). Recent genome searches revealed 10,000–20,000 ANK repeats among viruses, prokaryotes, and eukaryotes, usually with four to six (but up to 29) consecutive repeats in one protein (1,2). The main function of ANK repeat is to mediate protein-protein interactions. ANK repeat proteins are found in many different cellular roles, including those where mechanical stress and deformations may be involved. For example, in different cell types, ankyrins bind both to spectrin and to outer membrane proteins such as ion channels, thus connecting the membrane to the cytoskeleton (3). The spectrin-actin cytoskeleton provides mechanical support for the membrane bilayer (3). Recently, a chain of 17–29 ANK repeats in the mechanosensitive transduction channel TRPA1 has been proposed to be the gating spring that controls the opening and closing of ion channels in vertebrate hair cells (4–6).

ANK repeat proteins also represent an interesting class of models in protein folding, because their modular structure and lack of long-range interactions have led to the hypothesis that each ANK repeat in a protein could unfold and refold individually. Thus far, ensemble thermal and chemical denaturation experiments of ANK repeat proteins do not show this behavior (1,7–10). However, temperature and chemicals such as urea are global unfolding agents that affect the entire protein surface. On the other hand, the application of a stretching force in single-molecule atomic force microscopy (AFM) unravels a protein along a specific direction, and may be a physiologically relevant denaturant in some cases (11–17). In this study, we decided to test the hypothesis of individual ANK repeat unfolding by mechanically stretching a consensus ANK repeat protein, N6C, using single-molecule AFM.

N6C consists of an N-terminal cap, six identical copies of a designed consensus ANK repeat and a C-terminal cap that form a contiguous domain (S. Wetzel, K. Binz, and A. Plückthun, unpublished data; see Supplementary Material). N6C is an ideal system for single-molecule AFM because the presence of multiple copies of the same ANK repeat eliminates complications from the heterogeneous mixtures of ANK repeats in natural proteins (11). The N6C protein was adsorbed onto a gold-coated coverslip and randomly picked up and stretched by an AFM tip. Stretching N6C generates a uniform sawtooth pattern in the force-extension curve with as many as six peaks regularly separated by ~10 nm (Fig. 1 *b*). Using the worm-like chain model (18), we determined that successive peaks correspond to an increase in polymer contour length (ΔL_c) of 11.5 ± 0.7 nm (Fig. 1 *d*). Since an amino acid contributes 0.38 nm in contour length and each folded ANK repeat possesses a length of 0.8 nm, the unfolding of an ANK repeat is expected to increase the contour length by (33 × 0.38–0.8 nm) = 11.5 nm. The excellent agreement between the predicted and observed change in contour length and the regular sawtooth pattern are strong indications that ANK repeats in a N6C molecule unravel individually. The average unfolding force is 50 ± 20 pN (Fig. 1 *c*). Computer simulations have shown similar piecewise unfolding of ANK repeats in a four-repeat model (6). An ANK repeat protein is different from polyproteins such as titin, tenascin, fibronectin, and ubiquitin, because all the ANK repeats belong to a single protein domain and there are extensive hydrophobic interactions and hydrogen bonds

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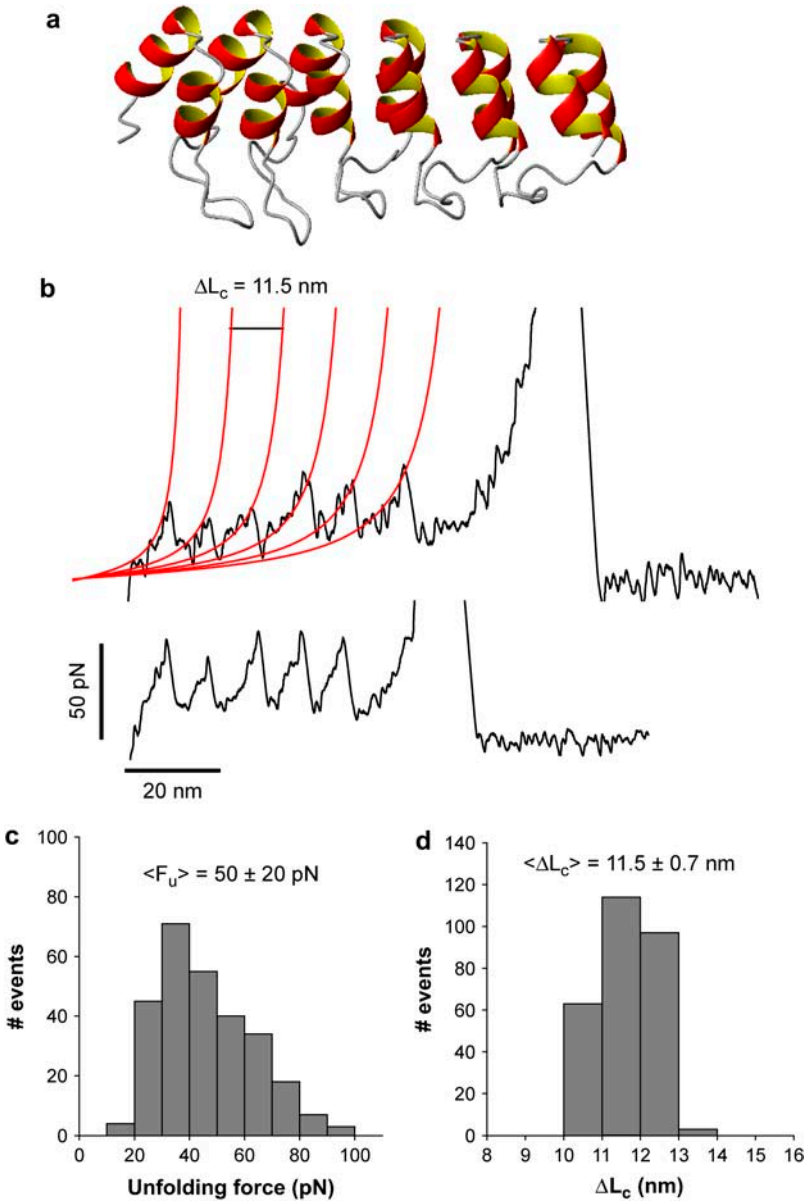

FIGURE 1 Mechanical unfolding of the ankyrin repeats in N6C with single-molecule AFM. (*a*) An ANK repeat protein structure containing six ANK repeats. This structure consists of the second to seventh repeats in human ankyrinR (19). (*b*) Representative force-extension curves for N6C. The AFM tip picked up the protein at random locations along the peptide backbone, resulting in a different number of peaks in each trace. The contour length increment, ΔL_c = 11.5 nm, is only observed at high forces. (*c*) The distribution of peak forces, with N = 277. (*d*) The distribution of ΔL_c, with N = 277.

between adjacent ANK repeats. In polyproteins, each protein domain is individually folded and spatially separated, and there may only be a small number of domain-domain contacts because of the short interdomain linker (two to four amino acids) and the relative rigidity of folded protein domains.

Here we demonstrate that, under a stretching force, the ANK repeats in a protein can be unraveled one at a time. In contrast, most bulk thermal and chemical unfolding studies of ANK repeat proteins, including N6C (S. Wetzel, K. Binz, and A. Plückthun, unpublished data), show highly cooperative transitions, with zero or one unfolding or folding intermediate (1,7–10). Why do the ANK repeats in N6C behave so differently between mechanical and chemical unfolding? In mechanical unfolding, the vectorial nature of the applied force compels the protein to unfold along a well-defined direction, whereas in chemical denaturation, the entire protein surface is exposed to unfolding agents. Therefore, the reaction coordinate is probably different between mechanical and chemical denaturation, resulting in different unfolding pathways, transition states, and unfolded states. For example, the mechanical stabilities of fibronectin domains do not follow their melting temperatures (14), and the mechanical stability of a protein depends on the direction of the applied force vector (12,13). The fact that we observed individual ANK repeat unfolding under a stretching force is an indication that the direction in which a protein moves on the unfolding free energy landscape is crucial to how a protein unfolds. Under a stretching force, the ANK repeats in N6C are better described as a linear sequence of unfolding units, with little communication or cooperation among various ANK repeats.

Some ANK repeat proteins may play mechanical roles in vivo. For example, a chain of 17–29 ANK repeats in the mechanosensitive transduction channel TRPA1 has been proposed to form a curved ANK superhelix, which is a possible candidate for the gating spring that transmits forces from sound waves or motion to ion channels (4–6). Molecular dynamics simulations postulated that chains of 12–24 ANK repeats respond to a stretching force in two phases (6). In the first phase, the ANK superhelix stretches and relaxes reversibly by ~11 nm without unfolding. During the second phase, individual ANK repeats begin to unfold, representing an attractive (albeit unconfirmed) safety mechanism against extreme stimuli. The piecewise unfolding of multiple ANK repeats in N6C (Fig. 1 *b*) show that individual ANK repeat can indeed be peeled off by mechanical stress. Therefore, our results support the idea that, in some biological systems, ANK repeats could behave like multiple buffers linked in series; to resist damagingly high forces, ANK repeats can be sacrificed and extended one at a time, without the whole protein losing its tertiary structure. Evolution may have selected the modular design of some ANK repeat proteins for mechanical reasons.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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