Viscoelastic Study of the Mechanical Unfolding of a Protein by AFM

Masaru Kawakami, Katherine Byrne, David J. Brockwell, Sheena E. Radford, and D. Alastair Smith
Institute of Molecular Biophysics, Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

ABSTRACT We have applied a dynamic force modulation technique to the mechanical unfolding of a homopolymer of immunoglobulin (Ig) domains from titin, (C47S C63S I27)$_5$, [(I27)$_5$] to determine the viscoelastic response of single protein molecules as a function of extension. Both the stiffness and the friction of the homopolymer system show a sudden decrease when a protein domain unfolds. The decrease in measured friction suggests that the system is dominated by the internal friction of the (I27)$_5$ molecule and not solvent friction. In the stiffness-extension spectrum we detected an abrupt feature before each unfolding event, the amplitude of which decreased with each consecutive unfolding event. We propose that these features are a clear indication of the formation of the known unfolding intermediate of I27, which has been observed previously in constant velocity unfolding experiments. This simple force modulation AFM technique promises to be a very useful addition to constant velocity experiments providing detailed viscoelastic characterization of single molecules under extension.

Received for publication 11 March 2006 and in final form 8 May 2006.

Address reprint requests and inquiries to Alastair Smith, E-mail: d.a.m.smith@ds.leeds.ac.uk.

How a polypeptide chain can spontaneously fold into its unique and highly ordered three-dimensional structure has been a fundamental question in biology for decades. Also, understanding how protein structure endows the molecule with its biochemical/biomechanical function is of great importance. This can only be fully answered by finding correlations between the structure and dynamic behavior of proteins. Until recently, almost all measurements of protein folding and protein dynamics required observation of an ensemble of molecules; the results therefore provide the average properties of the system, within which information about individual molecules is hidden. Rarely populated conformational states in the folding reaction, which might determine the pathway to the native state, and/or of functional relevance, are extremely difficult to characterize. Therefore techniques that can explore the behavior of single molecules are essential for developing new insights into the relationship between protein folding, dynamics, and function.

Single molecule techniques such as optical tweezers and the atomic force microscope (AFM) have been used to investigate the mechanical properties of various kinds of biomolecules. AFM has been used to mechanically unfold many proteins since the seminal work of Ikai (1) and the elastic behavior and mechanical resistance of proteins with a wide range of structural motifs have been investigated (2). Furthermore, the recent development of dynamic force spectroscopy has enabled us to probe the dynamical properties of single molecules in a quantitative manner (3–5).

Titin is a muscle protein mostly consisting of Ig and fibronectin type III domains linked to each other via their N- and C-termini. Titin's mechanical properties have been investigated extensively using AFM because of its relevance to the function of muscle. When a fragment (Ii-Ij) or a tandem-repeat of a single domain from titin (Ii)$_n$ is stretched, the resulting force-extension curve shows the now well-known saw-tooth pattern where sequential unfolding peaks of each folded domain are separated at fixed intervals. It has been previously reported (6) that with close inspection of each unfolding peak a slight deviation from the force-extension worm-like chain (WLC) model (7) is observed on the leading edge. This deviation is attributed to the transition from the native state of the protein to an unfolding intermediate, whose presence was predicted by steered molecular dynamics (8). This feature is most clearly seen in the first unfolding peak and becomes less evident with each consecutive unfolding event.

Recently we have developed a dynamic force AFM technique that is capable of the sensitive measurement of viscoelastic properties of a single molecule under extension. Here, a pentameric repeat of I27 domain from titin (C47S C63S), denoted here as (I27)$_5$ (9), was stretched at constant speed during which the cantilever was oscillated at fixed frequency (5 kHz) with an amplitude of 2 nm. The molecular viscoelasticity was calculated from the mechanical response of the cantilever-molecule system using a simple harmonic oscillator (SHO) model. (see Supplementary Material).

The force, stiffness, and friction of a single (I27)$_5$ molecule are plotted as a function of extension in Fig. 1. At a glance, both the stiffness and friction have the appearance of the saw-tooth pattern. Also, it is clear that the amplitude of the peaks in both the stiffness and friction decrease with each unfolding event. The reason for the decrease in the stiffness is that this property of (I27)$_5$ is dominated by the high compliance of the linker regions between the folded domains and of the length of unfolded polypeptide chain, which increases with each unfolding event. Previously we showed that the molecular friction of a polymer is dominated by internal friction, while solvent friction is negligibly small (3). The

doi: 10.1529/biophysj.106.085019

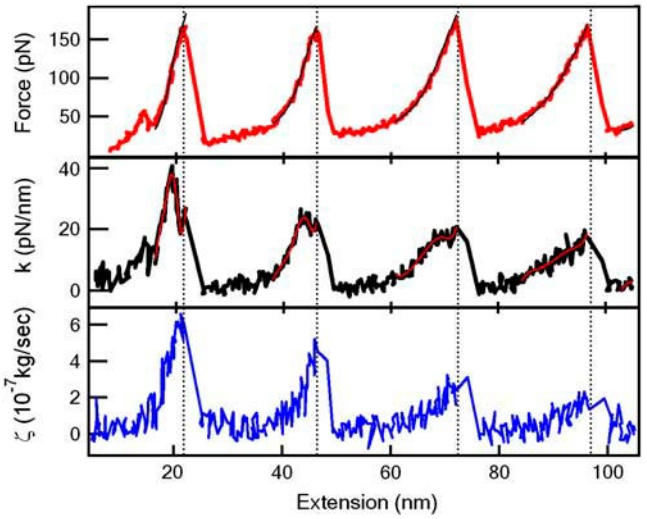

FIGURE 1 Viscoelastic data from the mechanical unfolding of $(I27)_5$. (*Top*) Force-extension curve. Dependence of the molecular stiffness (*middle*) and molecular friction (*bottom*) on the extension. Stiffness-extension curves in the middle panel have been fitted with polynomial functions (*red lines*) and these fitted curves have been converted to force-extension and drawn in the top panel (*black lines*). To clarify where unfolding events have occurred, dotted lines are drawn.

stepwise decrease in the friction of $(I27)_5$ in Fig. 1 indicates that the internal friction of the unfolded polypeptide chain is much smaller than that of the folded domains. Nevertheless, it would be possible to determine the friction or dissipative properties of a folded protein in the polymer from these data if we could determine the friction of unfolded polypeptide chain with accuracy and subtract its contribution. However, the signal/noise (S/N) ratio of the friction data is not yet sufficiently high to allow us to carry out this analysis. Work is currently underway using novel cantilevers to overcome this problem.

Close inspection of the force-extension curve in Fig. 1 (*top*) does not reveal significant deviation from WLC on the unfolding peaks for this construct of I27. However, in Fig. 1 (*middle*), a sudden decrease in the stiffness of $(I27)_5$ at an applied force of 100 pN is observed in the first peak, i.e., before the global unfolding event that in this case occurs at 160 pN. Similar features are also seen in the second and third peaks, each with decreasing amplitude. Interestingly, the clear decrease in the stiffness profiles before global unfolding do not give rise to obvious deviation in the force data at the same point. However, calculation of the force extension curve from the stiffness data (*black lines* in Fig. 1, *top*), demonstrates that such features can barely be seen. It is noticeable that there is excellent agreement between the force data and reconstructed force curve, indicating the accuracy and suitability of the SHO analysis.

The first unfolding peak in mechanical unfolding experiments is often affected by nonspecific interactions at the surface and therefore in Fig. 2 we show the data from four different molecules to demonstrate the reproducibility of the results with the first peak removed from the data. Clearly there is very good reproducibility in the molecular stiffness (Fig. 2, *middle*), and the unfolding intermediate is observed in the first and second peaks shown (which correspond to the second and third unfolding events). This gives us confidence that the observed sudden decrease in stiffness that occurs before the global unfolding event does not arise from artifacts due to nonspecific surface interactions. The force at which these features appear at 100 pN (101.7 $\pm$ 9.7 pN, $N = 32$) corresponds to the force at which the unfolding intermediate has previously been observed to become populated (6). So what gives rise sudden decrease in the molecular stiffness? According to simulation the hydrogen bonds between A and B β-strands are broken at around 100 pN and this disruption provides an additional elongation to the molecular length of 4 $\sim$ 7 Å (6,8). By contrast, an increase in length of 6.6 Å is obtained by experiment (6). The sudden decrease in the stiffness therefore implies that the released A-strand has considerable elasticity. We determined the contour lengths for each unfolding peak below 100 pN before any unfolding event by fitting the force extension curves to the WLC model (*blue lines* in Fig. 3, *top*). Using the resulting contour lengths, the stiffness of the WLC chain for each unfolding peak was reconstructed using the derivative of WLC model and is shown as blue lines in Fig. 3, *bottom* (see Supplementary Material). They show an excellent agreement with molecular stiffness before the partial unfolding event

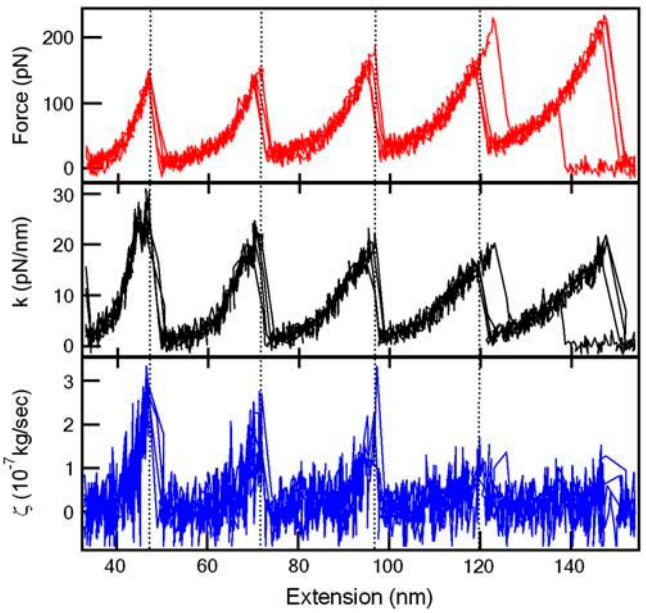

FIGURE 2 Superimposition of viscoelastic spectra from four different molecules of $(I27)_5$. Dependence of (*top*) force, (*middle*) molecular stiffness, and (*bottom*) molecular friction on extension. In these data the first unfolding peak has been removed to avoid any influence of nonspecific interactions near the surface.

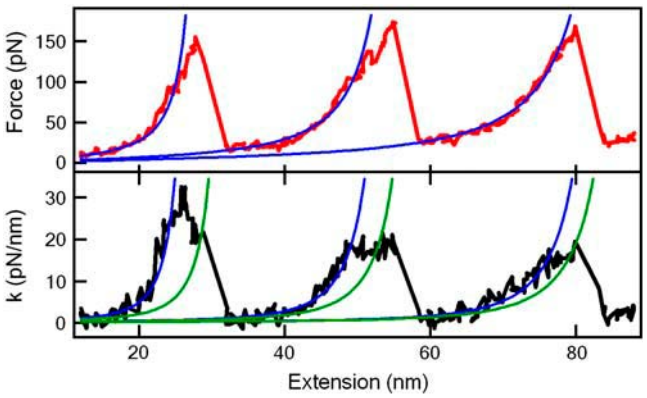

FIGURE 3 Dependence of the molecular force and stiffness of $(I27)_5$ on the extension. (*Top*) Force-extension curve (*red*) and fitted curves (*blue*) using the WLC model with a persistence length 0.4 nm. (*Bottom*) Stiffness-extension profile of $(I27)_5$ (*black*). The stiffness of WLC polymer simulated with contour length L_c of 30.1, 59.0, and 90.2 nm and L_c with an additional length corresponding to three amino acids per folded domain are also shown (*blue* and *green lines*, respectively).

obtained using the dynamic force technique (*black line* in Fig. 3, *bottom*). The stiffness of WLC fits were recalculated with an additional length. They are shown overlaid in Fig. 3, *bottom* (*green lines*). These curves coincide with the measured stiffness after the initial sudden decrease in the measured stiffness. These facts suggest that this initial decrease in the stiffness is caused by release of approximately three amino acids from the native structure from each folded domain.

Interestingly, no sudden changes in the internal friction are apparent at an extension corresponding to the formation of the unfolding intermediate (see first and second peaks in Figs. 1, *bottom*, and 2, *bottom*). Although a detailed discussion of these data is not possible due to poor S/N, these observations suggest that the dissipative dynamical properties of the I27 unfolding intermediate are not radically different from those of the native state, despite the separation of three amino acids of β-strand from the protein structure.

Very recently, using a novel frequency modulation AFM technique, Higgins et al. investigated the unfolding of I27 and also reported the detection of mechanical unfolding intermediates (10). Curiously, the intermediate appeared at around 30 pN, a much lower force than observed in constant velocity experiments (6,8) and in this study. In contrast, the results of the more straightforward force modulation technique described here show complete consistency with previous data and a much better S/N ratio obtained with a smaller oscillation amplitude (2 nm; cf. 4.5–26.5 nm in Higgins et al. (10)).

The final goal of our work is to obtain the viscoelastic properties of unfolded, partially folded, and folded domains and to investigate the frequency dependence of the response of native proteins to oscillatory forces. With improvements in the S/N of the friction data, this simple force modulation AFM technique promises to be able to provide detailed viscoelastic characterization of single protein molecules under extension.

SUPPLEMENTARY MATERIAL

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

ACKNOWLEDGMENTS

We thank T. Tezuka-Kawakami for preparation of the $(I27)_5$ sample.

This work was supported in part by the Engineering and Physical Sciences Research Council (EPSRC) and the Biotechnology and Biological Sciences Research Council (BBSRC). M.K. was a Japan Society for the Promotion of Science Research Fellow and is now supported by the EPSRC. D.J.B. is an EPSRC-funded White Rose Doctoral Training Centre Lecturer, and S.E.R. is a BBSRC Professorial Fellow.

REFERENCES and FOOTNOTES

1. Mitsui, K., M. Hara, and A. Ikai. 1996. Mechanical unfolding of alpha-2-macroglobulin molecules with atomic force microscope. *FEBS Lett.* 385:29–33.
2. Carrion-Vazquez, M., A. F. Oberhauser, T. E. Fisher, P. E. Marszalek, H. Li, and J. M. Fernandez. 2000. Mechanical design of proteins studied by single-molecule force spectroscopy and protein engineering. *Prog. Biophys. Mol. Biol.* 74:63–91.
3. Kawakami, M., K. Byrne, B. Khatri, T. C. B. Mcleish, S. E. Radford, and D. A. Smith. 2005. Viscoelastic measurements of single molecules on a millisecond timescale by magnetically driven oscillation of an AFM Cantilever. *Langmuir.* 21:4765–4772.
4. Janovjak, H., D. J. Müller, and A. D. L. Humphris. 2005. Molecular force modulation spectroscopy revealing the dynamic response of single bacteriorhodopsins. *Biophys. J.* 88:1423–1431.
5. Humphris, A. D. L., J. Tamayo, and M. Miles. 2000. Active quality factor control in liquids for force spectroscopy. *Langmuir.* 16:7891–7894.
6. Marszalek, P. E., H. Lu, H. Li, M. Carrion-Vazquez, A. F. Oberhauser, K. Schulten, and J. M. Fernandez. 1999. Mechanical unfolding intermediates in titin modules. *Nature.* 402:100–103.
7. Smith, S., Y. Cui, and C. Bustamante. 1996. Overstretching B-DNA: the elastic response of individual double-stranded and single-stranded DNA molecules. *Science.* 271:795–799.
8. Lu, H., B. Isralewitz, A. Krammer, V. Vogel, and K. Schulten. 1998. Unfolding of titin immunoglobulin domains by steered molecular dynamics simulation. *Biophys. J.* 75:662–671.
9. Zinober, R. C., D. J. Brockwell, G. S. Beddard, A. W. Blake, P. D. Olmsted, S. E. Radford, and D. A. Smith. 2002. Mechanically unfolding proteins: the effect of unfolding history and the supramolecular scaffold. *Protein Sci.* 11:2759–2765.
10. Higgins, M. J., J. E. Sader, and S. P. Jarvis. 2006. Frequency modulation AFM reveals individual intermediates associated with each unfolded I27 titin domain. *Biophys. J.* 90:640–647.