Understanding the elasticity of fibronectin fibrils: Unfolding strengths of FN-III and GFP domains measured by single molecule force spectroscopy

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Abstract

While it is well established that fibronectin (FN) matrix fibrils are elastic, the mechanism of fibril elasticity during extension is still debated. To investigate the molecular origin of FN fibril elasticity, we used single molecule force spectroscopy (SMFS) to determine the unfolding behavior of a recombinant FN-III protein construct that contained eight FN-III domains (1–8 FN-III) and two green fluorescent protein (GFP) domains. FN-III domains were distinguished from GFP domains by their shorter unfolding lengths. The unfolding strengths of both domains were determined for a wide range of pulling rates (50 to 1745 nm/s). We found that the mechanical stabilities of FN-III and GFP domains were very similar to each other over the entire range of pulling speeds. FN fibrils containing GFP remain brightly fluorescent, even when stretched, meaning that GFP domains remain largely folded. Since GFP and FN-III have equal unfolding strengths, this suggests that FN-III domains are not extensively unraveled in stretched FN fibrils. Our results thus favor an alternative model, which invokes a conformational change from a compact to an extended conformation, as the basis for FN fibril elasticity.

Keywords: Protein unfolding; Single molecule force spectroscopy; Wormlike chain

1. Introduction

The extracellular matrix (ECM) plays an important role for the elasticity of tissues and the regulation of cell adhesion (Oberhauser et al., 2002). The ECM contains a number of non-collagen adhesive glycoproteins that can assemble into fibrillar structures, which function to organize the ECM, that mediate cell attachment through specific binding sites and that guide cell migration in developing tissue (Alberts, 2002). Fibronectin (FN) is an ECM glycoprotein that is composed of tandem repeats of three distinct types (I, II and III) of individually folded domains (Potts and Campbell, 1996). The FN type III (FN-III) domain is a common motif of modular proteins and is found in about 2% of all animal proteins (Bork and Doolittle, 1992). The 15–17 FN-III domains of FN constitute about two thirds of the length of the molecule, and each FN-III domain contains seven β-strands that form a sandwich of anti-parallel β-sheets (Leahy et al., 1996).

Several studies have shown that FN matrix fibrils assembled in a cell culture are under tension (Halliday and Tomasek, 1995; Zhong et al., 1998); however, details of the molecular conformation of FN remain unclear. To study FN fibril elasticity, Ohashi et al. (1999, 2002) engineered cells that secreted FN that had a GFP domain spliced between 3FN-III and 4FN-III. This naturally fluorescent FN was assembled into matrix fibrils that could be observed at sequential times in living culture. One striking finding was
that the elastic matrix fibrils could stretch three to four times their rest length.

Two mechanisms for the deformation of FN molecules have been discussed recently (Erickson, 2002). The first mechanism proposes that stretching is achieved by force-induced unfolding of FN-III domains. Support for this model is provided by studies using the atomic force microscope (AFM) to stretch single molecules containing tandem repeats of FN-III domains (Oberhauser et al., 1998; Rief et al., 1998b). In these experiments, stretching produced a characteristic sawtooth wave of force, where each peak was interpreted to correspond to the sudden unfolding of one FN-III domain.

The second mechanism is based on observations that FN molecules, which can be considered as flexible filaments, can exist in two conformations. In the extended conformation the molecule is largely straightened, while in the compact conformation the molecule is folded back on itself, where the fold is likely stabilized by ionic bonds (Erickson and Carrell, 1983; Johnson et al., 1999). The proposed deformation mechanism is that FN molecules go from the folded, compact conformation to a more extended conformation when force is applied to a matrix fibril.

Two recent studies by Baneyx et al. (2001, 2002) used fluorescence resonance energy transfer (FRET) to investigate the conformation of FN molecules in FN fibrils. They cautiously interpreted their results in favor of the first deformation mechanism, i.e., force-induced domain unfolding. The observed FRET signal, however, may be reporting largely the conformational change from a compact to an extended conformation, and thus does not yet allow to distinguish between the two mechanisms (Erickson, 2002). Recent experiments by Ohashi et al. (2002) showed that FN-GFP fibrils remain brightly fluorescent even after extensive fibrillar stretching, which suggests that most GFP domains remain folded. This result could be interpreted in favor of the second deformation mechanism, assuming that under physiological conditions the average mechanical strength of FN-III domains is larger than the average strength of GFP domains. GFP is inserted in-line between FN-III domains three and four and, upon extension, sustains the same force as the FN-III domains. Since the GFP domains remain apparently folded under applied stress as observed by the FRET signal, one could conclude that most FN-III domains must remain folded also, as they would sustain similar forces.

To be able to further distinguish between the two deformation mechanisms, it now becomes important to determine the relative unfolding forces of GFP and FN-III domains in the same construct. To address this problem on the molecular level, we made a protein construct comprising FN-III domains 1–8, with a CFP and YFP (cyan and yellow variants of GFP) inserted between FN-III domains 3 and 4. We then used the AFM to stretch single molecules of this construct.
over a wide range of pulling rates to determine the relative unfolding strengths of FN-III and GFP domains.

2. Results and discussion

2.1. Pulling experiments on a recombinant construct containing FN-III and GFP domains

AFM pulling experiments were performed on a construct composed of $1^{\text{st}}$–$8^{\text{th}}$ FN-III domains with a pair of color-shifted GFPs inserted between the $3^{\text{rd}}$ FN-III and $4^{\text{th}}$ FN-III domains (Ohashi et al., 2002). The two GFP variants are yellow and cyan fluorescent proteins, and the mutations that shift the color are unlikely to cause major changes in the mechanical stability.

The construct also contained two cysteine residues, inserted just after the last FN-III domain, which can form links to a gold surface. However, non-specific adsorption of the recombinant construct cannot be ruled out (Rief et al., 1998b). Single molecules were picked up at random with an AFM cantilever tip and stretched for up to several hundred nanometers (Rief et al., 1998b). The resulting force-extension curves showed the characteristic sawtooth patterns that correspond to successive domain unfolding events (Fig. 1a–d). The representative examples shown in Fig. 1 suggest that GFP domains (contour lengths range from 40 nm to 82 nm) can unfold either before or after FN-III domains (contour lengths range from 5 nm to 32 nm) and at somewhat higher or lower force.

2.2. Fingerprints of FN-III and GFP domains

Examples of the unfolding length distributions at two pulling rates (436 and 870 nm/s) are shown in Fig. 2a and b. The unfolding lengths fall into two separate and statistically different distributions (Mann–Whitney rank sum test, $P<0.001$) that correspond to FN-III and GFP domains (gray and white fills, respectively). Within each of these two distributions, there appear three sub-peaks; the first two most likely correspond to partially folded intermediates and the third to the unfolding of a complete domain. Having a wide range of contour lengths due to the presence of partially folded intermediates is typical in SMFS of polyproteins (Meadows et al., 2003; Oberdorfer et al., 2000; Oberhauser et al., 2002). For both pulling rates, the third peak of the FN-III unfolding length distribution is located at about 28 nm, close to the expected 27 nm, and the third peak of the GFP distribution is located at about 75 nm, again close to the expected 79 nm. The unfolding lengths for all sub-peaks are reported in Tables 1 and 2. Our results for the unfolding of FN-III domains are in a good agreement with previous studies, which have reported contour length increases in the range of 28–32 nm (Rief et al., 1998a,b, 1999), of 28 nm (Oberhauser et al., 2002) and of 25 nm (Oberdorfer et al., 2000). We could now assign each peak of the sawtooth wave to FN-III or GFP based on the length of extension that followed it.

2.3. The mechanical stability of FN-III and GFP domains

An important and novel aspect of our study is that we have compared the unfolding strengths of FN-III and GFP domains...
in the same protein construct over a wide range of pulling rates. The unfolding forces over all pulling rates ranged from 29 to 399 pN for FN-III domains and from 35 to 271 pN for the GFP domains. Fig. 3a–h show the distribution of unfolding forces at all pulling rates. While the average unfolding strengths of FN-III and GFP span a similar range (Table 1), the GFP domains appear to be slightly weaker. The widths of the force distribution histograms are in good agreement with those observed separately for FN-III domains and GFP domains under similar experimental conditions (Dietz and Rief, 2004; Oberhauser et al., 2002). The unfolding forces of FN-III and GFP domains were statistically different at 103, 580 and 1745 nm/s (Mann Whitney rank sum test, \( P=0.002 \), 0.017 and 0.005, respectively).

Our values for the unfolding strengths of \( 1^{–8} \)FN-III domains at 580 nm/s (137.2±4.0 pN, \( n=159 \)) are in good agreement with those reported for the \( 2^{–14} \)FN-III domains at 600 nm/s pulling rate (145 pN) (Oberhauser et al., 2002). Also, our values for the unfolding forces of GFP molecules at 291 nm/s (104.2±5.4 pN, \( n=32 \)) are in excellent agreement with those reported recently by Dietz and Rief (2004) at 300 nm/s (104±40 pN). The data in Fig. 3 show that more FN-III than GFP domains unfolded, consistent with the fact that there are eight FN-III and only two GFP domains in each recombinant construct. Furthermore, the actual ratio likely depends also on where along its length the molecule was picked up. Although the number of domains of the two protein types differs in the recombinant protein, we have sufficiently many pulls for both domain types to evaluate our data in a statistically meaningful way. For example, Table 1 shows that the ratio of the number of FN-III domains unfolding to the number of GFP domains unfolding is about 4 to 1 at all investigated pulling rates, in good agreement with the ratio of the number of FN-III domains to GFP domains (8 to 2) in the construct.

### 2.4. The dependence of mechanical unfolding of FN-III and GFP domains on pulling rate

The unfolding strength of proteins typically depends on the pulling rate and varies for different protein domains (Li et al., 2000; Williams et al., 2003). We performed force pulling experiments over a wide range of pulling rates to compare the average unfolding strengths of FN-III and GFP domains in the same construct. The data in Fig. 4 shows (1) that at all pulling rates GFP domains have slightly lower unfolding strengths than FN-III domains; (2) that the unfolding strength of FN-III and GFP domains increases linearly with the logarithm of the pulling rate as predicted by Evans and Ritchie (1997); and (3) that the slopes of the lines are almost identical. This suggests that the mechanical stability of the two domain types is quite similar over a large range of pulling rates. Since the slopes of FN-III and GFP domains are similar, it is not unreasonable to extrapolate from the unfolding strengths obtained experimentally at high pulling rates to those at much lower pulling rates that apply in a FN fibril. Extension rates in FN fibrils in vivo may reach about 8 \( \mu m/h \) (i.e., movements that correspond to cell migration; Ohashi et al., 2002) and the corresponding pulling rates would be about 2 nm/s.

When extrapolating from the experimentally accessible, high pulling rates to the low pulling rates that occur in vivo, one must assume that only one mechanical energy barrier is present; i.e., the barrier observed with AFM at high pulling rates. The assumption of one major energy barrier over the range of rates of interest here is not unreasonable considering (1) the similarity in slopes for the rate dependence of FN and GFP domain unfolding; and (2) previous experimental evidence that showed that the extrapolation from a 100 nm/s pulling rate to low pulling rates for I27 domains is in good agreement with the unfolding rates of the protein measured by standard chemical denaturation techniques (Carrion-Vazquez et al., 1999b).
Our results of the rate dependence of FN-III domain unfolding agree well with those reported by Oberhauser et al. (2002). The unfolding strength of GFP was previously explored at only one pulling rate (Dietz and Rief, 2004). Our measurements of the unfolding strengths of FN-III and GFP domains in the same construct and over a range of pulling rates are essential for our conclusion that FN-III and GFP domains are approximately equal in strength, even at low extension rates.

2.5. Partially folded intermediates of FN-III and GFP domains

The increase in contour length upon unfolding FN-III and GFP domains spans a range, with a substantial number of...
events releasing less than the full length of polypeptide. This suggests the existence of partially unfolded intermediates, as observed in previous studies (Fisher et al., 2000b; Li et al., 2005; Oberhauser et al., 2002). We wanted to know if the partially unfolded intermediates might have unfolding forces that differ from those of the full-length domain (i.e., 27 nm for the FN-III domains and 79 nm for the GFP domains). We therefore determined the average unfolding force for all events within each sub-peak (Table 2). Our choice of intermediate unfolds was based on the increase in contour length upon domain unfolding. A contour length increase between 5 and 32 nm was attributed to FN-III domain unfolding, while a contour length increase between 40 and 82 nm was attributed to GFP domain unfolding. Since our choice of partial intermediates was based only on contour length differences, a doubt that an FN-III unfolding event may also be attributed to a partial unfolding of a GFP domain can be raised. However, since our FN-III contour length distribution looks the same with and without GFP, this effect is likely small.

The data summarized in Table 2 show (1) that the unfolding strengths of intermediates of different lengths were similar to those of full domains and (2) that the force for unfolding the full domains was in the same range as the global average, in good agreement with published results for partial FN-III domain unfolding (Fisher et al., 2000b; Li et al., 2005; Oberhauser et al., 2002; Rief et al., 1998b) and for partial GFP unfolding (Dietz and Rief, 2004).

2.6. Pulling experiments on native FN and on a construct of $7^{-14}$FN-III domains

As a control, SMFS experiments were also performed on native FN molecules and on a construct composed of $7^{-14}$FN-III domains. This recombinant fragment covers the part of the FN molecule not included in our $1^{-8}$FN-III-CFP-YFP construct. The force-extension curves obtained for both showed the characteristic sawtooth patterns (inserts in Fig. 5a and b). On average, the increase in unfolding distance of native FN and $7^{-14}$FN-III domains was $20 \pm 0.6$ (n = 168) and $21 \pm 0.5$ (n = 255), respectively. The peak force values for unfolding of native FN ranged from 35 pN to 202 pN (Fig. 5a) and those for $7^{-14}$FN-III domains ranged from 35 pN to 208 pN (Fig. 5b). Averaged over all events, the forces required to unfold the native FN and $7^{-14}$FN-III domains were $120 \pm 3$ pN (n = 163) and $125 \pm 3$ pN (n = 235), respectively. Importantly, the average unfolding forces for native FN and the $7^{-14}$FN-III domains are indistinguishable from the unfolding force for GFP (120 pN) and 1-FN-III (137 pN) at a pulling rate of 580 nm/s (Table 1). This observation implies that the unfolding of FN-III domains is independent of the presence of the GFP domains since on average the force required to unfold the FN-III domains in our construct was very similar to the force required to unfold the FN-III domains in the $7^{-14}$FN-III construct or in the native state.

Our average unfolding force of 120 pN for native FN is consistent with an average value of 145 pN (Oberhauser et al., 2002). Our average unfolding force of 137 pN for $1^{-8}$FN-III domains is consistent with the observation that domains $1^{-2}$FN-III are stronger than domains $10^{-12}$FN-III and $12^{-13}$FN-III (Oberhauser et al., 2002). We did not, however, see the pronounced hierarchy of unfolding strengths within single FN molecules that was reported previously (Oberhauser et al., 2002); our unfolding peaks were much more random.
2.7. Implications for FN elasticity

Previous studies showed that matrix fibrils stretch significantly from their original length. One example is the prominent fibril running between two cells in Fig. 5 of Ohashi et al. (2002), which stretches almost two-fold between 1:30 and 3:00 h. We showed previously that FN-GFP fibrils remain brightly fluorescent even when stretched and suggested that the GFP domains are mostly folded (Ohashi et al., 2002). We cannot, however, rule out the possibility that the observed fluorescence is produced by a small fraction of folded domains, with the majority being unfolded. To address this concern, we measured the integrated fluorescence of this fibril along its full length at four time points and found that the fluorescence remained constant relative to nearby immobilized fibrils (Ohashi et al., 2002). If GFP domains had unfolded during this stretch, it would have reduced the integrated fluorescence. It is possible that tension itself, without unfolding, could decrease fluorescence; however, since the fluorescence signal remained constant during stretching, we conclude that stretching does not unfold GFP domains and also not FN-III domains, since FN-III domains are of similar strength. While partial unfolding of GFP was observed by Dietz and Rief (2004), contributions from partially unfolded intermediates on the overall fluorescence signal could only have been small, as any intermediates that lost the hydrophobic core around the fluorophore would also have lost fluorescence.

Another important element in our analysis is the observation that FN fibrils in vivo appear to be stretched about four times their rest length. If unfolding of FN-III domains were the basis for this stretch, then it would require the majority of the FN-III domains to be unfolded. The reasoning is that each FN-III domain can contribute a maximum of a seven-fold extension, while FN-I and FN-II cannot contribute because they have internal disulfide links that would prevent their extension. If our analysis is correct that the majority of the GFP domains remain folded, then we conclude that the majority of the FN-III domains remain folded also. Recently, Li et al. (2005) observed unfolding intermediates when stretching a polyprotein of 10FN-III. According to their results, the force required to unfold the native state of the 10FN-III domain was 100±20 pN and the force required to unfold the intermediate was only 50±20 pN. Since these forces are lower than the forces reported here for both GFP and FN-III domains, unfolding or at least partial unfolding of the weak 10FN-III domains in native FN is possible. However, we showed that the average forces required to unfold domains in the native FN molecule and the 7–14FN-III domains in our constructs are indistinguishable from the unfolding force for GFP (120 pN) and 1–8FN-III (137 pN) at a pulling rate of 580 nm/s (Table 1). This observation implies that only a small fraction of the FN-III domains could actually have been unfolded since the average force required to unfold the FN-III domains remained constant with (in 7–14FN-III and native FN) and without (in 1–8FN-III) the presence of the 10FN-III domain. A small fraction of unfolded FN-III domains could not produce the four-fold stretch observed with FRET. Therefore, we conclude that the majority of the FN-III domains remain folded.

Fig. 6. A model illustrating how the change of FN from the compact to the extended conformation might produce fibril elasticity. (a) A FN dimer, where the two subunits are joined by disulfide bonds near their C-termini, is shown in the extended conformation. (b) The molecule folds in such a way that domains 2–3FN-III of one subunit form an electrostatic bond with domains 12–14FN-III of the other subunit. This is repeated to make a doubly folded molecule (Johnson et al., 1999). (c) The FN fibril is proposed to assemble by connecting molecules near their N-termini to make a longitudinal strand. With all molecules in the compact conformation, as shown in (c), the fibril would be in its relaxed, contracted state. Under tension, the electrostatic bonds would break and the molecules could be pulled to their extended conformation, resulting in a four-fold extension. Reprinted with permission from Erickson (2002).
Just as important as the unfolding force is the force that prevents significant domain refolding from occurring. While the forces developed during fibril extension may not be sufficient to cause major domain unfolding, they potentially could slow the refolding of already or spontaneously unfolded domains (Carrion-Vazquez et al., 1999b). For example, Li et al. (2002) have estimated that a static force of 13.7 pN would leave 50% of Ig domains unfolded. A similar force would probably apply to FN-III domains. However, the force that would prevent refolding of GFP domains is not known and will thus remain an important aspect for future research.

If, at present, we can exclude unfolding of FN-III domains as the dominant mechanism for fibril elasticity, this leaves the compact to extended conformational change as the favored model (Fig. 6). In this model, FN molecules are folded into the compact conformation in a relaxed fibril. As the fibril is stretched, molecules are pulled into the extended conformation. Depending on how the molecules are connected to each other (this is still not known), the extension could easily produce a four-fold stretch as was observed from the FRET analysis done on FN matrix fibrils assembled in cell culture (Ohashi et al., 2002). Studies of proteolytic and recombinant protein fragments indicated that the compact conformation is stabilized by an electrostatic contact between FN-III domains 2–3 of one subunit of the dimer and domains 12–14 of the other subunit (Johnson et al., 1999). If this connectivity, or another similar one, also prevailed in vivo, then the extension from the compact to the extended conformation could produce the four-fold stretch observed by fluorescence analysis (Ohashi et al., 2002). Finally, the compact conformation is stabilized by electrostatic bonds (Erickson, 2002; Johnson et al., 1999) that are likely broken at much weaker forces than those required to break the multiple hydrogen bonds that hold together FN-III domains.

3. Experimental procedures

3.1. Protein engineering

We began with the pAIPFN-YFP vector (Ohashi et al., 2002), and constructed a new vector pAIPFN/YFP-CFP, in which a CFP was inserted with a short, eight-amino acid linker immediately following the YFP. We then used PCR to amplify the fragment from FN-III domains 1–8, including restriction sites to clone it into pET15 (Novagen), which provides an N-terminal histag. Two cysteine residues were added to the C-terminus to enhance attachment to a gold substrate (presumably through covalent bonding). The final 1–3FN-III-YFP-CFP, 4–8FN-III amino acid sequence is: mgs-sshhhhhsglypgrshmsGPV, ...(FN1–3) ...ETTGT-ggr-MVSKG ...(YFP) ...(DELKgt-MVSKG ...(CFP) ...DELK-ggr-RPRSD ...(FN4–8) ...RQKTpgec (the FN, YFP and CFP sequences are underlined, and the sequences in lowercase are derived from the cloning site and linkers). Escherichia coli BL21 (DE3) was transformed with the vector and the recombinant protein was expressed at 20 °C to improve solubility (a large fraction of 1–3FN-III-YFP-CFP, 4–8FN-III was insoluble at 37 °C). The recombinant protein from the soluble fraction was purified with a cobalt-agarose column (TALON, Clonetech) using standard procedures. A FRET signal from the purified protein was confirmed with a spectro-fluorophotometer (Shimadzu, RF-5301PC), indicating that the GFP molecules are properly folded. FRET was demonstrated by the spectrum of a sample excited at 433 nm. A donor peak (CFP) was obtained at 488 nm and an acceptor peak (YFP) at 525 nm. The acceptor peak was about 50% higher in amplitude than the donor peak. The eight-amino acid linker had a thermolysin cut site and, after digestion, the donor peak increased about 50% and the acceptor peak fell, consistent with the loss of FRET. Native bovine FN and the recombinant fragment 7–14FN-III were prepared as described previously (Johnson et al., 1999).

3.2. Single molecule force spectroscopy experiments

All force measurements were performed using an atomic force microscope (AFM) (MultiMode with a low noise AFM head, Nanoscope III controller, Veeco, Santa Barabra, CA), commercially available “V"-shaped silicon nitride cantilevers (Veeco) were used in all experiments. The force constant of each cantilever was determined individually before the experiment from the power spectral density of the thermal noise fluctuations in solution (Hutter and Bechhoefer, 1993). The measured spring constant values for the cantilevers used in the experiments ranged between 50 and 80 pN/nm, in agreement with reported values using other methods and as reported by the manufacturer. A systematic error on the order of 10% can typically be expected in the determination of the spring constant values for AFM cantilevers. While this error largely impacts comparison with force data obtained from other laboratories, it does not impede the comparison between the unfolding strengths of FN and GFP domains in our experiments. A 100 μl aliquot of a 20 μg/ml mixture of the protein construct of interest (1–3FN-III-YFP-CFP, 4–8FN-III, native FN or 7–14FN-III) suspended in PBS was allowed to adsorb onto a freshly cleaned, evaporated gold surface for an hour. We found that this concentration produced about one successful “pick up" for every 50 touches, which yields a high probability for single molecule attachments (Evans and Ritchie, 1999). Our experiments were carried out in phosphate-buffered saline (PBS) buffer solutions to mimic physiological conditions, yielding pH values and ionic strengths close to those in vivo so as not to compromise the stability of the GFP domains. After adsorption, excess unbound proteins were removed by ampie rinsing with PBS. The protein sample substrate was then mounted onto the piezoelectric scanner of the AFM and covered with a drop of PBS before mounting the AFM head. During force pulling experiments, care was taken to minimize evaporative loss of solvent. Force pulling measurements were performed over a wide range of pulling rates (50, 103, 201, 291, 436, 580, 870 and 1745 nm/s) over a constant distance of 500 nm with a resolution of 4096 data points. All measurements were performed at room temperature and with a retraction (ramp) delay of 1 s.
3.3. Wormlike chain modeling

The wormlike chain (WLC) model of entropic elasticity (Eq. (1)) (Marko and Siggia, 1995) was applied to fit the force-extension profiles of individual protein domains. The force $F$ required for stretching an unfolded polypeptide chain to a length $X$ is given by (Bustamante et al., 1994; Oberhauser et al., 2002; Rief et al., 1997):

$$F = -k_B T \frac{X}{L_p} \left[ \frac{1}{L_c} + \frac{1}{4 \left( \frac{1 - X}{L_c} \right)^2} - \frac{1}{4} \right],$$

where $k_B$ is Boltzmann’s constant, $T$ is the absolute temperature, $L_p$ is the persistence length (which describes the bending rigidity of the protein) and $L_c$ is the contour length of the polypeptide. The change in contour length that results from unfolding of a protein domain is a measure for the unfolded length of the domain (Fisher et al., 1999). The applicability of the WLC to describe the unfolding force data of proteins has been demonstrated for a wide range of proteins (Best et al., 2001; Carrion-Vazquez et al., 1999a,b; Li et al., 2001; Marszalek et al., 1999; Ober dorfer et al., 2000; Oberhauser et al., 1998, 2002; Rief et al., 1997). In fitting the force-extension data, we used a constant value of 0.42 nm for the persistence length of all unfolded peptides, while the contour length was allowed to vary (Oberhauser et al., 1998). The persistence length value of 0.42 nm was chosen based on previous results of WLC fits to force-extension measurements on FN-III domains (Rief et al., 1998b), and a similar range between 0.3 and 0.5 nm found for a variety of proteins (Fisher et al., 2000a,b; Ober dorfer et al., 2000; Rief et al., 1998b). Keeping the persistence length constant allowed us to obtain a more consistent measure for the contour length of a protein module. The WLC model’s ability to fit the experimental data was gauged by the values of $r^2$ (coefficient of determination), obtained from non-linear curve fitting (TableCurve, SPSS).

3.4. Data analysis

For each domain, the unfolding force was taken as the maximum force of the unfolding peak with respect to the zero force base line. Different protein domains (FN-III or GFP) were distinguished based on their characteristic contour length increase upon domain unfolding (distance signature). Two methods have been applied to determine this distance signature. The first method considers the increase in contour length upon domain unfolding as the difference in the distance at the maximum force value between two successive unfolding events (Ober dorfer et al., 2000). The second method uses the increase in contour length as estimated from the fitting of two successive unfolding peaks to a polymer elasticity model (WLC model) (Oberhauser et al., 2002). In comparing both methods, one finds that the first method ignores the differences in the force magnitudes for successive peaks used to determine the increase in contour length, and the second method sometimes fails to give reliable fits due to the typically lower number of data points at larger forces. We used both methods to analyze a significant subset of our data and we found that they gave similar results (Fig. 7). Therefore, for simplicity, the data reported here were obtained by the first method unless otherwise mentioned.

Since we know the number of amino acids in each domain, we can match the experimentally observed unfolding length to the theoretically expected value. FN-III domains have 90 amino acids each and are expected to unfold to an overall length of 27 nm using an amino acid length of 0.34 nm, and subtracting a distance of 3.6 nm, corresponding to the size of the folded protein (Oberhauser et al., 1998). Although the maximal distance between adjacent α-carbon atoms is 0.38 nm, the tetrahedral geometry reduces this spacing to about 0.34 nm per residue when it is in a fully extended β-sheet conformation (Yang et al., 2000). Similarly, GFP is expected to unfold to a maximum distance of 79 nm since the protein consists of 239 amino acids and has a folded size of 2 nm (distance between N-and C-termini) (Ormo et al., 1996). Therefore, each unfolding event that resulted in an increase in contour length ranging between 5 and 32 nm was attributed to a FN-III domain, and any unfolding event that resulted in an increase in contour length ranging between 40 and 82 nm was attributed to a GFP.

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