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Mechanical Unfolding of a Titin Ig Domain: Structure of Unfolding Intermediate Revealed by Combining AFM, Molecular Dynamics Simulations, NMR and Protein Engineering

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The mechanical unfolding of an immunoglobulin domain from the human muscle protein titin (TI I27) has been shown to proceed *via* a metastable intermediate in which the A-strand is detached. The structure and properties of this intermediate are characterised in this study. A conservative destabilising mutation in the A-strand has no effect on the unfolding force, nor the dependence of the unfolding force on the pulling speed, indicating that the unfolding forces measured in an AFM experiment are those required for the unfolding of the intermediate and not the native state. A mutant of TI I27 with the A-strand deleted (TI I27 − A) is studied by NMR and standard biophysical techniques, combined with protein engineering. Molecular dynamics simulations show TI I27 − A to be a good model for the intermediate. It has a structure very similar to the native state, and is surprisingly stable. Comparison with a Φ-value analysis of the unfolding pathway clearly shows that the protein unfolds by a different pathway under an applied force than on addition of denaturant.

Keywords: force microscopy; immunoglobulin; fibronectin type III; protein folding; titin

Introduction

Many proteins, including those found in muscle and the cytoskeleton, experience mechanical stress *in vivo*. These proteins are typically composed of multimodular arrays of independently folded domains arranged in tandem.[1] It has been suggested that such proteins may undergo cycles of unfolding and refolding in the presence of extreme force under physiological conditions.[2–5] TI I27, the 27th immunoglobulin (Ig) domain of human cardiac titin is found in the region of the sarcomere responsible for passive elasticity in muscle.[6] Its structure has been determined[7] and the folding pathway of the isolated domain has been characterised in detail.[8] Tandem repeats of the single domain have been constructed, allowing its mechanical properties to be investigated using atomic force microscopy (AFM).[9–12] Force-induced unfolding generates a series of sawtooth peaks, each corresponding to the unfolding of one TI I27 domain. The resistance of TI I27 to force is significantly greater than that of the majority of proteins that have been investigated to date.[13–16] This makes TI I27 an ideal candidate for detailed investigation of the mechanism of forced unfolding and

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Abbreviations used: AFM, atomic force microscopy; MD, molecular dynamics; Ig, immunoglobulin; TI I27, the 27th Ig domain of the I-band of human titin; TI I27 − A, a mutant of TI I27 with the A-strand deleted; N, the native state; I, the unfolding intermediate; ‡, the unfolding transition state; RMSD, root mean square deviation; ppm, parts per million; $\Delta\Delta G_{D-N}$, change in free energy of unfolding on mutation; [GdmCl]-50%, the concentration of guanidinium chloride at which half the molecules are unfolded; r_{NC}, the distance between the N and C termini of the protein.

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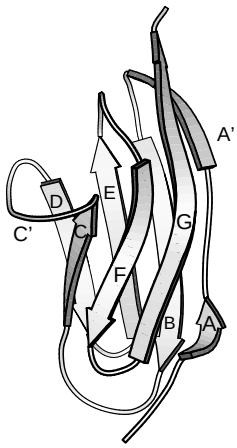

Figure 1. Structure of TI I27. MolScript[26] diagram of the structure of TI I27.[7] All residues from the A-strand were deleted upon construction of the TI I27 − A mutant.

for a comparison with unfolding in more conventional denaturant-induced unfolding experiments.

TI I27 has a β-sandwich fold with two antiparallel β-sheets packed against each other (Figure 1). Molecular dynamics (MD) and other simulations[17–20] have shown that detachment of the A-strand is the earliest step in forced unfolding. A metastable unfolding intermediate which appears to have the A-strand separated from the rest of the protein has been detected experimentally using AFM.[11] This was done by attributing the deviation of the force–extension curves from that expected for an ideal worm-like chain elasticity model to the detachment of the A-strand at a force of approximately 120 pN. However, the experimental results were not interpreted in terms of unfolding from this intermediate, but rather from the intact native state. Here we use analysis of a mutant protein to demonstrate that AFM measures the unfolding of the intermediate and not the native state. We use MD simulations, NMR and protein engineering techniques to characterise the structural and biophysical characteristics of this unfolding intermediate. We show that a mutant of TI I27 with the A-strand deleted (TI I27 − A) is a structural model for the intermediate. It is less destabilised relative to wild-type than might be predicted, and NMR spectroscopy and mutational analysis show that the structure

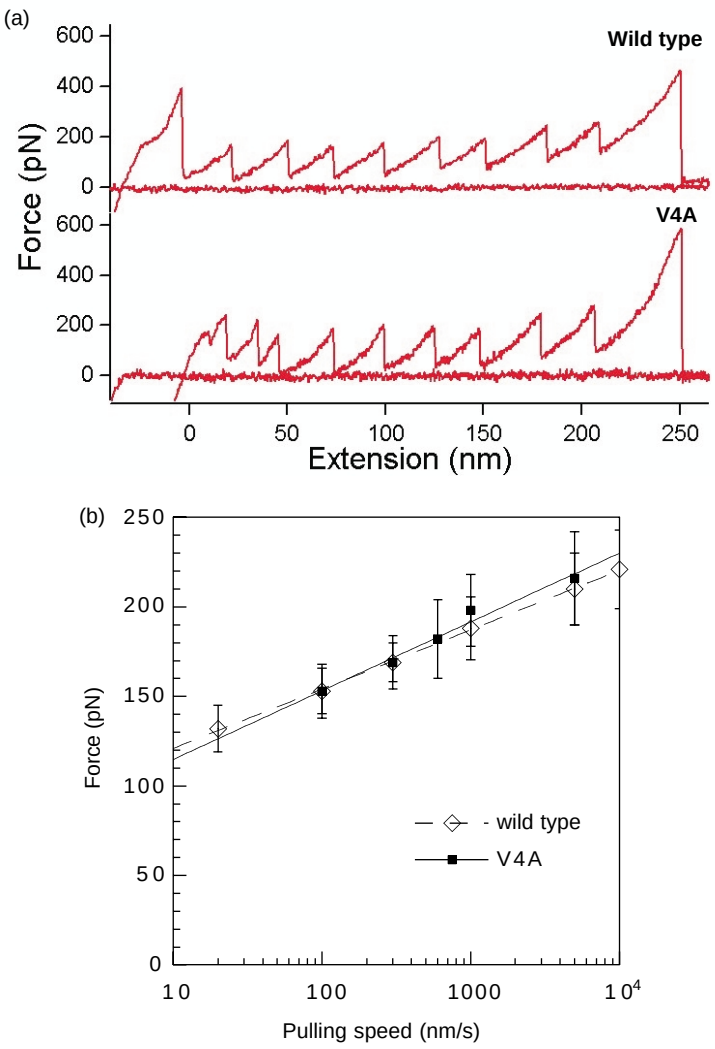

Figure 2. AFM data. (a) Force–extension curves showing the sequential unfolding of the wild-type TI I27 module and the V4A mutant at a pulling speed of 1000 nm s^{-1}. (b) Plot of the unfolding forces *versus* the logarithm of the pulling speed for wild-type TI I27 and V4A mutant. The mean of means of at least three separate experiments is shown with the standard deviation (~10%). All V4A data are within the error of the wild-type data at all pulling speeds.

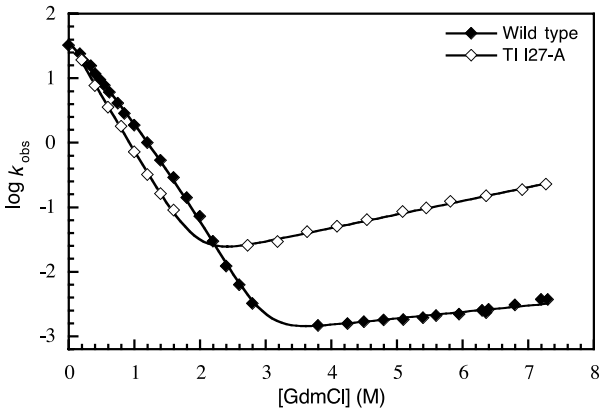

Figure 3. Folding kinetics of wild-type and TI I27 − A domains in denaturant. The stability lost upon deletion of this strand is mainly reflected in the unfolding kinetics and the unfolding rate constant of the mutant is significantly increased ($k_u^{H_2O}$(wt) = 4.9(±0.6) × 10^{-4} s^{-1}, $k_u^{H_2O}$(TI I27 − A) = 6.87(±0.03) × 10^{-3} s^{-1} where $k_u^{H_2O}$ is the unfolding rate constant in 0 M denaturant). The wild-type and mutant proteins have the same refolding rate constants (within error) at 0 M GdmCl, 35(±2) s^{-1} and 37(±3) s^{-1}, respectively, although the refolding intermediate that has been observed in wild-type refolding is destabilised upon mutation and folding becomes two-state in TI I27 − A, as is the case in many other mutants[8].

remains very similar to that of wild-type TI I27. MD simulations show that this mutant has essentially the same structure as the intermediate seen in forced unfolding. The results demonstrate that, in contrast to previous suggestions,[9] the mechanical unfolding pathway induced by AFM is different from that observed following addition of denaturant. This accords with MD comparisons of unfolding by high temperature and external forces.[20]

Results

Destabilisation of the A-strand has no effect on the force required to unfold TI I27

A mutant polyprotein was constructed containing eight TI I27 domains in tandem, each with a Val to Ala mutation at residue 4 in the A-strand (V4A) (Figure 1). This mutation results in a significant loss in thermodynamic stability ($\Delta\Delta G_{D-N}$ = 2.45(±0.10) kcal mol^{-1}).[8] Importantly, the mutation has the same effect on stability of both single domains and the 8-module construct in equilibrium denaturation experiments (data not shown). The loss in stability is mainly (but not exclusively) reflected in an increase in the unfolding rate constant.[8]

If the interactions made by the side-chain of Val4 were to contribute to the mechanical stability, the mutation would be expected to result in lower unfolding forces. AFM was used to study force-induced unfolding of both wild-type and V4A 8-module proteins at a range of pulling speeds. A characteristic sawtooth pattern was observed, where each peak corresponds to the unfolding of a single domain (Figure 2(a)). The dependence of the mean unfolding force on the logarithm of the pulling speed for wild-type and mutant is shown in Figure 2(b). The mutant data fall within the same range as repeated measurements of the wild-type protein. There is no significant difference in the force required to unfold V4A and wild-type TI I27, nor in the dependence of the force on pulling speed.

A model for the mechanical unfolding intermediate

AFM experiments and simulation show that formation of the unfolding intermediate results from early detachment of the A-strand and suggest this structure has significant mechanical stability.[11] To determine whether this is a structurally reasonable model, a mutant of TI I27 was constructed with the whole A-strand deleted (the first seven residues of the protein, LIEVEKP, which pack directly on the B- and G-strands (Figure 1). This "model" of the intermediate is soluble and folded in solution. The free energy for unfolding (ΔG_{D-N}) of TI I27 − A is 4.8(±0.1) kcal mol^{-1} at 25 °C, a loss in stability of only 2.8 kcal mol^{-1} compared to wild-type (7.6(±0.1) kcal mol^{-1}), which is surprisingly low considering the amount of secondary and tertiary structure removed. Deletion of the A-strand is primarily reflected in a significant increase in the unfolding rate constant (Figure 3)†.

Structure of the model intermediate

(a) NMR studies. To ascertain the extent of structural rearrangement, ^{15}N HSQC spectra of the wild-type and TI I27 − A mutant were obtained. Changes in chemical shift are a sensitive indicator of structural change. Only residues that directly contact the deleted strand or form a charged surface network that has been disrupted on removal of three charged residues (Glu3, Glu5 and Lys6) show significant changes in chemical shift (Figure 4). These include residues in the B–C, D–E and F–G loops (Figure 4(b)). The fact that only local

† Note that wild-type TI I27 folds *via* a marginally stable folding intermediate in folding studies.[8] This is not the same as the unfolding intermediate described in this manuscript. We have previously shown that most mutations destabilise the folding intermediate so that folding moves to a 2-state pathway. Removal of the A-strand has the same effect, resulting in the loss of "roll over" in the folding arm of the data (Figure 3). This is consistent with previous studies showing that, in the isolated domain, the A-strand is partly structured in the folding transition state of TI I27 (transition state ϕ-values ≈ 0.3–0.4).[8]

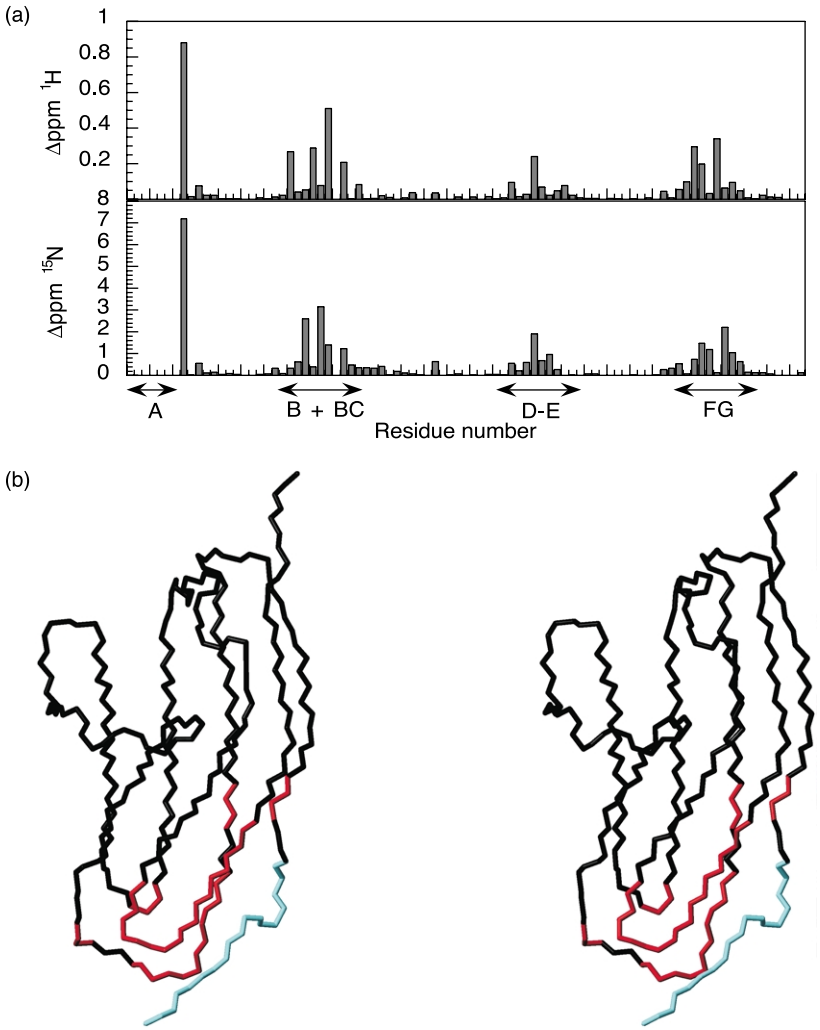

Figure 4. NMR chemical shift analysis indicates little structural rearrangement in TI I27 − A. (a) ^{15}N and ^{1}H shift changes (Δppm) for each residue upon deletion of the A-strand. (b) Stereo diagram of TI I27. Residues in red are those with a Δppm > 0.1 in the ^{1}H and/or >1.0 in the ^{15}N dimensions upon deletion of the A-strand. These changes are local or attributed to the disruption of charged surface interactions. Residues deleted in TI I27 − A are shown in cyan.

Table 1. Mutations in TI I27 − A affect the stability in the same way as mutations in wild-type TI I27

Mutation	Position in TI I27	Position of non-local contacts[a] deleted in TI I27	$\Delta\Delta G_{D-N}$[b] in TI I27 (kcal mol^{-1})	$\Delta\Delta G_{D-N}$[c] in TI I27 − A (kcal mol^{-1})
V13A	A′-strand	B, E-F, G	2.15 ± 0.1	2.37 ± 0.1
I23A	B-strand	A, C, E, F, G	2.70 ± 0.1	2.89 ± 0.1
L41A	C′-strand	C, D	2.70 ± 0.1	2.67 ± 0.1
L58A	D-strand	B, C, D, F	3.23 ± 0.1	3.61 ± 0.1
L60A	D-strand	B, C, C′ D, E-F, F, G	4.88 ± 0.1	5.27 ± 0.1
F73L	F-strand	B,C,D,E,F	3.83 ± 0.1	3.06 ± 0.1
V86A	G-strand	A′ E-F, F, G	4.45 ± 0.1	4.78 ± 0.1

[a] Single letter represents β-strand, hyphenated letters represent loops between strands.
[b] Data taken from Ref. 8.
[c] Calculated using equation (1) and a mean *m*-value of 3.25 kcal mol^{-1} M^{-1} for TI I27 − A and all mutants.

effects are observed shows that there has been no overall change in structure.

(b) Mutation studies. A number of mutations have been made in the TI I27 − A construct covering the majority of structural elements. The loss of free energy on mutation was compared with the effect of the same mutation in the wild-type protein. All mutations with the exception of F73L result in a very similar loss of stability, $\Delta\Delta G_{D-N}$, as in the wild-type, confirming that the structural rearrangement is small (Table 1). The mutation F73L is slightly less destabilising in TI I27 − A than the same mutation in the wild-type structure although it has no contact with the deleted strand (Table 1).

(c) MD simulations. MD simulations were used to compare the structure of the AFM unfolding intermediate with that of TI I27 − A. To model the intermediate, a number of MD simulations up to 5 ns in length were performed on TI I27 by applying a constant pulling force of 400 pN, 350 pN or 300 pN along a reaction coordinate defined as the distance between the N- and C termini, r_{NC}. In general, the larger the force, the faster the unfolding, but only at 400 pN did all simulations result in an unfolding event on the 5 ns timescale. At

300 pN full unfolding was not observed in any simulation over a 5 ns timescale. Figure 5(a) shows r_{NC} of TI I27 as a function of time for three simulations, each with a different constant force.

In all simulations with all these forces the first event, even with the lowest force, is an extension of the native state. In simulations of native TI I27 in the absence of applied force, r_{NC} oscillates between 43 Å and 46 Å, but in the presence of a force the r_{NC} oscillates between 46 Å and 49 Å without unfolding. This extension results mainly from changes in the N-terminal region. Overall, the native state under force is essentially identical with that in the absence of a force, the RMSD from the native structure is less than 2 Å, and essentially all native state contacts are maintained. In all simulations at all forces studied, the protein then unfolds partly to populate a metastable intermediate (I) with $r_{NC} \approx 53$ Å (i.e. at an extension of about 7 Å relative to the native state). This intermediate is stable on the 3 ns timescale with forces of 300 pN (or lower). The lifetime of I decreases as the magnitude of the applied force increases; i.e. at 400 pN, I unfolds further in all simulations but at 350 pN it is unfolded further in only some of the simulations. The last 2 ns of a 3 ns simulation with an applied force of 300 pN (Figure 5(a)) were used to determine the properties of the intermediate. The A-strand is essentially detached from rest of the protein (Figure 5(b)), but in spite of an elongation of ~7 Å, the protein preserves most of the structural characteristics of the native state. The RMSD from the native structure for the rest of the protein is ~2 Å, the same as that found for the native state in the presence of applied force (see above). Analysis shows that I and N are indistinguishable in terms of the native-like side-chain contacts made in the two states for most of the protein (Figure 5(b) and (c)). Major losses of native contacts are concentrated around strand A, and around the B–C, D–E and F–G loops, precisely the regions identified in the NMR experiments on TI I27 − A, where contacts with residues in the A-strand are lost.

Simulations of the TI I27 − A mutant in the absence of applied force resulted in a stable structure over 2 ns that is very similar to I (Figure 5(d)) with a RMSD of 1.6 Å (excluding residues in the flexible C′–D loop). In both states, residues Leu25, in the B-strand, V30 in the B–C loop and Asn77 and Ser80 in the F–G loop lose most of their native contacts. The first residue is hydrogen bonded to the A-strand in the native state, while the others make side-chain contacts with it. Interestingly, the simulations show that a number of residues make more contacts in both the TI I27 − A mutant and I than in the native state. This is most evident for residues Thr18, Leu60, His61, Leu65 and Met67 that form a cluster involving both sheets in the C-terminal region. The L60A mutant was, indeed, observed to be slightly more destabilising in TI I27 − A than in TI I27 (Table 1). These new contacts are likely to stabilise both the mutant and the intermediate and suggest there is some repacking on removal of the A-strand. This may explain the observation that deletion of the A-strand results in a loss of stability (2.8 kcal mol^{-1}) that is similar to that observed in the mutation of Val4 to Ala alone (2.5 kcal mol^{-1}).[8]

In the simulations at 400 pN and the simulations at 350 pN that unfolded, the transition state for mechanical unfolding appears to be around $r_{NC} \approx 59$ Å (Figure 5(a)), after which unfolding proceeds rapidly and without hindrance, irrespective of the magnitude of the force. This result and its relevance to the AFM experiments will be discussed elsewhere.

Discussion

The mechanical unfolding pathway of TI I27

Both experiment and MD simulations indicate that the mechanical unfolding of TI I27 proceeds *via* a metastable intermediate, $N \rightarrow I \rightarrow \ddagger \rightarrow D$. Marszalek *et al.*[11] have shown that the intermediate is populated at forces >120 pN, significantly below the force required for the unfolding of TI I27 (~200 pN at a pulling speed of 1 μm s^{-1}). In confirmation of this we have measured the mechanical unfolding of a mutant protein with the destabilising A-strand mutation V4A. Although the mutation of Val4 to Ala results in significant destabilisation of the native state, the unfolding forces for V4A are the same as for wild-type within the error of the experiment (Figure 2). The probability of the protein unfolding at any given force and any given pulling speed is related to the height of the energy barrier between the initial state and the transition state for unfolding in the rate-limiting step. This result shows that the mutation V4A does not change the height of the energy barrier for mechanical unfolding. These data are consistent with the three-state model in which the unfolding of I is the rate-determining step (Figure 6).

An important corollary of these results is that AFM experiments measure the unfolding of the intermediate and not the native state. Thus, an essential step in the analysis of the mechanical unfolding pathway of TI I27 is characterisation of the unfolding intermediate.

The properties of the unfolding intermediate

TI I27 − A construct maintains its structural integrity without the A-strand with a small loss of stability, (2.8 kcal mol^{-1}) similar to that resulting from point mutations in this strand; in a previous study[8] Ile2 and Val4 were both mutated to Ala with losses in stability of 1.55(±0.09) kcal mol^{-1} and 2.45(±0.10) kcal mol^{-1}, respectively. To establish that TI I27 − A is a good model for the unfolding intermediate, MD simulations of the forced unfolding intermediate were compared with simulations of TI I27 − A in the absence of external

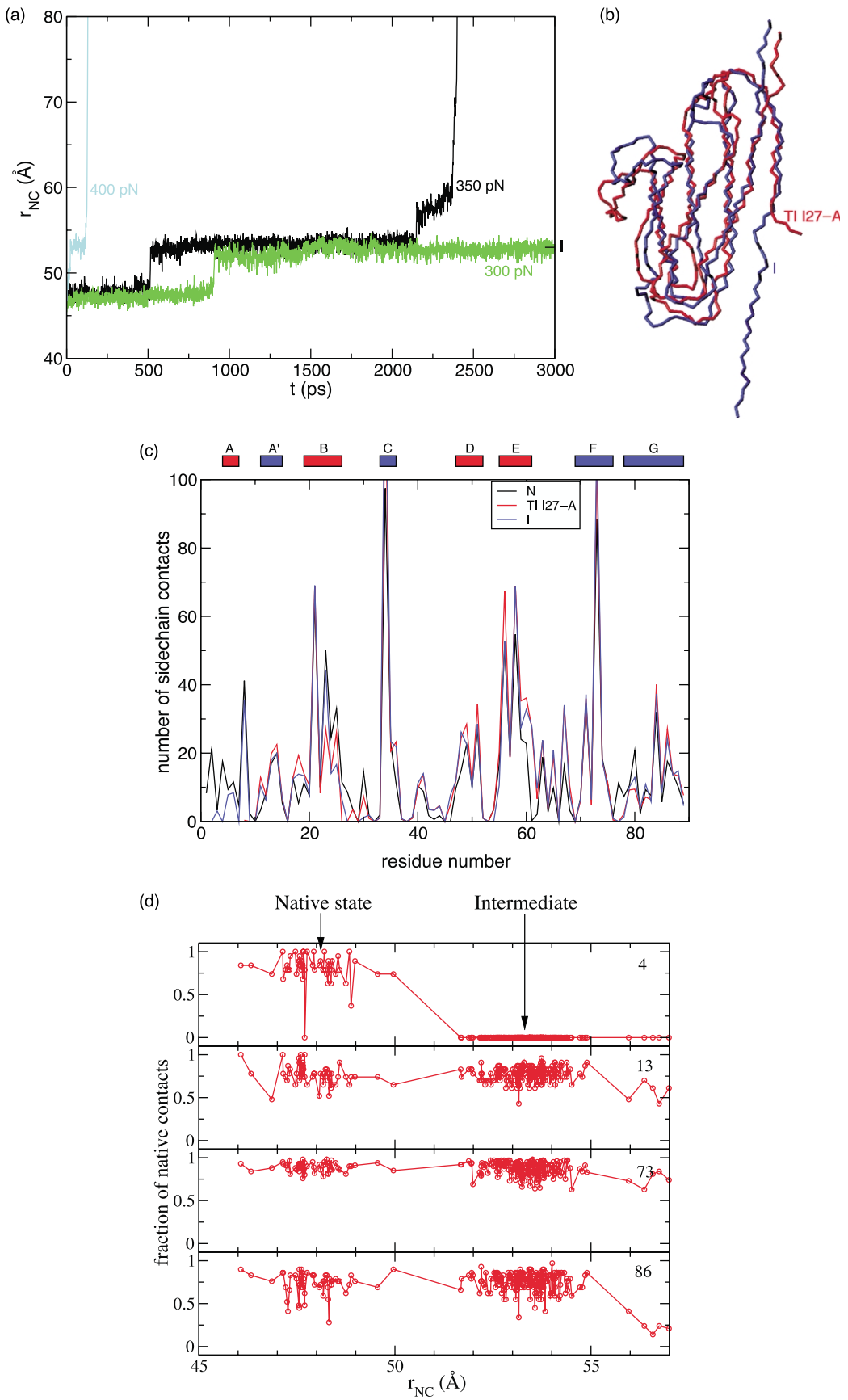

Figure 5. MD simulations. (a) The length r_{NC} of the TI I27 domain as a function of time for three simulations. In all three simulations a metastable intermediate (I) is observed. At 300 pN (green) I is stable for >3 ns. At an applied force of 350 pN (black) I is seen to unfold further after ~ 2.7 ns, while at 400 pN (cyan) I is short-lived and the transition state is crossed at $r_{NC} \approx 59$ Å. (b) Representative structures from MD simulations of TI I27 − A (red) and I

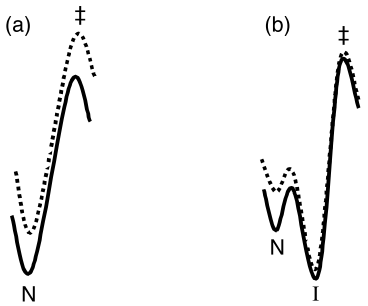

Figure 6. V4A mutant. The observed unfolding force is the force required to unfold the intermediate and not the native state. (Continuous line represents wild-type, dashed line is V4A mutant.) There are two plausible models to explain the observation that the unfolding forces for V4A are the same as wild-type. At any given pulling speed the probability of the protein unfolding at any given force is related to the height of the energy barrier between the starting state and the transition state for unfolding in the rate-limiting step. This result means that the mutation does not change the height of the energy barrier for mechanical unfolding. (a) Consider a 2-state system, N → ‡ → D. If a mutation that destabilises N has no effect on the force required to unfold the protein, then the mutation has no effect on the height of the unfolding energy barrier and must therefore destabilise the transition state (‡) to the same extent as N. This explanation requires that Val4 is as structured in ‡ as in N. This is most unlikely, since MD and other simulations and previous AFM experiments have clearly indicated that the detachment of the A-strand is the first step in forced unfolding and in denaturant-induced unfolding this strand is partly detached early in unfolding even in the absence of force ($\phi \approx 0.4$). (b) Consider a 3-state system, N → I → ‡ → D. At a low force (below the observed unfolding force) the unfolding intermediate, I is more stable than N. (The transition between I and N has been observed to occur at ≈120 pN.) Now the barrier for unfolding is I → ‡. If the mutation that destabilises N is in part of the protein that is fully unstructured in I and in ‡, this mutation will have no effect on the force required to unfold the protein. The results obtained for V4A are fully consistent with such a model.

force. They showed that the structures are indeed very similar, in terms of RMSD and the average number of side-chain contacts for each residue. Since the AFM experiments are monitoring the unfolding of an intermediate that has the same structure as TI I27 − A, we now have biophysical and kinetic data on the properties of this AFM unfolding "ground state" and its response to denaturant and mutation.

The NMR studies indicate, in accord with the MD simulations, that removal of the A-strand does not significantly perturb the structure of TI I27. The MD simulations also suggest that the relatively small $\Delta\Delta G$ found on deletion of the A-strand is due to the formation of a small number of new (non-native) interactions in TI I27 − A that compensate for the loss of the A-strand. The overall structural integrity is confirmed by the effect of conservative side-chain deletion mutations that probe a number of regions of the molecule; they affect the stability of TI I27 and TI I27 − A in the same way. In response to denaturant, TI I27 − A folds at the same rate as the wild-type protein, but it unfolds significantly more rapidly.

The mechanical and denaturant-induced unfolding pathways are different

Previous studies that compared the unfolding of titin domains using AFM and chemical denaturants suggested that the unfolding pathways are similar, on the basis of two lines of evidence:[9,12] (1) the unfolding rate, extrapolated to zero force estimated by analysis of the AFM data, was the same as the unfolding rates at 0 M denaturant estimated from kinetic stopped-flow experiments; (2) in both cases the transition state for unfolding was very close to the state (presumed to be the native state) from which unfolding occurred. By combining protein engineering with AFM and MD simulation we show here that there are, in fact, significant differences between the unfolding pathway induced by force and that induced by denaturant in the absence of external forces. First, an extensive protein engineering Φ-value analysis on single TI I27 domains by conventional methods has shown that the only region of structure that is completely unfolded in the transition state is the A′–G region.[8] Residues in the A-strand still make some native contacts in this transition state and there is no detectable unfolding intermediate. Second, it is now clear that the correspondence between the unfolding rates at zero force and 0 M denaturant was misleading. Since it has now been established that unfolding of the intermediate, and not the native state, is the rate-determining step being measured in the AFM experiment, the appropriate comparison is with the unfolding of the intermediate, I, that unfolds significantly faster than the wild-type protein in response to denaturant.

(blue). (c) Comparison of the average number of side-chain contacts for the native state (N—black), TI I27 − A (red) and the intermediate (I—blue). I and TI I27 − A lose contacts with the A-strand but have a similar number of contacts as N in the rest of the structure. (d) Detailed analysis of number of native contacts for certain residues along the trajectory (at 350 pN). Each data point represents a single structure sampled at 10 ps intervals. V4 (A-strand) loses all contacts in I but other residues including V13 (A′-strand), F73 (F-strand) and V86 (G-strand) maintain all native contacts in I. Other simulations at different forces show corresponding behaviour although the time of forming the intermediate and unfolding is different in different simulations as expected (see the text).

Conclusions

MD simulations show that a stable mutant with the A-strand deleted is a good model for the mechanical unfolding intermediate of TI I27 observed by AFM. This model for the intermediate has been characterised experimentally and been shown to be stable and to have a structure very similar to the wild-type protein. The forces measured in the AFM experiments relate to the unfolding of this intermediate where the A-strand has already detached, and residues in this strand play no role in the mechanical transition state structure or in maintaining mechanical stability.[11,17–20] Simulation shows that upon deletion/detachment of the A-strand, some new, non-native contacts are formed. This may be an important factor in maintaining the stability of the intermediate under force and preventing total unfolding of the domain directly from the native state. Such an unfolding intermediate has also been observed in the 28th Ig domain of the I-band, TI I28, which has a significantly lower thermodynamic stability than TI I27, yet is more resistant to force.[11,12] It has been suggested that the presence of unfolding intermediates plays a significant role in the elasticity of titin.[11] As shown here, in the intermediate the domain retains its structural integrity, remains stable (both thermodynamically and mechanically), and yet allows a significant lengthening of the titin molecule to be achieved if the same unfolding mechanism under force applies to all Ig molecules in the I-band.

Methods

Production of proteins

TI I27 and all mutants were expressed as described.[8] Deletion of the A-strand (first seven residues) was performed using standard PCR techniques on the parent plasmid (pTI I27) and the mutations were introduced using the Quik Change kit from Stratagene. All proteins were purified using Ni^{2+} affinity chromatography. The 8-module AFM constructs (both wild-type and mutant) were produced as described.[21] In brief, eight identical repeats of TI I27 or the mutant TI I27 V4A were cloned using nine different restriction sites into an expression vector which encodes a $(His)_6$ tag at the N terminus for easy purification. This tag is left on the protein after purification, which possibly explains why on occasion AFM traces with eight unfolding peaks are observed. At the C terminus, two cysteine residues were incorporated for attachment of the protein to the AFM surface.

Chemical denaturation studies

Thermodynamic and kinetic properties of wild-type and mutant proteins were determined as described[8] using guanidinium chloride (GdmCl) as a denaturant. All experiments were carried out in phosphate buffered saline, pH 7.4 with 5 mM DTT at 25 °C. The change in free energy upon mutation, $\Delta\Delta G_{D-N}$ was determined according to equation (1):

$$\Delta\Delta G_{D-N} = \langle m \rangle \, \partial[\mathrm{GdmCl}]_{50\%} \quad (1)$$

where $\langle m \rangle$ is the mean m-value of wild-type and all mutants and $\partial[\mathrm{GdmCl}]_{50\%}$ is the difference in [GdmCl] at which 50% of the protein is denatured.[22] For mutants of TI I27 − A each was compared to the wild-type TI I27 − A and not to native TI I27.

Atomic force microscopy

All AFM experiments were carried out using a molecular force probe (Asylum Research, Santa Barbara, CA). The cantilevers were Si_3N_4 microlevers (Thermomicroscopes, Sunnyvale, CA) and calibration was carried out in solution. These have a spring constant of approximately 30 pN nm^{-1}; the exact value was calibrated from the response of the cantilever to thermal noise. The protein was placed in PBS for 10–20 min onto a freshly evaporated gold surface on a microscope slide, and then washed thoroughly. All experiments were performed at room temperature (20–24 °C) in PBS. Data were analysed using Igor Pro software (Wavemetrics Inc., Lake Oswego, OR). At least 40 unfolding events were measured at each pulling speed and data were collected on at least three days using a different cantilever each time. The mean pulling speed was determined each day and the mean of these means (± standard error) are reported in Figure 2.

NMR spectroscopy

^{15}N HSQC spectra of ^{15}N labelled TI I27 wild-type and TI I27 − A mutant proteins were recorded on a Bruker (Karlsruhe, Germany) AMX-500 spectrometer at 308 K in deuterated acetate buffer pH 4.5.

Simulations

MD simulations were performed using the CHARMM program[23] and potential[24] plus a continuum representation of the solvent.[25] The method used in the simulation has been described.[20] The protein was energy minimised and slowly heated to 300 K temperature, and then equilibrated during 2 ns; the RMSD from native was 2.5 Å on average during equilibration. Unfolding was induced by applying a constant pulling force on a reaction coordinate which is the distance between the N and the C termini (r_{NC}).[20] Three simulations up to 5 ns in length were performed at each of three pulling forces, 300 pN, 350 pN and 400 pN. The force is applied parallel with r_{NC} and directed in the direction of increasing r_{NC}. All simulations were performed at constant 300 K temperature using the Nosé–Hoover thermostat and using a 2 fs integration timestep. Conformations for further analysis were saved every picosecond.

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