questions concern the mechanism of transfer, the frequent co-insertion of non-coniguous mitochondrial insertions and the presence of mitochondrial insertions in the vicinity of LTRs and tRNA genes.

Methods

Experimental methods

The two haploid strains used are derivatives of FYB12-3D (MAT-a, ura3Δ1, leu2Δ2, trp1Δ63), in which YK1222 (position 3507–3621) or YK9086 (position 632659–634809) have been replaced by the UR3-I scr cassette. Both strains are isogenic to the FY1679 (S288C) strain whose nuclear genome has been sequenced, except for the markers indicated. Both mutants retained the wild-type phenotype of the haploid cell. Yeast cells were transformed with the replicative I- scr expression plasmid, pPE7, by the lithium acetate method. Analysis of repair was performed by PCR directly on cells using 20-mer oligonucleotides whose 5’-end coordinates are 2725 and 6106 for FYB12-3D Δykr098c, 3451 and 635483 for FYB12-3D Δykr098c:URA1-I-scr (see Fig. 1). In both cases the primer distal to the centromere was biotinylated. Polymere chain reaction products were separated on 1.5% agarose gels. Under these conditions insertions or deletions of at least 50 bp are detectable. These PCR-amplified DNA fragments were cut out from the gel and purified with QiAquick PCR Purification kit (Qiagen). The appropriate single-strand DNA was separated with streptavidine and then sequenced.

The Δyme1 mutant of the strain FYB12-3D Δykr098c:URA1-I-scr was obtained with the one-step gene disruption technique by replacing YPR024w (YME1) with the KANMX module12. The presence of the KANMX module instead of YPR024w was molecularly checked by PCR amplification of the specific fragment and by the restriction pattern of the amplified fragment with different restriction enzymes. As described for the yme1 mutants14, the double mutant Δyme1 Δykr098c constructed here did not form p+ or p- colonies and was unable to grow on glucose at 14°C and on glycerol at 37°C. The p+ and p- mutants of the FYB12-3D Δykr098 strain were obtained as described15. By definition, p- strains retain only a fraction of the wild-type mitochondrial genome, often in an amplified and rearranged configuration, and p+ strains have no mitochondrial DNA.

Sequences with probability > 90% for the longer sequences) suggests that some of these may correspond to size and that a third of them are present in coding regions of chromosomes (compared to genome. Although statistically significant, the fact that these sequences have such a small sequences originate from both coding and non-coding regions of the mitochondrial genome. Although statistically significant, the fact that these sequences have such a small size and that a third of them are present in coding regions of chromosomes (compared with only 9% for the longer sequences) suggests that some of these may correspond to random incidence rather than actual transfer. These sequences have not been taken into consideration in our conservative analysis.

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Mechanical unfolding intermediates in titin modules

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The modular protein titin, which is responsible for the passive elasticity of muscle, is subjected to stretching forces. Previous work on the experimental elongation of single titin molecules has suggested that force causes consecutive unfolding of each domain in an all-or-none fashion1–9. To avoid problems associated with the heterogeneity of the modular, naturally occurring titin, we

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The modular protein titin, which is responsible for the passive elasticity of muscle, is subjected to stretching forces. Previous work on the experimental elongation of single titin molecules has suggested that force causes consecutive unfolding of each domain in an all-or-none fashion1–9. To avoid problems associated with the heterogeneity of the modular, naturally occurring titin, we
engineered single proteins to have multiple copies of single immunoglobulin domains of human cardiac titin. Here we report the elongation of these molecules using the atomic force microscope. We find an abrupt extension of each domain by \( \sim 7 \) Å before the first unfolding event. This fast initial extension before a full unfolding event produces a reversible 'unfolding intermediate'. Steered molecular dynamics simulations show that the rupture of a pair of hydrogen bonds near the amino terminus of the protein domain causes an extension of about 6 Å, which is in good agreement with our observations. Disruption of these hydrogen bonds by site-directed mutagenesis eliminates the unfolding intermediate. The unfolding intermediate extends titin domains by \( \sim 15\% \) of their slack length, and is therefore likely to be an important previously unrecognized component of titin elasticity.

The complex mechanical design of the elastic protein titin is still not understood\(^1\). Recent in vitro studies using optical tweezers and atomic force microscopy (AFM) have revealed at least two components, where titin elasticity is governed by the entropic behaviour of its segments and the unravelling of its folded domains\(^2\). The force–extension relationship of titin was explained by models of simple entropic elasticity\(^3\), \(^5\) in which module unfolding increased the contour length of the protein in a stepwise fashion\(^5\). In the experiments using an atomic force microscope (AFM), extension of titin domains generated a force–extension relationship with a characteristic sawtooth pattern of equally spaced peaks that result from the sequential unfolding of its modules\(^2\). In these recordings, the force–extension relationship of titin leading up to an unfolding event is described by the worm-like chain (WLC) model of polymer elasticity\(^2\), \(^3\), \(^5\). However, close examination of the rising phases of the force peaks shows that they deviate from the shape predicted by the WLC model, raising the possibility that there are additional components in the elasticity of titin modules which have been missed. This is particularly evident in the rising phase of the first force peak which shows the elasticity of the protein before any unfolding event\(^5\).

Figure 1a shows that the force–extension curve of a native titin fragment strongly deviates from the expected entropic elasticity (WLC), revealing a pronounced 'hump' that tends to disappear on unfolding of all the modules. Native titin is a highly heterogeneous polymer, and therefore it is difficult to relate its elastic properties to individual structural elements. Hence, to examine the elastic components of titin modules at high resolution we engineered polyproteins composed of identical tandem repeats of either the I\(^28\) module, revealing fine mechanical details at high resolution.

Force–extension curves for the I\(^28\) and I\(^27\)\(_12\) polyproteins showed the characteristic sawtooth pattern, corresponding to the sequential unfolding of their modules (Fig. 1b–d). Fits of the WLC to the force–extension curves leading up to the first peak (Fig. 1b–d; thin lines on the first peak) show a large deviation from simple entropic elasticity that becomes apparent at 108 \( \pm 19 \) pN (127, \( n = 87 \)) and 151 \( \pm 16 \) pN (128, \( n = 30 \)), well before the unfolding events occur (210 \( \pm 27 \) pN, 127, \( n = 87 \); 264 \( \pm 49 \) pN, 128, \( n = 30 \)). When the force reaches \( \sim 100–150 \) pN (Fig. 1b–d), the slope of the force–extension curve decreases sharply creating the shape of a 'hump' that deviates from the line predicted by the WLC model. After this deviation, the slope of the curve increases again, after which the force reaches a maximum of about 200–300 pN where domain unfolding occurs.

Abrupt slope changes in the force–extension curve of a molecule, such as those shown in Fig. 1b–d, have been observed in polysaccharides and have been shown to correspond to force-induced transitions that elongate the molecule by a small amount\(^2\), \(^3\). Hence, it is likely that the 'hump' observed in the force–extension curves of I\(^28\) and I\(^27\)\(_12\) corresponds to a previously undetected force-driven transition that elongates folded modules by a small amount at forces of 150 pN (I\(^28\)) and 100 pN (I\(^27\)). This elongation is a property of folded modules only. The hump in the force–extension relationship is most evident in the first peak, but it diminishes gradually with module unfolding and disappears completely when all the modules have unfolded and the protein is fully extended (Fig. 1; thin lines on the last peak).

The structure of the I\(^27\) module of titin is known, and its mechanical topology has been examined by molecular dynamics\(^5\), \(^2\) and single-molecule AFM\(^7\). Thus, the I\(^27\) module is ideal to examine the molecular origin of the 'hump'. In Fig. 2, we analyse in detail the transitional extension observed at \( \sim 100 \) pN in the I\(^27\)\(_12\) polyprotein. We measured the width of this transition as a contour length increment, \( \Delta L_\text{c} \), obtained by fitting the WLC model before and after the transition, as shown in Fig. 2a. These measurements were done on the transition observed in the first peak where the transition is most prominent. Although the I\(^27\)\(_2\) polyprotein is engineered with 12 tandem modules, the AFM tip rarely picks up the protein from its termini. Hence, the AFM tip picks up proteins of various lengths, from very short proteins (2 modules long) up to their maximum length (12 modules long). We found that \( \Delta L_\text{c} \), measured in the first unfolding peak, increases linearly with the total number of folded modules of the polyprotein fragments picked by the AFM tip (Fig. 2b). A linear regression of the data in Fig. 2b gives a slope of 6.6 Å per module.

This result indicates that at \( \sim 100 \) pN, a folded domain undergoes a transitional extension of 6.6 Å; this extension contrasts with the much larger 284 Å extension caused by the full unfolding of the I\(^27\) module observed at \( \sim 200 \) pN (ref. 7). Figure 2c shows a histogram of the force at which the first transitional extension is observed compared with the unfolding force. A gaussian fit of the data (Fig. 2c; solid lines) gives a force of 108 pN for the small transitional extension, and 210 pN for the full unfolding event (\( n = 87 \)).
However, the unfolding forces are dependent on the rate at which the protein is pulled\textsuperscript{7,12}. Hence it is likely that the small transitional extension observed here may also occur at much lower forces.

Although the small transitional extension of the I27 module is most prominent in the first unfolding peak of the force–extension curve, this transition is also observed in consecutive peaks, albeit with decreasing amplitude (Fig. 1). These observations put together suggest that the transition observed in the first peak corresponds to the accumulated transitional extension of all the folded domains. The reappearance of a progressively smaller transition in consecutive peaks suggests that the transition is reversible upon relaxation of the force (peak to trough transition), and that a small transitional extension of the modules that remain folded occurs again at a force of \( \sim 2 \) A\textsubscript{\text{N}} at forces larger than 150 pN (Fig. 3b). The discontinuity observed between 50 and 100 pN corresponds to an abrupt extension of the module by 4–7 A\textsubscript{\text{N}} caused by the rupture of the AB hydrogen bonds, and the subsequent extension of the partially freed polypeptide segment. Figure generated using the program VMD\textsuperscript{30}.

Simultaneous rupture of these hydrogen bonds is the critical event that allows the full unravelling of the module under an applied force\textsuperscript{23}. These simulations showed that the rupture of the two hydrogen bonds bridging the A and B \( \beta \)-strands was a minor event that preceded the rupture of the A’G patch\textsuperscript{29}. We examined whether the small extension that precedes the main unfolding event was due to the rupture of two hydrogen bonds bridging the AB \( \beta \)-strands (K6-E24). For this purpose, SMD simulations were performed in which forces of 20, 50, 100, 150, 200 and 500 pN were applied, each for 1 ns, to the termini of a single I27 domain. Upon application of these forces, the I27 domain extended within less than 200 ps to a quasi-equilibrium value that is plotted in Fig. 3b. Full unfolding within the 1-ns time window was observed only for the strongest force (500 pN). At forces of less than 50 pN the I27 module was forced to extend by \( \sim 2 \) A\textsubscript{\text{N}}, whereas for forces larger than 100 pN the quasi-stationary extensions ranged from 6 to 9 A\textsubscript{\text{N}}. Analysis of the SMD trajectories showed that at forces of 20 pN and 50 pN the hydrogen bonds between the A and B \( \beta \)-strands are maintained (Fig. 3b, line I) and that the 2-A\textsubscript{\text{N}} extension arising in this case is due to straightening of the terminal residues. At larger forces these hydrogen bonds break (Fig. 3b, line II), producing a discontinuous extension from 2 A\textsubscript{\text{N}} at lower forces to an additional 4–7 A\textsubscript{\text{N}} (at forces \( \geq 100 \) pN; Fig. 3b). The discontinuous elongation caused by the rupture of these hydrogen bonds is in good agreement with the 6.6-A\textsubscript{\text{N}} elongation observed with the AFM. This event occurs at low force and precedes the full unravelling of the I27 module.

Furthermore, an estimate of the mechanical work done to extend

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**Figure 2** The size of the hump-like deviation depends linearly on the number of folded modules. \( F_{hump} \) is the hump force, \( F_{unfold} \) is the force required to completely unfold the module, and \( F_{ref} \) is the reference force. The thin lines are fits of the WLC model to the data before and after the hump. The contour length of the second fit is \( L_\text{c} = 46.5 \) Å longer than the one before the hump. The dashed area represents the work done by extending the protein through its intermediate. The relationship between \( F \) (force) and \( L_\text{c} \) (contour length) is given by the equation \( F = kL_\text{c}^2 \), where \( k \) is the force constant. The relationship between \( F \) and \( L_\text{c} \) is shown in the inset graph, with the solid line representing a linear regression to the data. The contour length of the second fit is \( L_\text{c} = 46.5 \) Å longer than the one before the hump. The dashed area represents the work done by extending the protein through its intermediate.

**Figure 3** Steered molecular dynamics simulations of I27 extensibility under constant force. \( F_{hump} \) is the hump force, \( F_{unfold} \) is the force required to completely unfold the module, and \( F_{ref} \) is the reference force. The thin lines are fits of the WLC model to the data before and after the hump. The contour length of the second fit is \( L_\text{c} = 46.5 \) Å longer than the one before the hump. The dashed area represents the work done by extending the protein through its intermediate. The relationship between \( F \) (force) and \( L_\text{c} \) (contour length) is given by the equation \( F = kL_\text{c}^2 \), where \( k \) is the force constant. The relationship between \( F \) and \( L_\text{c} \) is shown in the inset graph, with the solid line representing a linear regression to the data. The contour length of the second fit is \( L_\text{c} = 46.5 \) Å longer than the one before the hump. The dashed area represents the work done by extending the protein through its intermediate.
Calibration of the spring constant of each individual cantilever was done in solution using elsewhere5. The I27-K6P point mutation monomer was generated by polymerase chain sawtooth force pattern that ended with a high-force peak that corresponds to stretching a coverslips. For the analysis of the polyproteins, we chose only recordings showing a their carboxy terminus to facilitate the attachment of the molecules to the gold-coated reaction using mutagenic primers. All these recombinant proteins have two Cys residues in

the modules at low force gives a value of 10.1 ± 0.63 kcal per mol per module (Fig. 2a, hatched area) which agrees well with the work required to rupture a pair of hydrogen bonds.

These observations strongly suggest that the unfolding intermediate that we observe during the extension of the I27 module corresponds to the rupture of the pair of hydrogen bonds bridging the A and B 3-strands together. In contrast to the wild-type form of the I27 module, the rising phase of mutant I27 module where lysine in position 6 was replaced by a proline) where the mutation interferes with the formation of these hydrogen bonds. Figure 4 shows a force−extension curve for the (I279-K6P) polyprotein. The figure shows that, in contrast to the wild-type form, the mutant polyprotein does not show deviations from the WLC model. Hence, induction of the K6P mutation eliminates the unfolding intermediate that we have described.

A folded I27 module has a length of 44 Å, as determined by NMR13. We have shown that a force of ~100 pN can extend the module by 6.6 Å without causing full domain unfolding. This rapidly reversible unfolding intermediate corresponds to an extension of a titin module by 15% of its resting length. Our data therefore provide direct evidence for a new source of extensibility in human cardiac titin, caused by a force-driven conformational change in its immunoglobulin domains.

Methods

Protein engineering

We constructed polyproteins containing 12 direct tandem repeats of a single immunoglobulin domain no. 27, and 8 tandem repeats of a single immunoglobulin domain no. 28 from the I band of human cardiac titin, according to methods described elsewhere17. We also expressed and purified the I27-134 construct of human cardiac titin synthesized elsewhere18. The I27-K6P point mutation monomer was generated by polymerase chain reaction using mutagenic primers. All these recombinant proteins have two Cys residues in their carboxy terminus to facilitate the attachment of the molecules to the gold-coated coverslips.

Force spectroscopy of single proteins

Our custom-made AFM apparatus and its mode of operation have been described20. Calibration of the spring constant of each individual cantilever was done in solution using the equipartition theorem as described in ref. 27. The proteins were suspended in PBS buffer at a concentration of ~10 μg ml⁻¹ and adsorbed onto freshly evaporated gold coverslips. For the analysis of the polyproteins, we chose only recordings showing a sawtooth force pattern that ended with a high-force peak that corresponds to stretching a fully denatured protein (all modules unfolded). This allowed us to count the number of modules within the fragment of the protein that were actually stretched.

Steered molecular dynamics simulation

The steered molecular dynamics (SMD) simulations of I27 were performed with the programs NAMD21 and XPLOR22 with the CHARMM22 (ref. 29) force field. The structure of I27 has been reported23 and is available elsewhere (Brookhaven PDB databank; PDB code 1TTT). The structure was solvated in water and equilibrated following the procedure described elsewhere24. SMD simulations with constant forces were performed by fixing one terminus of the domain and applying the force to the other terminus. The direction of the force was chosen along the vector from the N terminus to the C terminus. The constant stretching force F (pN) was determined by adding a harmonic potential between the C terminus and a point relatively far away (400 nm) from it along the force direction with the spring constant set to F(pN)/400(nm). The fluctuation of the magnitude of the force is less than 0.2%. The simulation adopted a timestep of 1 fs, a uniform dielectric constant of 1, and a cut-off of Coulomb and van der Waals interactions.

The modules at low force gives a value of 10.1 ± 0.63 kcal per mol per module (Fig. 2a, hatched area) which agrees well with the work required to rupture a pair of hydrogen bonds.

These observations strongly suggest that the unfolding intermediate that we observe during the extension of the I27 module corresponds to the rupture of the pair of hydrogen bonds bridging the A and B 3-strands of the I27 module. To verify this view, we engineered a point mutant polyprotein (I279-K6P; 9 repeats of a mutant I27 module where lysine in position 6 was replaced by a proline) where the mutation interferes with the formation of these hydrogen bonds. Figure 4 shows a force−extension curve for the (I279-K6P) polyprotein. The figure shows that, in contrast to the wild-type form, the mutant polyprotein does not show deviations from the WLC model. Hence, induction of the K6P mutation eliminates the unfolding intermediate that we have described.

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