THE ROYAL SOCIETY

Published online 25 February 2002

Role of titin in vertebrate striated muscle

L. Tskhovrebova and J. Trinick*

School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK

Titin is a giant muscle protein with a molecular weight in the megaDalton range and a contour length of more than 1 μm. Its size and location within the sarcomere structure determine its important role in the mechanism of muscle elasticity. According to the current consensus, elasticity stems directly from more than one type of spring-like behaviour of the I-band portion of the molecule. Starting from slack length, extension of the sarcomere first causes straightening of the molecule. Further extension then induces local unfolding of a unique sequence, the PEVK region, which is named due to the preponderance of these amino-acid residues. High speeds of extension and/or high forces are likely to lead to unfolding of the β-sandwich domains from which the molecule is mainly constructed. A release of tension leads to refolding and recoiling of the polypeptide.

Here, we review the literature and present new experimental material related to the role of titin in muscle elasticity. In particular, we analyse the possible influence of the arrangement and environment of titin within the sarcomere structure on its extensible behaviour. We suggest that, due to the limited conformational space, elongation and compression of the molecule within the sarcomere occur in a more ordered way or with higher viscosity and higher forces than are observed in solution studies of the isolated protein.

Keywords: muscle elasticity; titin; protein fold

1. MUSCLE ELASTICITY

One of the important properties of striated muscle is its elasticity (see review by Gajdosik 2001). Muscle fibres can be extended more than two-fold without loss of structural order. Extension is more or less uniform and all sarcomeres undergo the same relative elongation. Lengthening is accompanied by an increase in resistance or passive tension. This tension is responsible for spontaneous retraction to the rest length on release. Passive tension can be measured directly in relaxed muscle, while in activated muscle it comprises a part of the total measured force. Passive tension at a given muscle length depends on the rate of extension, demonstrating elastic behaviour in slow stretches and viscous behaviour in fast stretches. It is influenced by the extensible properties of both intra- and extracellular structures. The contribution of the extracellular, as well as exosarcomeric components is noticeable at large extensions beyond overlap of thin and thick filaments. Within the working range of sarcomere lengths, the contribution of the endosarcomeric components is thought to dominate (Magid & Law 1985; Brady 1991; Wang *et al.* 1993; Granzier & Irving 1995; Mutungi & Ranatunga 1996). Different types of muscles exhibit differences in force–extension behaviour reflecting, on the one hand, variations in the content of extracellular and exosarcomeric contributors and, on the other hand, differences in the extensible properties of the endosarcomeric proteins (Granzier & Irving 1995).

The generally accepted view is that the elasticity of striated muscle is largely determined by the giant protein titin, one of the major components of the sarcomere (see reviews by Trinick 1994, 1996; Wang 1996; Maruyama 1997; Linke & Granzier 1998; Gautel *et al.* 1999; Horowits 1999; Trinick & Tskhovrebova 1999; Linke 2000). Extensibility of titin in the I-band, and its relation to passive tension, was clearly demonstrated by *in situ* immunofluorescence and immuno-electron microscopy, and by mechanical measurements on fibres and myofibrils (Wang *et al.* 1991, 1993; Horowits 1992; Granzier *et al.* 1996; Linke *et al.* 1996; Trombitás *et al.* 1998, 2000).

2. TITIN IN THE SARCOMERE

The titin molecule spans half of the sarcomere (see, for example, figure 1 in Trinick & Tskhovrebova (1999)); its C-terminus is an integral component of the M-line (Furst *et al.* 1988; Obermann *et al.* 1997) and its N-terminus is part of the Z-disc (Furst *et al.* 1988; Gregorio *et al.* 1998). In the A-band, titin is an integral component of the thick filament; estimates based on different methods indicate that six titin molecules are bound to each half of the thick filament (Cazorla *et al.* 2000; Liversage *et al.* 2001). The major titin-binding proteins within the thick filament are probably myosin and C-protein (Soteriou *et al.* 1993*b*; Houmeida *et al.* 1995; Freiburg & Gautel 1996). Some self-association of titin molecules here cannot be excluded, although the limited data currently available do not provide any evidence for this (Politou *et al.* 1995).

The I-band section of titin forms flexible connections between the ends of the thick filament and the Z-disc. These connections centre thick filaments in the sarcomere and they are the main mechanical connections through

* Author for correspondence (j.trinick@leeds.ac.uk).

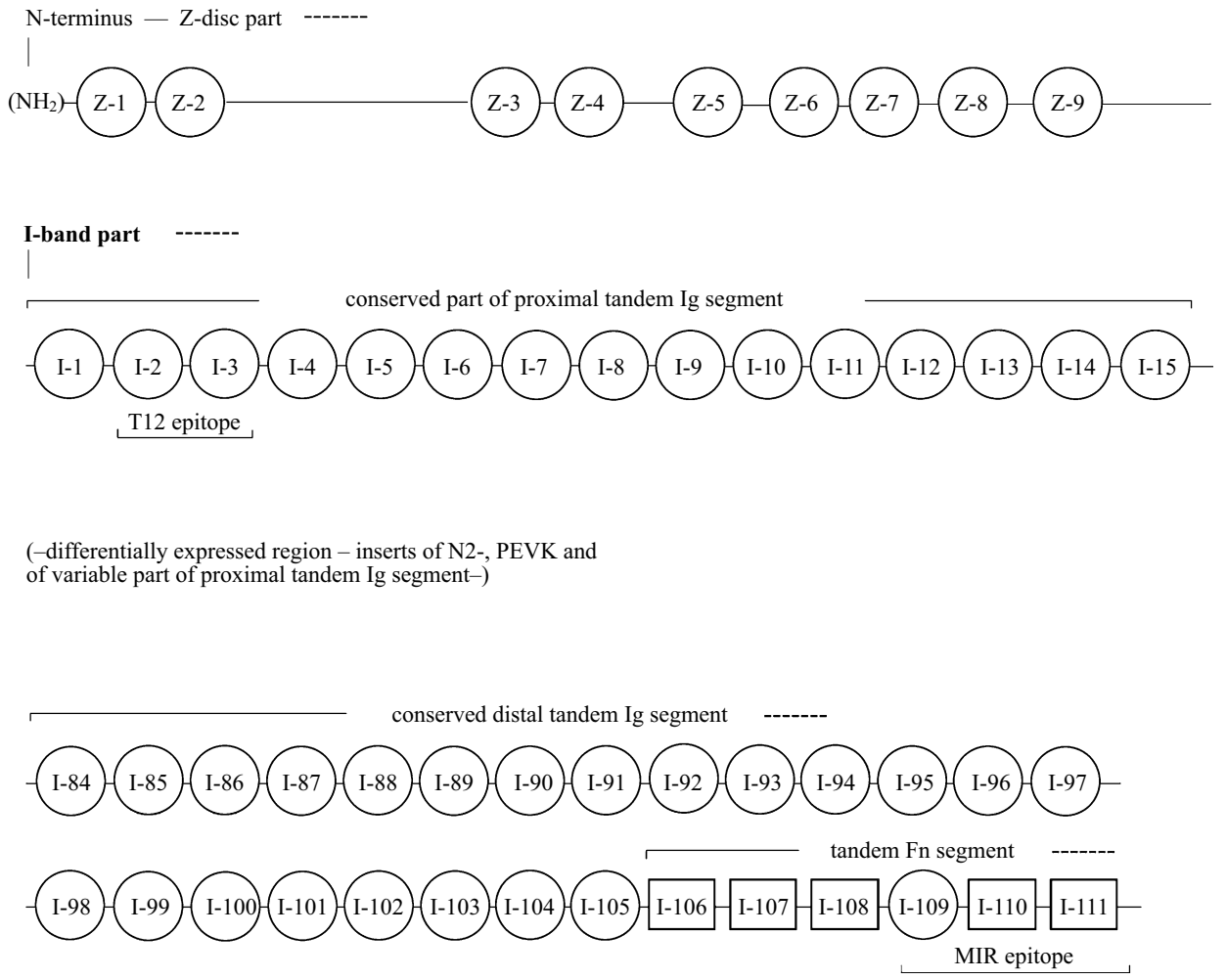

Figure 1. The domain structure of the I-band region of the titin molecule. The Z-disc structure corresponds to the cardiac isoform. Other isoforms vary in the length and structure. The T12 and MIR epitopes mark approximate edges of the I-band region that is not bound to thin or thick filaments. The I-band region of the smallest cardiac isoform comprises the conserved portion of the proximal tandem Ig segment that has 15 domains and is *ca*. 60 nm long. This is separated from the conserved distal Ig segment by the N2B and PEVK regions formed mainly by unique sequences. The N2B and PEVK polypeptides contain, in total, approximately 1200 amino-acid residues. In skeletal muscle isoforms the length of the proximal tandem Ig segment is extended by the inclusion of a variable portion of up to 53 Ig domains. They also comprise a different version of the N2 region (i.e. N2A) and a significantly longer PEVK polypeptide. Numbering of domains is given according to Freiburg *et al*. (2000).

relaxed muscle fibres (Horowits & Podolsky 1988). The spatial organization of I-band titin is complex (Tskhovrebova & Trinick 2000). There are three major regions: near the end of the thick filament six titin molecules are associated into a single bundle, the end filament (Trinick 1981; Bennett *et al*. 1997); in the middle of the I-band, titin probably branches either into smaller bundles or single molecules (Funatsu *et al*. 1993); and near the Z-disc, the molecules are associated with thin filaments (Linke *et al*. 1997; Trombitás *et al*. 1997). This complex spatial organization correlates with the structural heterogeneity of the molecule (Labeit & Kolmerer 1995; Freiburg *et al*. 2000).

The disposition of titin within the sarcomere predetermines the configurational and conformational transitions in its I-band portion during contraction or passive extension of muscle. The following estimates illustrate the magnitude of the changes in contour length of the I-band titin during normal muscle function. The maximum sarcomere length working range is estimated to be 1.9–2.5 μm in cardiac muscle, 2.0–3.4 μm in psoas muscle and 2.2–3.8 μm in soleus muscle (Millman 1998). This gives working ranges for the I-bands of 0.05–0.35, 0.12–0.82 and 0.21–1.01 μm, respectively. The corresponding I-band contour lengths of titin isoforms estimated from the sequence are 0.2, 0.37 and 0.49 μm. Thus, the I-band section of titin in each muscle can be expected to afford at least a two-fold reversible increase and a more than two-fold decrease in contour length. Estimates based on mechanical measurements on muscle fibres and myofibrils indicate that the extension of titin beyond its contour length produces forces up to a few tens of picoNewtons per molecule (Wang *et al*. 1991; Granzier & Irving 1995; Linke *et al*. 1996).

3. TITIN STRUCTURE

The titin molecule is more than 1 μm long and is formed by a single polypeptide with a molecular weight ranging from *ca*. 3.0 to 3.7 MD, depending on the isoform

(Freiburg *et al.* 2000). Despite its giant size, the tertiary structure of the molecule is relatively simple and sequence analysis predicts that more than 90% of the polypeptide is folded into a chain of immunoglobulin- and fibronectin-like domains. This multidomain structure implied by sequence correlates with the interdomain periodicity of *ca.* 4 nm observed in the electron microscope (Trinick *et al.* 1984). The A-band section of the molecule is conserved between isoforms. Approximately 65% of the length of the A-band is formed by Fn and 30% by Ig domains (Labeit *et al.* 1992; Labeit & Kolmerer 1995). The domains are arranged into patterns or super repeats reflecting the thick filament structure.

In contrast to the A-band section, the I-band section of titin reveals large size variations between isoforms (Freiburg *et al.* 2000). These variations correlate with the structure and elastic properties of the different muscle types, emphasizing the structural and functional role of the protein. In general, the I-band section comprises three major regions, two composed of conserved tandem Ig segments, flanking a central region formed by unique sequences and variable tandem Ig segments (figure 1). The unique sequence regions, in turn, contain two sub-regions: N2, consisting of alternating Ig domains and unique sequences, and the PEVK region named from its preponderance of these amino-acid residues. The N2 region is represented in two versions: all skeletal isoforms usually contain the N2A region, whereas cardiac isoforms contain either the N2B, or both the N2A and N2B regions. While the distal and Z-line bordering parts of proximal tandem Ig segments are conserved between isoforms, the variable tandem Ig segment and PEVK regions vary in size. The shortest cardiac isoform contains only the conserved portion of the proximal tandem Ig segment, which is immediately followed by the N2B region and a relatively short PEVK region. In all other cardiac isoforms between the N2B and PEVK regions there are inserts of additional Ig domains and N2A segments. In skeletal muscle isoforms the conserved portion of the proximal tandem Ig segment is directly followed by the variable Ig segment and then by the N2A and relatively long PEVK regions. In the largest soleus muscle isoform, the primary structure of the variable tandem Ig segment reveals two types of super-repeats of six and 10 domains, respectively (Gautel 1996; Witt et al. 1998). This suggests two types of periodicity in the tertiary structure of the molecule of 20–24 and 35–40 nm.

4. FLEXIBILITY AND EXTENSIBILITY OF THE ISOLATED MOLECULE

Electron microscopy of isolated titin molecules at high ionic strength, which favours solubility, reveals a highly flexible configuration (Maruyama *et al.* 1984; Trinick *et al.* 1984; Wang *et al.* 1984). Dynamic light scattering (Fugime & Higuchi 1993; Higuchi *et al.* 1993) and electron microscopy studies (Tskhovrebova & Trinick 2001) indicate an average value of the persistence length of 13–15 nm. Electron microscopy also demonstrates that the equilibrium configuration can be easily deformed by small mechanical forces, resulting in straightened or even partially unfolded molecules (Nave *et al.* 1989; Tskhovrebova & Trinick 1997). Figure 2 illustrates some examples

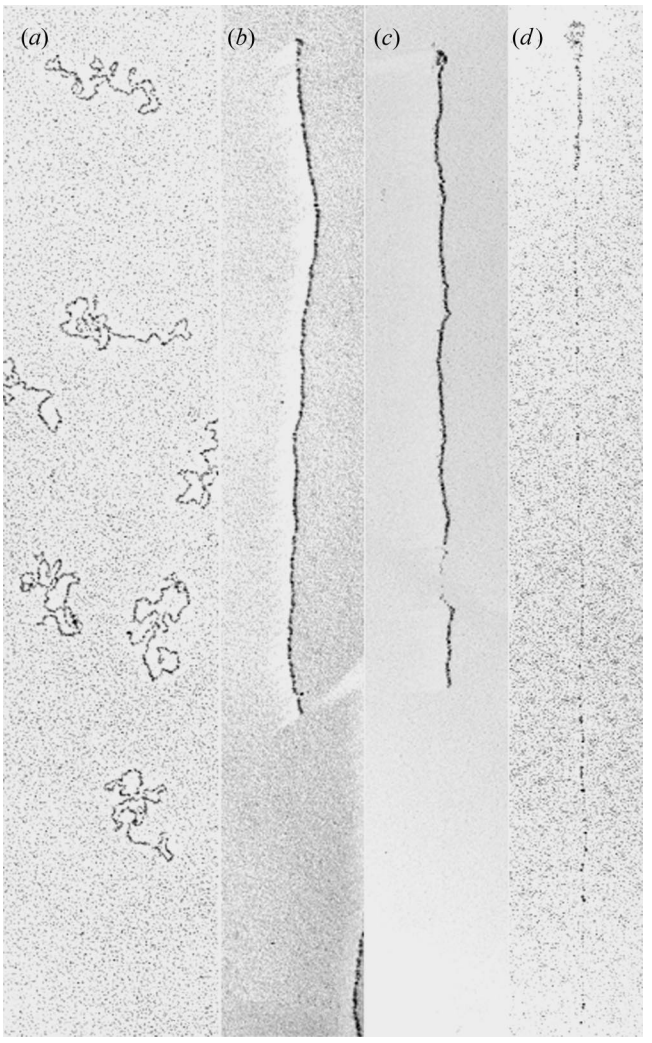

Figure 2. Electron micrographs illustrating titin molecules purified from rabbit back muscle and metal-shadowed after drying on a mica substrate in the (*a*) coiled, (*b*) straightened, and (*c, d*) partially unfolded configurations. (*a*) The coiled configuration closely reflects the equilibrium state adopted by the isolated molecule in a solution of high ionic strength. The equilibrium configuration, in most cases, is distorted by hydrodynamic forces, which are related to drying. These forces are responsible for straightening or 'molecular combing' of the molecules. The maximum measured length of rabbit back muscle isoform in a straightened configuration is *ca.* 1.3 μm (*b*), which is close to the length of the longest isoform estimated from primary structure. Straightening of the molecule is often accompanied by local unfolding at the PEVK region (*c*), leading to separation of the apparently intact A-band (larger segment) and I-band (smaller segment) regions. In some cases, the Ig–Fn containing A-band segment may also be observed in a partially unfolded state (*d*). The differences in the appearance reflect differences in the magnitude of hydrodynamic forces exerted on the molecules during specimen preparation.

of typically observed coiled, straightened and partially unfolded molecules. The pattern of events during extension agrees with conclusions reached from primary structure analysis (Labeit & Kolmerer 1995), immuno-electron microscopy (Granzier *et al.* 1996; Trombitás *et al.* 1998, 2000) and mechanical measurements (Granzier *et al.* 1996; Linke *et al.* 1996, 1998*a*,*b*). These results suggest that straightening of the molecule is followed by local

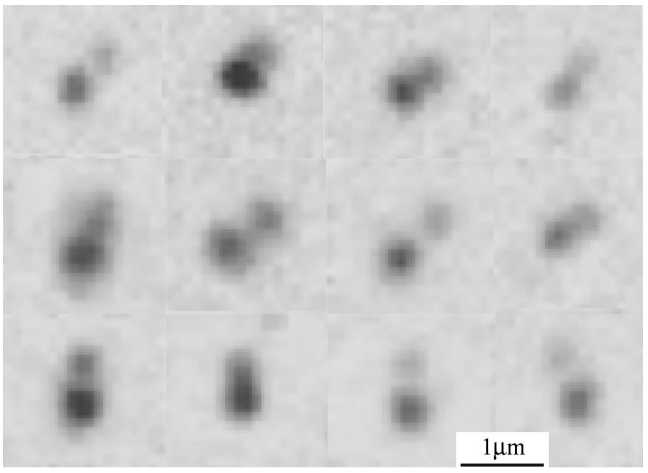

Figure 3. Optical microscopy of titin molecules labelled with lysine-reactive tetrametyl rhodamine isothiocyanate and dried on a mica substrate from a glycerol-containing buffer. To label the molecules, 0.5 ml of titin solution in 0.5 MKCl (pH 9.0–9.5) at 1.0 mg ml^{-1} was mixed with 10–30 ml of the dye dissolved at a concentration of 1 mg ml^{-1} in DMSO. The protein–dye mixture was left at room temperature for 30 min and then passed through a Sepharose CL-2B column (*ca.* 40 × 0.5 cm). Binding of the dye to the protein was detected spectroscopically. Electron microscope examination showed that labelling did not change significantly the flexibility of the protein, which was readily combed out by hydrodynamic forces. Here titin molecules are seen as elongated fluorescent spots *ca.* 1 μm long with increased fluorescence towards both ends. The shape of the fluorescent spots suggests a straightened configuration of the molecules and an uneven binding of dye along the length. The latter is in agreement with a significantly higher concentration of lysine residues at the PEVK region, which is close to the N-terminus, and the high affinity of the C-terminal to M-line proteins, which are usually present in the purified protein. Scale bar = 1 μm.

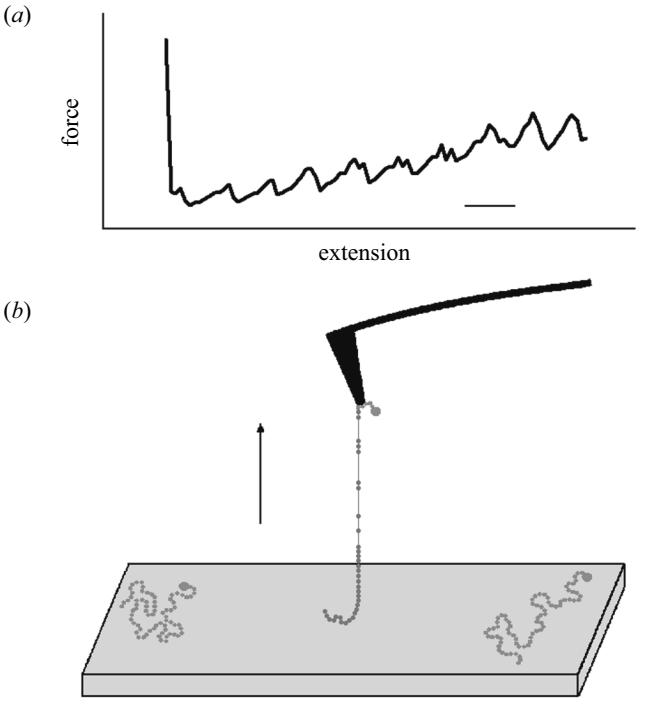

Figure 4. (*a*) An example of the characteristic sawtooth pattern observed during the stretching of titin, using an atomic force spectroscope cantilever. Titin, 10 μg ml^{-1} in 0.1 MKCl, was non-specifically adsorbed to gold-coated mica. (*b*) Force measurements were carried out with a commercial AFM (Molecular Imaging) in 0.1 MKCl buffer. Silicon nitride cantilevers used for the measurements were purchased from Digital Instruments. The tip was brought into contact with the surface and kept there for a few seconds to allow titin adsorption. The extension rate was 1 μm s^{-1}. The distance between peaks in the sawtooth pattern is *ca.* 25 nm, close to the length of unfolded Ig and Fn domains. Scale bar, 25 nm.

elongation at the PEVK region and then by unfolding of the Ig/Fn segments (Soteriou *et al.* 1993*a*).

Labelling titin molecules with lysine-reactive fluorescent dyes does not change their extensible properties (figure 3; see also Zhuang *et al.* (2000)). The labelled molecules reveal increased fluorescence at their ends. Increased fluorescence towards the C-terminus of the molecule correlates with an increased concentration of lysine residues in the PEVK region, suggesting that at least part of the PEVK region is present in the purified protein.

Mechanical experiments with single molecules using optical tweezers or atomic force spectroscopy show qualitative similarity with the force–extension relationship measured *in situ* (Kellermayer *et al.* 1997, 1998; Tskhovrebova *et al.* 1997). Simulations of the experimental curves using entropic WLC polymer models indicate the probable presence of two serially linked springs, which were identified as the Ig–Fn and PEVK regions.

Reversible unfolding of titin domains at high forces was also directly illustrated (Rief *et al.* 1997; Kellermayer *et al.* 1997; Tskhovrebova *et al.* 1997). Unfolding of the domains occurs sequentially, leading to a characteristic sawtooth-like pattern (figure 4). Computer simulations based on the structure of I27,[1] one of the few domains solved to atomic resolution (Improta *et al.* 1996), indicate that the sawtooth pattern can arise due to the presence of a potential barrier in the domain structure (Lu & Schulten 2000). The barrier reflects the necessity (after an initial stretch from an original 4 nm to *ca.* 7 nm) to simultaneously break a set of hydrogen bonds. As soon as this barrier is overcome, the polypeptide chain unravels according to the WLC chain behaviour (Rief *et al.* 1997). A release of the tension leads to a collapse of the chain and refolding of the domains. The refolding properties of the molecule can be strikingly demonstrated in the electron microscope when, after a complete chemical denaturation–renaturation cycle, the renatured molecules appear indistinguishable from native ones (Tskhovrebova & Trinick 2000). Biochemical studies indicate the refolding time of single domains to be of the order of seconds (Carrion-Vazquez *et al.* 1999).

5. TITIN AND MUSCLE ELASTICITY

The conclusions reached from the *in vitro* studies can be summarized as follows: the end-to-end distance of the isolated molecule (contour length *ca.* 1 μm) can be reversibly and quickly changed from *ca.* 0.15 μm (compact relaxed conformation) to several microns (extended and unfolded). These changes result from a highly flexible native structure and from the fast kinetics of folding–

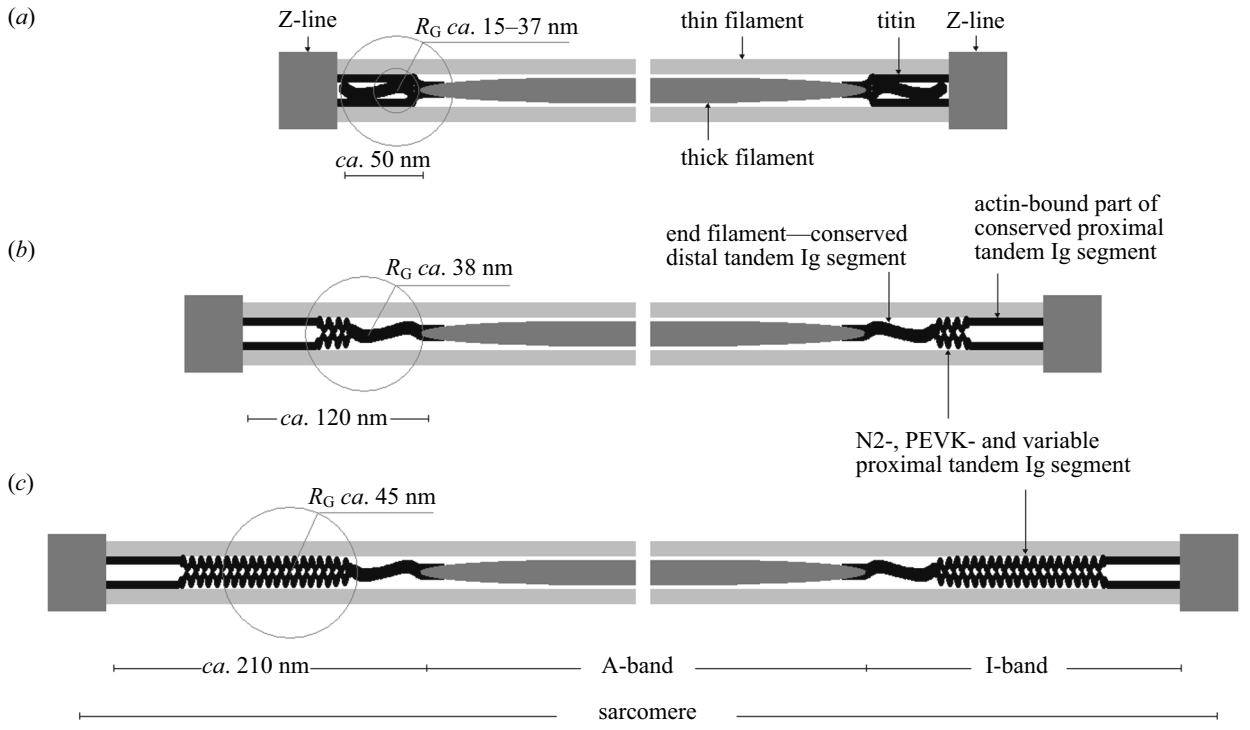

Figure 5. The effect of confinement on the configuration of the titin I-band region within (*a*) cardiac, (*b*) psoas and (*c*) soleus muscles at slack sarcomere lengths. The scheme is drawn at a scale proportional to the known dimensions and relative positions of the major contractile filaments. (*a*) In cardiac muscle the I-band length in the slack sarcomere is approximately equal to the length of the actin-bound section of titin and also to the length of end filament. The contour length of the remainder of the I-band titin polypeptide, comprising the N2B and PEVK regions, is also *ca.* 50 nm long (this estimate is based on an assumption that the folding density of the N2B–PEVK polypeptide and the diameter of the molecule in this region are similar to the rest of the protein). This implies that the N2B–PEVK segment is stretched between the tip of the end filament, which is set against the Z-disc, and the actin-bound section which has moved towards the A-band. For the smallest cardiac isoform of titin, a further decrease of the sarcomere length during muscle contraction may lead to over-extension of the I-band segment and thus initiate an increase in sarcomere-length restoring force (Helmes *et al.* 1996; Wu *et al.* 2000). For psoas (*b*) and soleus (*c*) muscles the I-band lengths in slack sarcomeres are *ca.* 120 and 210 nm, respectively, whereas the contour lengths of the I-band regions of the corresponding titin isoforms are 320 and 440 nm (excluding actin-bound portions). The isolated I-band titin segment in solution will occupy a volume of approximately spherical shape with a radius of 38–45 nm, as can be judged from estimates of radii of gyration. However, within the sarcomere the average space per single I-band segment is restricted by a volume of an elongated shape with a length equal to the I-band length and a radius of 8–10 nm. The latter value is based on thick filament spacing of 43 nm (Millman 1998) and the estimate of six titin molecules per half thick filament (Cazorla *et al.* 2000; Liversage *et al.* 2001). Thus, there is an apparent discrepancy between the space required and that actually available. This implies that titin strands in the I-band at slack sarcomere length are either arranged in a more ordered way than can be predicted from solution observations or, they are highly interwired.

unfolding transitions of unique sequences and β-structure domains.

In muscle, the I-band section of titin tends to shorten its end-to-end distance due to the flexible nature of the molecule. In the absence of external tension this decreases the sarcomere to a slack length. Starting from slack length, extension of the sarcomere first causes straightening of the elastic I-band section of titin. Further extension induces local unfolding of the PEVK region, but high speeds of extension and/or high forces probably lead to unfolding of Ig domains (see e.g., Minajeva *et al.* 2001). The differences in mechanical stability between PEVK and Ig domains are thought to be related to the differences in their tertiary structure. As mentioned above, the relative resistance of Ig domains to mechanical unfolding is due to particular intradomain bonds that give rise to a potential energy barrier. In the PEVK region, studies of its primary structure (Greaser 2001) and folding properties of expressed fragments (Gutierrez-Cruz *et al.* 2001; Ma *et al.* 2001) indicate the presence of a 28-residue long 'polyproline helix-coil' motif as the probable structural repeat. This would be radically different from the β-sandwich domains and could result in a lower potential energy barrier and lower resistance to mechanical stress.

In cardiac muscle, with the shortest isoform, the I-band section of titin probably acts as a bi-directional spring. This extends and gives rise to a tension not only during extension of the sarcomere but also during contraction below the slack length (Helmes *et al.* 1996; Wu *et al.* 2000). In the latter case, the tension developed will tend to restore the sarcomere rest length. This rest-length restoring function may be a general function of titin, although due to large variations in the I-band section of the molecule between isoforms, details of the mechanisms will vary.

The above scheme is a general one and the latest data indicate that extensibility might be more finely tuned and dependent on fibre type than previously thought. Thus, in

cardiac isoforms, the N2B region is also involved in multiphase extension of the molecule (Linke *et al.* 1999; Trombitás *et al.* 1999). The PEVK region, in addition to its involvement in the mechanism of elasticity, might have additional functions deriving from its high negative charge. For instance, it may be involved in regulatory mechanisms through binding sarcoplasmic proteins or cations.

Quantitative comparisons of data obtained by *in vitro* and *in situ* mechanical experiments is complicated by several factors that still need to be resolved. One major factor is the titin arrangement in the sarcomere. The approach used in single-molecule experiments for studying titin extensible properties (Kellermayer *et al.* 1997; Rief *et al.* 1997; Tskhovrebova *et al.* 1997) is of particular interest since it mimics the situation with titin extension in muscle, by anchoring both ends of the molecule and applying a mechanical force to one of its ends. The essential difference, however, is that, in contrast to *in vitro* experiments, I-band titin in the sarcomere is located within an ordered filament lattice. Thus, all conformational changes occur within the confined lattice space.

Figure 5 schematically illustrates the probable configuration of titin molecules in the I-band region at slack sarcomere length. It is obvious that the I-band section of the molecule cannot adopt the equilibrium conformation of the isolated molecule in solution, which can be approximated as a sphere of radius R_G *ca.* 15–45 nm for different isoforms (estimations are based on the assumption of six titin molecules per half thick filament; Cazorla *et al.* (2000); Liversage *et al.* (2001)). Instead, the average conformational space per molecule is restricted by the elongated and narrow volume, with a radius of *ca.* 8.5 nm and length of *ca.* 50, 120 and 210 nm in cardiac, psoas and soleus muscles, respectively. The difference between the space required and that actually available implies that transverse motions of titin in muscle are likely to be significantly inhibited. Thus, the configurational deformation resulting from changes in sarcomere length may not occur as a rod–coil transition in solution, but rather in a more ordered way maintaining the elongated shape of the conformational space. As a consequence of this confinement, compression of the molecule may involve higher viscosity and extension may have different dynamics in comparison with a free chain.

The above analysis is based on the assumption that six titin molecules, bound to each half of the thick filament in the A-band and interacting with each other within the end filament, desegregate in the middle of the I-band into six separate strands forming six independent elastic connections with the Z-disc. If the molecules desegregate, not into monomers but into dimers or trimers, this will change quantitative estimates. However, the effect of confinement on the conformation and dynamics of the I-band titin is likely to remain.

Regardless of the details of the arrangement in the I-band, the roles of titin in muscle passive tension are now well established. The bending and extensible properties of the molecules are at the heart of the molecular mechanisms controlling the functionally important long-range elasticity in striated muscle. The rich variety of isoforms also provides a simple basis for the variation of sarcomere-length working range observed in different types of muscle fibres.

We thank The Wellcome Trust for financial support.

ENDNOTE

[1]Corresponds to I91 in the latest numbering of domains (Freiburg *et al.* 2000).

REFERENCES

Bennett, P. M., Hodkin, T. E. & Hawkins, C. 1997 Evidence that the tandem Ig domains near the end of the muscle thick filament form an inelastic section of the I-band titin. *J. Struct. Biol.* **120**, 93–104.

Brady, A. J. 1991 Length dependence of passive stiffness in single cardiac myocytes. *Am. J. Physiol.* **260**, H1062–H1071.

Carrion-Vazquez, M., Oberhauser, A. F., Fowler, S. B., Marszalek, P. E., Broedel, S. E. & Fernandez, J. M. 1999 Mechanical and chemical unfolding of a single protein: a comparison. *Proc. Natl Acad. Sci. USA* **96**, 3694–3699.

Cazorla, O., Freiburg, A., Helmes, M., Centner, T., McNabb, M., Wu, Y., Trombitás, K., Labeit, S. & Granzier, H. 2000 Differential expression of cardiac titin isoforms and modulation of cellular stiffness. *Circ. Res.* **86**, 59–67.

Freiburg, A. & Gautel, M. 1996 A molecular map of the interactions between titin and myosin-binding protein C. Implications for sarcomeric assembly in familial hypertrophic cardiomyopathy. *Eur. J. Biochem.* **235**, 317–323.

Freiburg, A. (and 11 others) 2000 Series of exon-skipping events in the elastic spring region of titin as the structural basis for myofibrillar elastic diversity. *Circ. Res.* **86**, 1114–1121.

Fugime, S. & Higuchi, H. 1993 An analysis of the dynamic light scattering spectra of worm-like chains: β-connectin from striated muscle. *Macromolecules* **26**, 6261–6266.

Funatsu, T., Kono, E., Higuchi, H., Kimura, S., Ishiwata, S., Yoshioko, T., Maruyama, K. & Tsukita, S. 1993 Elastic filaments *in situ* in cardiac-muscle—deep-etch replica analysis in combination with selective removal of actin and myosin-filaments. *J. Cell Biol.* **120**, 711–724.

Furst, D. O., Osborn, M., Nave, R. & Weber, K. 1988 The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immuno-electron microscopy—a map of 10 non-repetitive epitopes starting at the Z-line extends to the M-line. *J. Cell Biol.* **106**, 1563–1572.

Gajdosik, R. L. 2001 Passive extensibility of skeletal muscle: review of the literature with clinical implications. *Clin. Biomech.* **16**, 87–101.

Gautel, M. 1996 The super-repeats of titin/connectin and their interactions: glimpses at sarcomeric assembly. *Adv. Biophys.* **33**, 27–37.

Gautel, M., Mues, A. & Young, P. 1999 Control of sarcomeric assembly: the flow of information on titin. *Rev. Physiol. Biochem. Pharmacol.* **138**, 97–137.

Granzier, H. L. & Irving, T. C. 1995 Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. *Biophys. J.* **68**, 1027–1044.

Granzier, H., Helmes, M. & Trombitás, K. 1996 Nonuniform elasticity of titin in cardiac myocytes: a study using immunoelectron microscopy and cellular mechanics. *Biophys. J.* **70**, 430–442.

Greaser, M. 2001 Identification of new repeating motifs in titin. *Proteins* **43**, 145–149.

Gregorio, C. C. (and 11 others) 1998 The NH terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J. Cell Biol.* **143**, 1013–1027.

Gutierrez-Cruz, G., van Heerden, A. H. & Wang, K. 2001 Modular motif, structural folds and affinity profiles of the

PEVK segment of human fetal skeletal muscle titin. *J. Biol. Chem.* **276**, 7442–7449.

Helmes, M., Trombitás, K. & Granzier, H. 1996 Titin develops restoring force in rat cardiac myocytes. *Circ. Res.* **79**, 619–626.

Higuchi, H., Nakauchi, Y., Maruyama, K. & Fujime, S. 1993 Characterization of β-connectin (titin 2) from striated mucle by dynamic light scattering. *Biophys. J.* **65**, 1906–1915.

Horowits, R. 1992 Passive force generation and titin isoforms in mammalian skeletal muscle. *Biophys. J.* **61**, 392–398.

Horowits, R. 1999 The physiological role of titin in striated muscle. *Rev. Physiol. Biochem. Pharmacol.* **138**, 57–96.

Horowits, R. & Podolsky, R. J. 1988 Thick filament movement and isometric tension in activated skeletal muscle. *Biophys. J.* **54**, 165–171.

Houmeida, A., Holt, J., Tskhovrebova, L. & Trinick, J. 1995 Studies of the interaction between titin and myosin. *J. Cell Biol.* **131**, 1471–1481.

Improta, S., Politou, A. S. & Pastore, A. 1996 Immunoglobulin-like modules from titin I-band: extensible components of muscle elasticity. *Structure* **4**, 323–337.

Kellermayer, M. S. Z., Smith, S. B., Granzier, H. L. & Bustamante, C. 1997 Folding–unfolding transitions in single titin molecules characterized with laser tweezers. *Science* **276**, 1112–1116.

Kellermayer, M. S. Z., Smith, S. B., Bustamante, C. & Granzier, H. 1998 Complete unfolding of the titin molecule under external force. *J. Struct. Biol.* **122**, 197–205.

Labeit, S. & Kolmerer, B. 1995 Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* **270**, 293–296.

Labeit, S., Gautel, M., Lakey, A. & Trinick, J. 1992 Towards a molecular understanding of titin. *EMBO J.* **11**, 1711–1716.

Linke, W. A. 2000 Stretching molecular springs: elasticity of titin filaments in vertebrate striated muscle. *Histol. Histopathol.* **15**, 799–811.

Linke, W. A. & Granzier, H. 1998 A spring tale: new facts on titin elasticity. *Biophys. J.* **75**, 2613–2614.

Linke, W. A., Ivemeyer, M., Olivieri, N., Kolmerer, B., Ruegg, J. C. & Labeit, S. 1996 Towards a molecular understanding of the elasticity of titin. *J. Mol. Biol.* **261**, 62–71.

Linke, W. A., Ivemeyer, M., Labeit, S., Hinssen, J. C., Ruegg, J. C. & Gautel, M. 1997 Actin–titin interaction in cardiac myofibrils: probing a physiological role. *Biophys. J.* **73**, 905–919.

Linke, W. A., Ivemeyer, M., Mundel, P., Stockmeier, M. R. & Kolmerer, B. 1998*a* Nature of PEVK titin elasticity in skeletal muscle. *Proc. Natl Acad. Sci. USA* **95**, 8052–8057.

Linke, W. A., Stockmeier, M. R., Ivemeyer, M., Hosser, H. & Mundel, P. 1998*b* Characterizing titin's I-band Ig domain region as an entropic spring. *J. Cell Sci.* **111**, 1567–1574.

Linke, W. A., Rudy, D. E., Centner, T., Gautel, M., Witt, C., Labeit, S. & Gregorio, C. 1999 I-band titin in cardiac muscle is a three-element molecular spring and is critical for maintaining thin filament structure. *J. Cell Biol.* **146**, 631–644.

Liversage, A. D., Holmes, D., Knight, P. J., Tskhovrebova, L. & Trinick, J. 2001 Titin and the sarcomere symmetry paradox. *J. Mol. Biol.* **305**, 401–409.

Lu, H. & Schulten, K. 2000 The key event in force-induced unfolding of titin's immunoglobulin domains. *Biophys. J.* **79**, 51–65.

Ma, K., Kan, L. S. & Wang, K. 2001 Polyproline II helix is a key structural motif of the elastic PEVK segment of titin. *Biochemistry* **40**, 3427–3438.

Magid, A. & Law, D. J. 1985 Myofibrils bear most of the resting tension in frog skeletal muscle. *Science* **230**, 1280–1282.

Maruyama, K. 1997 Connectin/titin, giant elastic protein of muscle. *Federation Am. Soc. Exp. Biol. J.* **11**, 341–345.

Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H. & Kikuchi, M. 1984 Molecular size and shape of beta-connectin, an elastic protein of striated muscle. *J. Biochem.* (*Tokyo*) **95**, 1423–1433.

Millman, B. M. 1998 The filament lattice of striated muscle. *Physiol. Rev.* **78**, 359–391.

Minajeva, A., Kulke, M., Fernandez, J. M. & Linke, W. A. 2001 Unfolding of titin domains explains the viscoelastic behavior of skeletal myofibrils. *Biophys. J.* **80**, 1442–1451.

Mutungi, G. & Ranatunga, K. W. 1996 The viscous, viscoelastic, and elastic characteristics of resting fast and slow mammalian (rat) muscle fibres. *J. Physiol.* **496**, 827–836.

Nave, R., Furst, D. O. & Weber, K. 1989 Visualization of the polarity of isolated titin molecules—a single globular head on a long thin rod as the M-band anchoring domain. *J. Cell Biol.* **109**, 2177–2187.

Obermann, W. M., Gautel, M., Weber, K. & Furst, D. O. 1997 Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. *EMBO J.* **16**, 211–220.

Politou, A. S., Thomas, D. J. & Pastore, A. 1995 The folding and stability of titin immunoglobulin-like modules, with implications for the mechanism of elasticity. *Biophys. J.* **69**, 2601–2610.

Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J. M. & Gaub, H. E. 1997 Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science* **276**, 1109–1112.

Soteriou, A., Clarke, A., Martin, S. & Trinick, J. 1993*a* Titin folding energy and elasticity. *Proc. Roy. Soc. Lond.* B **254**, 83–86.

Soteriou, A., Gamage, M. & Trinick, J. 1993*b* A survey of interactions made by the giant protein titin. *J. Cell Sci.* **104**, 119–123.

Trinick, J. A. 1981 End-filaments: a new structural element of vertebrate skeletal muscle thick filaments. *J. Mol. Biol.* **151**, 309–314.

Trinick, J. 1994 Titin and nebulin: protein rulers in muscle? *Trends Biochem. Sci.* **10**, 405–409.

Trinick, J. 1996 Titin as a scaffold and spring. Cytoskeleton. *Curr. Biol.* **6**, 258–262.

Trinick, J. & Tskhovrebova, L. 1999 Titin: a molecular control freak. *Trends Cell Biol.* **10**, 377–380.

Trinick, J., Knight, P. & Whiting, A. 1984 Purification and properties of native titin. *J. Mol. Biol.* **180**, 331–356.

Trombitás, K., Freiburg, A., Centner, T., Labeit, S. & Granzier, H. 1999 Molecular dissection of N2B cardiac titin's extensibility. *Biophys. J.* **77**, 3189–3196.

Trombitás, K., Greaser, M., Labeit, S., Jin, J. P., Kellermayer, M., Helmes, M. & Granzier, H. 1998 Titin extensibility *in situ*: entropic elasticity of permanently folded and permanently unfolded molecular segments. *J. Cell Biol.* **140**, 853–859.

Trombitás, K., Greaser, M. L. & Pollack, G. H. 1997 Interaction between titin and thin filaments in intact cardiac muscle. *J. Muscle Res. Cell Motil.* **18**, 345–351.

Trombitás, K., Redkar, A., Centner, T., Wu, Y., Labeit, S. & Granzier, H. 2000 Extensibility of isoforms of cardiac titin: variation in contour length of molecular subsegments provides a basis for cellular passive stiffness diversity. *Biophys. J.* **79**, 3226–3234.

Tskhovrebova, L. & Trinick, J. 1997 Direct visualization of extensibility in isolated titin molecules. *J. Mol. Biol.* **265**, 100–106.

Tskhovrebova, L. & Trinick, J. 2000 Extensibility in the titin molecule and its relation to muscle elasticity. In *Elastic filaments of the cell* (ed. H. Granzier & G. H. Pollack), pp. 163–178. New York: Kluwer.

Tskhovrebova, L. & Trinick, J. 2001 Flexibility and extensi-

bility in the titin molecule: analysis of electron microscope data. *J. Mol. Biol.* **310**, 755–771.

Tskhovrebova, L., Trinick, J., Sleep, J. A. & Simmons, R. 1997 Elasticity and unfolding of single molecules of the giant muscle protein titin. *Nature* **387**, 308–312.

Wang, K. 1996 Titin/connectin and nebulin: giant protein rulers of muscle structure and function. *Adv. Biophys.* **33**, 123–134.

Wang, K., Ramirez-Mitchell, R. & Palter, D. 1984 Titin is an extraordinarily long, flexible, and slender myofibrillar protein. *Proc. Natl Acad. Sci. USA* **81**, 3685–3689.

Wang, K., McCarter, R., Wright, J., Beverly, J. & Ramirez-Mitchell, R. 1991 Regulation of skeletal muscle stiffness and elasticity by titin isoforms—a test of the segmental extension model of resting tension. *Proc. Natl Acad. Sci. USA* **88**, 7101–7105.

Wang, K., McCarter, R., Wright, J., Beverly, J. & Ramirez-Mitchell, R. 1993 Viscoelasticity of the sarcomere matrix of skeletal muscles. The titin–myosin composite filament is a dual-stage molecular spring. *Biophys. J.* **64**, 1161–1177.

Witt, C. C., Olivieri, N., Centner, T., Kolmerer, B., Millevoi, S., Morelli, J., Labeit, D., Labeit, S., Jockush, H. & Pastore, A. 1998 Survey of the primary structure and the interspecies conservation of the I-band titin's elastic elements in vertebrates. *J. Struct. Biol.* **122**, 206–215.

Wu, Y., Cazorla, O., Labeit, D., Labeit, S. & Granzier, H. 2000 Changes in titin and collagen underlie diastolic stiffness diversity of cardiac muscle. *J. Mol. Cell Cardiol.* **32**, 2151–2161.

Zhuang, X. W., Ha, T., Kim, H. D., Centner, T., Labeit, S. & Chu, S. 2000 Fluorescence quenching: a tool for single-molecule protein-folding study. *Proc. Natl Acad. Sci. USA* **97**, 14 241–14 244.