Characterisation of native titin

J. TRINICK, P. KNIGHT and A. WHITING

AFRC Meat Research Institute, Langford, Bristol BS18 7DY, UK

Introduction

Titin is a new myofibrillar protein that escaped discovery until comparatively recently because its extremely high chain weight (approximately 10^6 daltons, Wang et al., 1979) precludes entry into normal SDS/polyacrylamide gels. It makes up approximately 10% of the protein mass of myofibrils and has been proposed to form part of a set of longitudinal elastic filaments running through the sarcomere (Wang, 1982). The precise disposition of such filaments is still uncertain, but one plausible arrangement would be as connections between the ends of thick filaments and the Z-line. Here they might serve to centralize the A-band in the sarcomere, the mechanism of which is not otherwise obvious (Magid, 1983). Locker (1984) has proposed that such filaments are a major factor affecting meat tenderness.

Previously Maruyama and co-workers had studied the properties of a preparation called connectin which has recently been shown to contain titin, although this material also contained other proteins (Maruyama et al., 1981; Maruyama et al., 1983).

Until now, titin and connectin have been studied under denaturing conditions, so their properties in the native state were not known. Recently we have developed a method for the isolation of titin without exposure to denaturing solvents and we report here some of its properties.

Preparation of purified native titin

A volume of fresh myofibril suspension (prepared by the method of Knight and Trinick, 1982) containing approximately 1 g protein was centrifuged for 5 min at 5000 x g and the supernatant removed. To the pellet was added a quantity of an ice-cold extracting solution containing 0.6 M KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10 mM imidazole-HCl, pH 7 at 0°C sufficient to bring the final protein concentration of the suspension to about 5 mg/ml. After thoroughly dispersing the pellet with a glass rod (which took about 20 s), the suspension was immediately centrifuged for 1 h at 15000 x g. The clarified extract was then carefully removed with a large syringe, since the upper portion of the pellet was easily dislodged. It was then dialysed for several hours against 2 volumes of water, following which precipitated myosin was removed by centrifugation at 6000 x g for 30 min. The supernatant was dialysed against 2 changes of a solution containing 0.1M KCl, 1 mM EDTA, 0.3 mM DTT, 50 mM Tris-HCl, pH 7.9 at 4°C and pumped on to a column (1.6 cm x 42 cm) containing DEAE-cellulose (Whatman DE52) equilibrated in this buffer. The flow rate was 30 ml/h and 8 ml fractions were collected. Bound protein was eluted by an approximately linear salt gradient running from 0.1 M to 0.4 M KCl in a total volume of 2 l. The titin peak fractions were concentrated by the addition of solid ammonium sulphate (Schwarz-Mann, Special Enzyme Grade) to 35% saturation followed by centrifugation at 8000 x g for 30 min, after which the pellets were resuspended and dialysed overnight against a solution containing 0.5 M KCl, 1 mM EDTA, 0.3 mM DTT, 50 mM Tris-HCl, pH 7.9 at 4°C. The dialysed protein was then passed through a gel filtration column (1.6 cm x 84 cm) containing Bio Gel A5U-M equilibrated in the dialysis buffer and run at a rate of 15 ml/h. The titin-containing fractions were again concentrated by the addition of solid ammonium sulphate to 35% saturation.

(For details of other procedures see Trinick et al. (1984).

Results

(i) Purification and solubility

Native titin was extracted with 0.6 M KCl from washed myofibrils. Subsequent purification involved selective precipitation of myosin at an ionic strength of about 0.2, followed by ion exchange and gel filtration chromatography. Initial extracts generally showed two titin bands (designated T_1 and T_2) on SDS/polyacrylamide gels, but during the purification the leading band (T_2) appeared to become more intense at the expense of the trailing one, presumably due to proteolysis. The purified protein usually showed only T_2 (Fig. 1).

The solubility properties of titin were similar to those of myosin and C-protein in that it aggregated in low salt (<0.2M) or pH (<7.0).

(ii) Sedimentation velocity centrifugation

Sedimentation velocity analytical ultracentrifugation of the purified protein showed most of the protein migrating as a single hypersharp peak (Fig 2). The sedimentation coefficient ($S_{20,w}$) of this boundary when extrapolated to zero concentration was 13.4S.

(iii) Circular dichroism spectroscopy

Circular dichroism spectra of these preparations indicate a very low α-helix content and a largely random coil conformation. These data may be significant in view of the possible elastic character of titin and the high random coil content of elastic proteins such as elastin.

(iv) Electron microscopy

(a) After spraying and shadowing

Titin specimens after spraying and shadowing revealed two types of structure; long, thin strings and small, globular structures; frequently the two were associated (Fig. 3a).
The strings were about 40 A^o in diameter and were heterogeneous in length up to maximum of about 8000 A^o. Their tortuous tracks were suggestive of considerable flexibility. Sometimes aligned sections of the strings could be seen and very occasionally such sections could be seen to be joined by very thin connections that may be single polypeptide chains (Figs 3b and c).

(b) After layering and shadowing

Some reduction in the complexity of these images was observed if the specimens were layered rather than sprayed onto mica, excess liquid being removed by centrifugation (Fig. 4).

Now only the long strings could be seen and the globular particles were largely absent. Similarly the aligned sections of the strings were no longer observed. We tentatively attribute these differences in appearance found after spraying or layering to elasticity in titin revealed by the high shearing forces accompanying spraying.

(c) After negative staining

Negatively stained titin again shows long thin strings of diameter about 40 Å, but these can now be seen to have a beaded appearance, the spacing between successive beads being about 40 Å (Fig. 5).

(d) Negatively stained thick filaments

Beaded strings very similar to negatively stained purified titin can also be seen associated with separated native thick filaments. These can be seen emanating from the ends of the filaments in coils (Fig. 6a) or running alongside the cross-bridge regions (Fig. 6b).

We have observed both these appearances on a single half filament but have never seen more than one strand alongside a cross-bridge region at any one point.

Discussion

Although titin monomers are extremely large they are too small for the longest of the string-like structures seen by electron microscopy to consist of only one such polypeptide chain. Since a cylindrical protein molecule of mass 10^6 daltons and diameter of 40 Å would have a length of about 1500 Å, some sort of polymer seems likely, possibly an end-to-end one. If titin constitutes 10% of myofibril and there was one titin filament 40 Å wide per thick filament it would have a length of about 2 μm.

Interestingly, only about a half of the titin present in myofibrils is capable of being liberated by high ionic strength. That fact that

SDS/polyacrylamide gels of titin often show a doublet indicates that some proteolysis, in addition to the high salt concentration, may be necessary for solubilization. However, this does not necessarily mean that all the liberated titin chains are degraded, since proteolytic cleavage at the ends of a linear polymer could still leave undegraded monomers in the middle.

The fact that titin strands are found at the ends of thick filaments, together with their approximately 40 Å periodicity, suggests that they are associated with thick filaments *in vivo*. A small part of the titin present in myofibrils may well form end-filaments, recently identified structures at the tips of thick filaments (Trinick, 1981). End-filaments have a similar periodicity to titin (43 Å), although they appear slightly thicker and more rigid and therefore may involve more than one titin strand.

Precisely how titin and end-filaments are arranged *in vivo* remains unclear. Although several possible structural arrangements have been put forward, there is not yet sufficient evidence to distinguish between them. Locker (1984) has suggested that elastic filaments (termed gap-filaments by him) connect the ends of thick filaments to the Z-line, whereas Wang (1982) has proposed that titin filaments are part of a system largely independent of thick filaments. More definite conclusions will require antibody labelling data, preferably obtained with antibodies to the native protein and using muscle where the titin has not suffered any proteolytic degradation.

References

Locker, R.H. (1984). J. Food Microstructure, in press.
Knight, P.J. & Trinick, J.A. (1982). Methods in Enzymology, 85B, 9-12.
Magid, A. (1983). Biophys. J. 41, 35a.
Maruyama, K., Kimura, S., Ohashi, K. & Kuwano, Y. (1981). J. Biochem. (Tokyo), 89, 701-709.
Maruyama, K., Kimura, S., Toyota, N. & Ohashi, K. (1983) In 'Muscular Dystrophy: Biomedical aspects' (Ebashi, S. & Oosawa, E. eds) pp.201-208. Japan Sci. Soc. Press, Tokyo/Springer Verlag, Berlin.
Trinick, J. (1981). J. Mol. Biol. 151, 309-314.
Trinick, J., Knight, P. & Whiting, A. (1984). J. Mol. Biol. In press.
Wang, K., McClure, J. & Tu, A. (1979). Proc. natl. Acad. Sci. USA 76, 3698-3702.
Wang, K. (1982). In 'Muscle Development - molecular and cellular control' (Pearson, M.L. and Epstein, H.F. eds). pp 439-452. Cold Spring Harbor Laboratories.

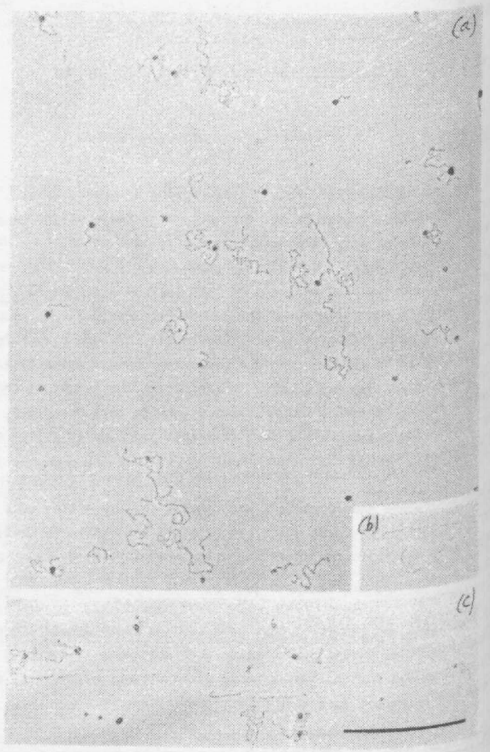

Fig. 3 Electron microscopy of titin after spraying and shadowing.
(a) Field of molecules
(b) Aligned sections
(c) Aligned sections with thin connections
Scale bar in (c) indicates 5000 Å

Fig. 1 SDS/polyacrylamide gels of purified native titin (3% acrylamide gels loaded with 4 µgm, 40 µgm and 80 µgm

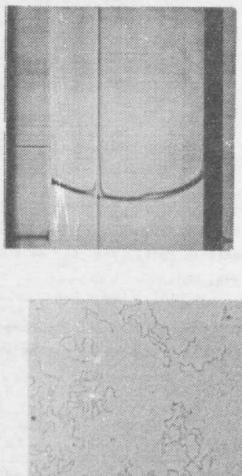

Fig. 2 Sedimentation velocity ultracentrifugation of titin

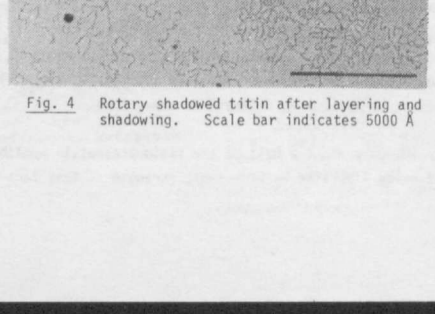

Fig. 4 Rotary shadowed titin after layering and shadowing. Scale bar indicates 5000 Å

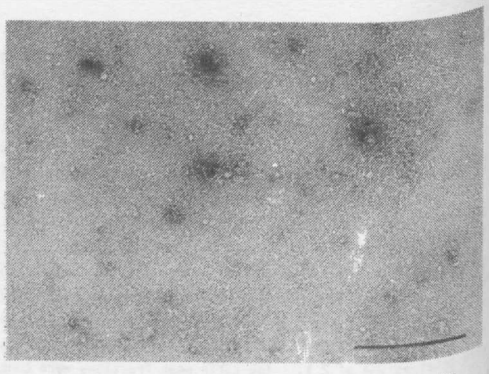

Fig. 5 Negatively stained titin. Scale bar indicates 2000 Å

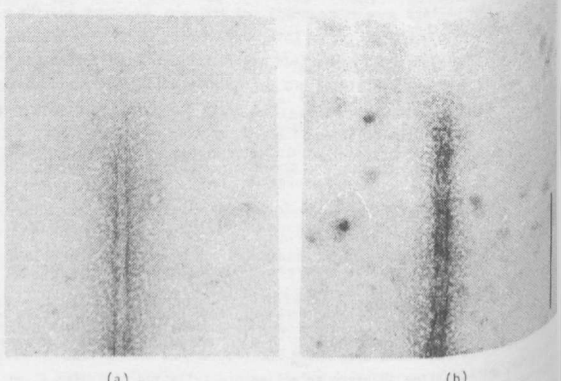

Fig. 6 Titin associated with negatively stained native thick filaments.
(a) Strands at the end of a filament
(b) Strand running alongisde the cross-bridge region
Scale bar in (b) indicates 2000 Å.