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Titin: quantitative mass measurements by scanning transmission electron microscopy and structural implications for the sarcomere matrix of skeletal muscle

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Scanning transmission electron microscopy has been used to investigate mass and linear mass density of native titin-2, a large soluble fragment of intact titin, from rabbit skeletal muscle. Dark field images of unstained, freeze-dried titin-2 appeared as either compact globules or looser and larger balls of string. Direct mass measurements indicated that the compact forms have an average mass of $2.40 \pm 0.50 \times 10^6$ Da. The mass to length ratio, determined from well-spread portions of titin strands (3–5 nm wide) from the ball of string forms, averaged 2.7 ± 0.9 kDa/nm. Thus a single native intact titin molecule has a calculated contour length of well above ~ 1 μm, sufficient to span unidirectionally between the Z line and M line region in a resting-length sarcomere.

Titin; Connectin; Scanning transmission EM; Sarcomere matrix; Elastic filament; (Skeletal muscle)

1. INTRODUCTION

Titin, a giant protein that is the third most abundant component of striated muscle sarcomere, is thought to constitute a set of elastic longitudinal filaments that impart structural continuity to the sarcomere by linking thick filaments, along their length, to the Z line (see reviews [1,2]). Despite intensive antibody localization analysis, much of titin's architectural arrangement in the sarcomere remains speculative. As a complementary approach, we have initiated studies of molecular morphology and assembly properties of purified titin. We [3] and others [4,5] have reported that purified native titin-2 (a large soluble fragment of intact titin (titin-1)), when rotary-shadowed or negatively stained, appears as extremely long, flexible strings of beads about 4 nm wide. These beaded-strings however exhibited a broad and heterodisperse length distribution from 0.2 μm to 1.2 μm [3], and it was not possible to assign a definitive contour length for native rabbit titin (see also [4]). Molecular mass and size analysis of chicken titin by sedimentation and hydrodynamic techniques also suffered from such heterodispersity [5].

In this paper, we report that by applying scanning transmission electron microscopy (STEM), which is well-suited to evaluate mass and dimensional parameters of individual molecules or entities even in a heterodisperse specimen [6], we have measured the mass and linear mass density (mass to length ratio) of native rabbit titin-2. These values now allow the contour length to be calculated. Portions of these data have been presented at the 1984 ASBC annual meeting [7].

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2. EXPERIMENTAL

2.1. *Native titin-2*

Rabbit skeletal muscle titin-2 was purified as described [3]. The second titin peak in the included volume of a Sephacryl S1000 column was dialyzed exhaustively against 1.0 M am-

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monium formate/20 mM imidazole/2 mM NaN_3/0.1 mM dithiothreitol (pH 7.8).

2.2. *Scanning transmission electron microscopy*

The Brookhaven Biotechnology Resources STEM was used, with its 40 keV electron probe focused to a 0.25 nm diameter [8]. Native titin was applied to glow-discharged carbon film by the 'drop-injection' technique [9] with coabsorbed Tobacco mosaic virus (TMV). The grids were allowed to stand for 5–10 min before washing 10 times with 1.0 M ammonium formate (pH 7.8 by NH_4OH) and then quickly frozen in liquid N_2 slush and dried in vacuum. Specimens were maintained at −130°C during imaging and low dose, first scan images were obtained in the dark field mode.

2.3. *Determination of mass and linear mass density*

The particle mass was measured by integrating the intensity of the dark field scattering, off-line, on a Brookhaven VAX 11/750 computer using TMV as the internal standard and subtracting local background intensity [6,8]. The linear mass density was determined on short segments (10–50 nm) of extended titin strands or of the background by measuring similar segments in representative nonparticle areas.

3. RESULTS

3.1. *STEM of unstained titin-2*

Dark field images of unstained, freeze-dried titin-2 on a thin carbon film (fig.1) revealed two predominant types of morphology: one type appears as compact globules with stringy perimeters and dense cores roughly from 25 to 35 nm in overall diameter; the other type appears as a loose ball of string that spread well over 80 nm. In favorable cases individual strings can be discerned as 3–5 nm wide filaments. Variations of protein concentration from 5 to 30 μg/ml, and ionic strength from 0.1 M to 1.0 M ammonium formate in the sample buffer did not alter their general appearance. These images of tangled or aggregated filaments are in contrast to that well-spread, beaded-string appearance of rotary-shadowed titin on a mica surface [3–5]. This is presumably due to the more pronounced surface-mediated interactions on the hydrophilic mica surface.

3.2. *Mass and linear mass density of titin-2*

Initial analysis of these images indicated that only the compact globular forms were suitable for reliable particle mass measurements, because the ill-defined boundaries of the ball of string forms made it difficult to assign areas for mass integration. The mass distribution curve for 29 particles from two independent protein preparations

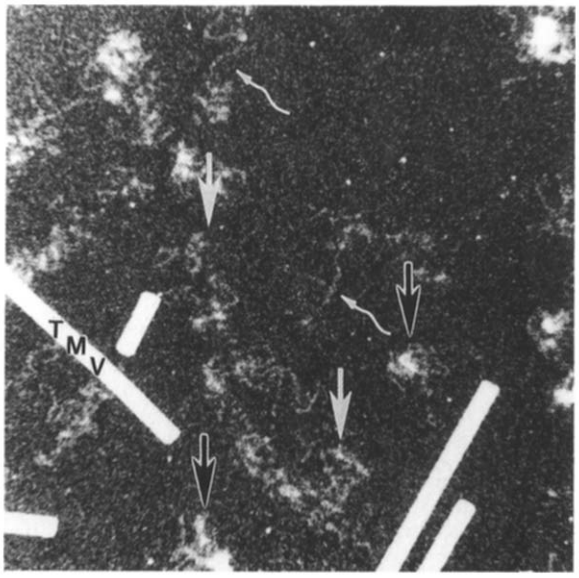

Fig.1. Dark field STEM images of unstained, freeze-dried native titin-2. Native titin molecules, freeze-dried on carbon film, appear as either compact globules (black arrows), with dense centers and stringy perimeters; or ball of strings (white arrows) which can be discerned as comprising 3–5 nm wide filaments. Single well-spread titin strands (curved arrows) are also visible. The straight rods are TMV particles.

(fig.2A) indicated that, despite some scattering resulting probably from degraded or aggregated titin, most particles are within a reasonable narrow range around an average value of 2.40 ± 0.50 × 10^6 Da.

The linear mass density was measured on extended titin strands from the ball of string forms. The distribution curve of linear mass density, uncorrected for the background (fig.2B), ranged from 1.9 to 6.1 kDa/nm, with the majority around an average value of 3.5 ± 0.9 kDa/nm (n = 44). The sizeable variation stemmed from several factors: (i) the background mass due to residual salts or denatured proteins averaged to 0.8 ± 0.2 kDa/nm (n = 14) (fig.2B) and represented a significant fraction (10–25%) of the fairly low linear mass density of titin; (ii) titin strands were either curvilinear or had irregularly spaced nodules of various sizes [3,4]. Both features would lead to an overestimate of linear mass; (iii) a portion of the titin strand may have been stretched thin ($\ll$4 nm) (cf. fig.2f of [3]), leading to an underestimation. Titin strands with such features were excluded whenever clearly identifiable, but

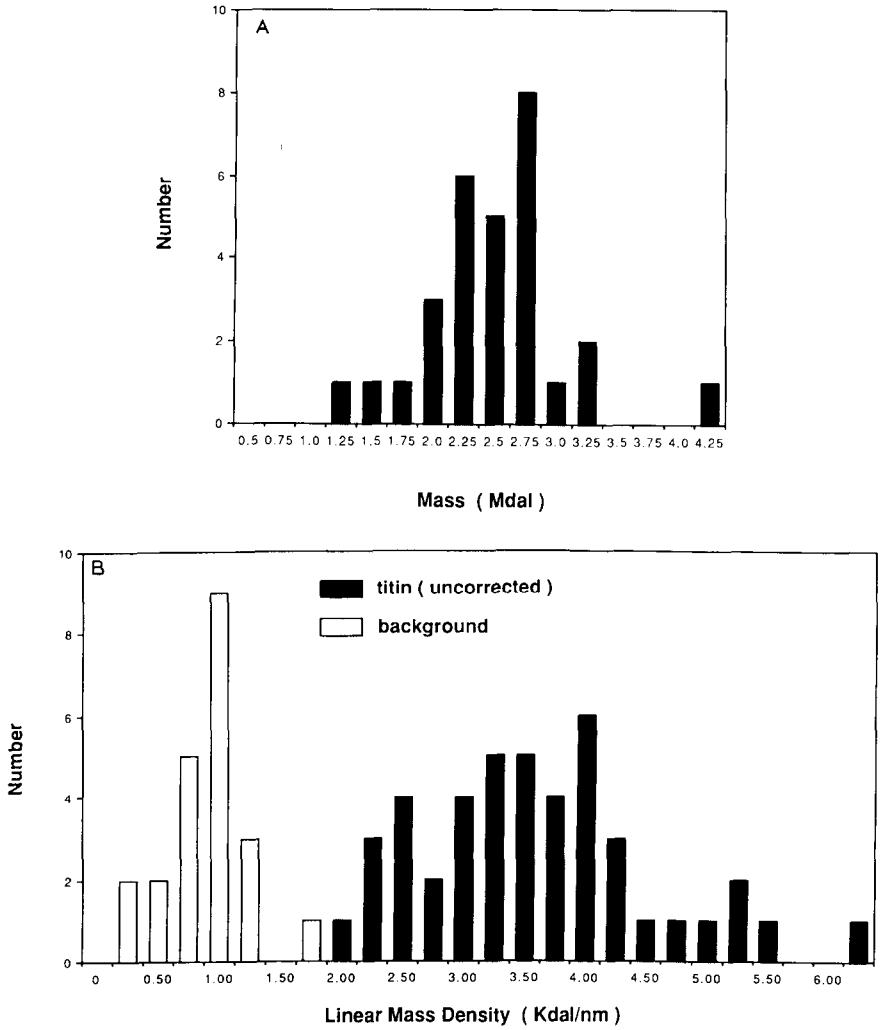

Fig.2. Histograms of distribution of mass and linear mass density of native titin-2 molecules. (A) Mass distribution. Values after subtracting for background mass are plotted. (B) Linear mass distribution. Both uncorrected values and background values are plotted.

some may have inadvertently entered into the final measurements. After subtracting a background value of 0.8 ± 0.2 kDa/nm, the linear mass density of titin strand has an average value of 2.7 ± 0.9 kDa/nm.

4. DISCUSSION

4.1. *Mass, linear mass density and contour length of native titin-2*

The present data, although considerably below the high precision that STEM can potentially offer [6], have provided important data that are missing links in a complete description of the basic molecular characteristics of native titin: first, a molar mass of 2.40 ± 0.50 × 10^6 Da for native titin-2 is very similar to the subunit chain mass of 2.4 to 2.6 × 10^6 [10], indicating that native titin-2 in high ionic strength solutions exists mainly as monomers of a multimegadalton polypeptide; second, the contour length of a native titin-2 molecule with a linear mass density of 2.7 ± 0.9 kDa/nm would be about 0.9–1.0 μm. This value corresponds to the longest of the peak values of the heterodisperse length distribution curve of rotary-shadowed titin that we have described (cf.

fig.2i of [3]), indicating that shorter filaments represent degraded titin, instead of assembly-intermediates of titin polypeptides; third, the linear mass density imposes an important constraint that has to be considered in evaluating proposals of morphology and polypeptide folding of titin. For example, two alternative models of titin molecules, a cylindrical rod of 4 nm diameter, and a string of spherical beads of 4 nm diameter, predict linear mass values of 10.3 kDa/nm and 5.3 kDa/nm, respectively (assuming spec. vol. = 0.73 cm^3/g [3]). These are significantly higher than the measured value, suggesting that titin may have a noncircular cross-section or a diameter smaller than 4 nm.

Table 1

Linear mass densities of cytoplasmic filaments

Filaments	kDa/nm
Titin (pure)[a]	2.7 ± 0.9
Thin filament[b]	24
F-actin (pure)[c]	15.5 ± 2.2
Intermediate filaments (pure)[b]	37 ± 4
Thick filament[d]	87 ± 23
Microtubule (pure)[e]	210 ± 19

[a] This work
[b] Steven et al. (1983) [12]
[c] Abei et al. (1986) [13]
[d] Lamvik (1978) [14]
[e] Johnson and Wall (1983) [15]

4.2. *Titin: a filamentous molecule spanning half a sarcomere*

We have previously suggested, based on immunoelectron microscopy with monoclonal antibodies directed to several unique nonrepeating epitopes of titin, that a single titin molecule might span unidirectionally the entire distance between the Z line and the M line region [11]. The calculated contour length of intact titin (titin-1, which is ~15% larger and presumably proportionally longer than titin-2), 1.1 to 1.2 μm, corresponds closely to the half sarcomere length of a resting muscle. Therefore our mass analysis lends credence to this novel idea. This unidirectional arrangement in turn suggests that the contour length of titin may be an important parameter in determining the rest length of the sarcomere.

The linear mass density of native titin now allows us to estimate the upper limit of extension of titin filaments in sarcomeres when subjected to stretch. Assuming that the most extended polypeptide conformation can be approximated by the geometry of β-sheet (with a displacement of 3.47 Å per residue and a linear mass of 0.32 kDa/nm), a 8–9-fold extension of the contour length of titin filaments is theoretically possible.

A comparison of the linear mass density of titin with those of other cytoplasmic filaments (table 1) is informative: it is merely 3.1% of that of thick filaments and 14% of that of thin filaments. Given the slenderness, the extreme flexibility and the low mass density of titin strands, there is little wonder that titin filaments in the sarcomere have successfully evaded detection by many morphological techniques which generally favor large size, unique form and dense mass.

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