Direct Visualization of Surface-adsorbed Single Fluorescently Labeled Titin Molecules

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Abstract

The properties of titin, a giant, structurally and mechanically complex filamentous protein were explored here by using single-molecule techniques. Fluorescently labeled titin molecules deposited on a glass substrate surface were directly visualized by using confocal microscopy. The molecules appeared as bright particles with a spatially distributed Gaussian fluorescence intensity profile. The physical dimensions of the space occupied by the 1-µm-long molecule on the surface are below the 0.25 µm resolution limit of the instrument used here, thereby directly demonstrating the flexible nature of the titin filament. The kinetics of titin’s surface adsorption revealed that the process is governed by diffusion and the molecules are irreversibly bound to the substrate surface. From the kinetics data the molecule’s translational diffusion constant was calculated.

Introduction

Titin is a giant, ~3.5 MDa muscle protein of modular construction, whose structure may serve as a molecular template for sarcomeric organization, and whose elasticity defines the passive mechanical properties of striated muscle [1, 2, 3, 4]. The I-band segment of titin is constructed of serially-linked immunoglobulin (Ig) type C2 domains interspersed with unique sequences including a proline-, glutamate-, valine- and lysine-rich (i.e., PEVK) segment and the N2A and N2B segments that lend tissue specificity to the molecule [5]. Upon stretch of the sarcomere, passive tension is generated by the extension of titin’s I-band segment [6, 7, 8]. The A-band segment of titin is composed primarily of super-repeats of different Ig- and fibronectin (FN3)-type domains [5]. This portion of titin appears to provide a structural scaffold for thick filament assembly [9].

Due to its large size, structural complexity and elastic nature it is difficult to explore titin’s properties by conventional bulk-solution methods. Single-molecule techniques, on the other hand, may provide unique information about titin’s characteristics. Titin, given its conveniently large size, has even been used as a protein-folding model system in various recent single-molecule studies [10, 11]. Single-molecule-mechanics experiments described titin as an entropic chain in which domain unfolding was inferred to occur at high forces and refolding at low forces [12, 13, 14, 15]. However, the spatial distribution of the structural changes remained hidden. It
would be desirable to be able to visualize the molecule while being manipulated in aqueous solution. As a step towards this objective, in this work we directly imaged surface-adsorbed, fluorescently labeled individual titin molecules.

Materials and Methods

Protein Preparation and Labeling

Titin was prepared from rabbit *longissimus dorsi* muscle using previously established protocols [16, 17] with modifications. Titin was purified by gel filtration and fluorescently labeled with the sulfhydryl reagent tetramethylrhodamine-5-(and-6)-iodoacetamide (TMRIA). The labeling ratio varied between 80-140 TMRIA molecules per titin molecule. Multi-molecular aggregates were removed by centrifugation [18].

Imaging of Surface-adsorbed Titin

Fluorescently labeled titin molecules were allowed to bind to the surface of a glass coverslip and visualized by using confocal microscopy (Bio-Rad MRC-1024 laser scanning confocal system, Nikon Eclipse TE300 inverted microscope, Nikon PlanApo 100x/1.4NA Oil objective). Samples were excited by using the 514-nm line \(0.120\) mW of an Ar-ion laser (Ion Laser Technology) at 0.3-1% intensity. The typical scan time for a 512 x 512 (11.3 x 11.3 µm) pixel image was 1.2 s, which corresponds to an integrated exposure time of ~0.55 ms for a single surface-adsorbed molecule. Fluorescence was detected through a 550-nm longpass filter. To reduce photobleaching, the solution contained 140 mM ß-mercaptoethanol. Microscopic images were imported into “Image” software (v.1.61, Wayne Rasband, NIH) and analyzed with custom-written Pascal routines. For measuring surface-adsorption kinetics, a 10-µl titin sample, diluted to a protein concentration of 19 ng/ml, was pipetted onto the coverslip surface and incubated for various amounts of time. Following incubation the coverslip surface was rinsed with buffer.

Theory and Calculations

Polymer Configuration Statistics

The mean-square end-to-end distance \(\langle R^2 \rangle_{2D} \) of a polymer chain deposited by equilibration on a substrate is

\[
\langle R^2 \rangle_{2D} = 4PL \left( 1 - \frac{2P}{L} \left( 1 - e^{-\frac{L}{2P}} \right) \right)
\]  

where \(L\) is the contour length of the chain, and \(P\) is the persistence length, the measure of the chain’s bending rigidity [19].

A flexible, fluorescently labeled polymer, for which \(P<<L\), may contract into a configuration in which its chain segments cannot be optically resolved, and the images of the chain segments coalesce into a single, diffraction-limited spot. The mean radius of the spot can be related to the radius of gyration \(R_g\). For a polymer chain unperturbed by excluded volume effects, in the limit of infinite chain length, the end-to-end distance and the radius of gyration are directly related as ([20])

\[
\langle R^2 \rangle = \frac{\langle R_g^2 \rangle}{6}
\]  

For point emitters under illumination with coherent light source the minimum resolved distance is given by the Rayleigh criterion

\[
d_{\text{min}} = \frac{0.61\lambda}{\text{NA}}
\]

where \(\lambda\) is the wavelength of the light used and \(\text{NA}\) is the objective numerical aperture. For the 572 nm emission maximum of TMRIA and the 1.4 NA used here \(d_{\text{min}}\sim0.25\) µm.

Deposition of Titin onto Substrate Surface

If the transfer process is driven solely by diffusion, then the fraction of titin molecules attached to the surface at any time \(t\) is

\[
n_s = n_0 \left( \frac{4D}{\pi} \right)^{1/2} \sqrt{t}
\]

where \(n_s\) is the number of surface-adsorbed molecules per cm², \(n_0\) is the number of molecules in unit volume (cm³) of solution at \(t=0\), and \(D\) is the diffusion coefficient [19, 21]. The relationship holds true provided that a) the titin molecules are irreversibly bound to the surface, b) convection currents do not contribute to the transport from solution to the surface, and c) the top layer of the deposition drop is not significantly depleted of titin molecules during the deposition time. If either condition a) or b) is not true, \(n_s/n_0\) will be proportional to a power of \(t\) higher than 0.5 [21]. By fitting the power function

\[
f(x) = k_1x^{k_2}
\]

to the experimental data of the fraction of deposited molecules versus time we obtain the coefficients \(k_1\) and \(k_2\). Whereas \(k_1\) allows the calculation of the diffusion coefficient of titin, \(k_2\), the exponent of the time variable, reveals the
mechanism that governs the deposition process. The diffusion constant of the statistical polymer chain can be related to its contour \((L)\) and persistence \((P)\) lengths as

\[
D = \frac{k_B T}{3\eta L} \left( 1 + 1.84 \left( \frac{L}{2P} \right)^2 \right)
\]

where \(\eta\) is solution viscosity, \(k_B\) is Boltzmann’s constant, and \(T\) is absolute temperature \([18]\).

Results and Discussion

Appearance and Dimensions of Surface-Adsorbed Titin Molecules

Individual molecules of the giant muscle protein titin were visualized following fluorescent labeling and surface adsorption. Titin molecules appeared as bright spots under the confocal microscope (Fig. 1A). In the first approximation the particles were treated as ellipses for which the major and minor axes were determined. We observed a nearly one-to-one correspondence between the two axes, indicating that the particle images were essentially circular. The minor axis values were therefore used to describe the diameter of the particles. For some image particles, however, there was no correlation between the minor and major axes, suggesting that they represent two or more closely spaced, and therefore unresolved, molecules. As Fig. 1B suggests, the fluorescence intensity distribution across each particle image resembles an Airy disc, the image of a diffraction-limited self-luminous point.

**Fig. 1.** Image of surface-adsorbed titin molecules. (A) Confocal microscopic image and dimensions of fluorescently labeled titin molecules adsorbed to a microscope coverslip. Scale bar, 2 µm. (B) Surface plot of a portion of titin-coated glass coverslip. X and Y axes are spatial dimensions (scale bar, 1 µm), and the Z-axis is fluorescence intensity.

**Fig. 2.** Particle analysis of the surface-adsorbed titin molecules with consideration of their Gaussian fluorescence intensity profile. (A) Fluorescence intensity profile across the center of a surface-adsorbed titin molecule, with Gaussian fit. (B) Distribution of the diameter of the particles measured at their half-maximal grayscale intensity. The mean particle diameter is 0.289 µm (±0.04 SD, \(n=873\)). (C) Distribution of the grayscale intensity maximum of the particles.
In support of this notion, the grayscale intensity profile across such a particle image can be fitted well with a Gaussian function (Fig. 2A). The diameters of the particle images were determined as the half-maximal width of the Gaussian intensity profile across the particle. The histogram of particle image diameter calculated accordingly (Fig. 2B) reveals a narrow distribution around a mean value of 0.289 μm (±0.04 SD, n=873). For comparison, an average diameter of 0.3-0.5 μm has recently been reported for titin labeled with Cy3 [22]. The mean particle image diameter (0.289 μm) is comparable to \(d_{\text{min}}\) of our instrument (0.25 μm), but slightly exceeds it because of the smoothing convolution step that degrades the effective image resolution (i.e., increases \(d_{\text{min}}\)). The narrow distribution of the diameter suggests that the physical dimensions of the area occupied by any of the particles fall below \(d_{\text{min}}\) regardless of their peak fluorescence intensity (Fig. 2C). The wide distribution of the peak fluorescence intensity may be explained with several factors: a) presence of multi-molecular aggregates, b) presence of both T1 and T2 form of titin (mother molecule and proteolytic fragment, respectively) which have different molecular weights and corresponding chain lengths, c) molecule-to-molecule variation in the fluorophore labeling ratio, and d) molecule-to-molecule variation in the fraction of non-fluorescent rhodamine dimers. Thus, the physical size of the area occupied by a titin molecule equilibrated or trapped to the glass surface cannot be resolved by this method. Accordingly, the radius of gyration \(R_g\) of a surface-adsorbed titin molecule must be smaller than the radius of the smallest resolvable particle (~0.125 μm). According to equation 2, the root-mean-square end-to-end distance \(<R^2>\) of the surface-adsorbed titin chain may not exceed ~0.3 μm. Considering the contour length (~1μm, [23]) and the persistence length (15 nm, [18]) of the native titin molecule, according to equation 1 its \(<R^2>\) is ~0.24 μm, which indeed falls below the upper limit calculated here.

### Kinetics of Surface-adsorption of Titin

To determine the diffusion constant of the fluorescently labeled titin molecules and to explore the mechanism of their transfer from solution to the substrate surface, we measured the kinetics of surface adsorption. The fraction of molecules attached to the substrate surface was determined after varying periods of incubation time. Fig. 3 shows our results. The experimental data were fitted well with equation 5 (\(r=0.93\)). The exponent of the time variable was 0.48, and a diffusion constant of \(4.8\times10^{-8} \text{ cm}^2/\text{s}\) was calculated. The exponent of time is comparable to that for a diffusion-driven process \((0.5, [21])\), indicating that the transfer of titin molecules from the solution to the substrate surface is governed by diffusion, and that the molecules bound irreversibly to the surface (see Theory and Calculations). The diffusion constant calculated here \((4.8\times10^{-8} \text{ cm}^2/\text{s})\) is comparable to but somewhat lower than the temperature-corrected value of the earlier-determined diffusion constant of titin \((6\times10^{-8} \text{ cm}^2/\text{s} [18])\). Possible reasons for discrepancy are that a) our samples contained a greater proportion (~50%) of T1, the larger, mother molecule of titin, and b) a low number of contaminating aggregates were present in spite of the clarifying centrifugation procedure. Both of these factors contribute to a decrease of the apparent diffusion constant. The overall similarity of our result to the one previously determined [18] confirms titin’s diffusion constant and suggests that the fluorescent modification did not significantly alter the molecule’s hydrodynamic properties. The persistence length of the native titin molecule calculated according to equation 6 using the diffusion constant determined above and a contour length of 1 μm [23] is 26 nm. This persistence length exceeds that calculated earlier for native β-connectin (or T2) (15 nm [18]), but can still be reconciled with the upper limit of the physical dimensions of the area occupied by the surface-adsorbed molecule. The calculated persistence length must be treated with caution, however, for the same reasons as the diffusion constant determined here. Conceivably, by using a more efficient method to prevent the aggregation of titin, or by precisely determining the titin-molecule concentration, measuring the kinetics of molecular deposition may, in the future, yield better estimates of the physical parameters of the titin molecule.

![Fig. 3. Kinetics of surface adsorption of fluorescently labeled titin molecules. The ratio of surface-bound molecules (per cm²) and initial concentration (molecules per cm³ solution at t=0) is shown in a log-log plot as a function of incubation time. Inset shows the same dataset on a linear scale. The data were fitted with equation 5 to obtain the parameters of equation 4. An exponent of 0.48 was determined and a diffusion constant of 4.8x10-8 cm²/s was calculated from the fit.](image-url)
Implications

Individual titin molecules have, in the past, been visualized with higher-resolution methods [24, 25, 26]. However, examining fluorescently labeled titin molecules is advantageous over these techniques because the molecules remain in the aqueous phase, and can be detected even in molecular crowds. Even though structural detail of the molecule cannot be resolved, the molecules can be detected, which lends itself to measuring titin’s hydrodynamic properties. The method employed here may offer unique advantages over the bulk solution method used before [18]. Considering that individuals of molecules are examined, the data may be segregated according to user-prescribed image criteria, and associations of titin and other, suitably labeled, proteins may be distinguished. Ultimately, the interaction between titin and other sarcomeric proteins may be studied by the technique at the single-molecule level.

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References