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Up-and-down movement of a sliding actin filament in the *in vitro* motility assay

Itsuki Kunita [a], Shigeru Sakurazawa [b,*], Hajime Honda [c]

[a] Molecular and System Life Science Unit, Riken Advanced Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
[b] School of Systems Information Science, Future University Hakodate, 116-2 Kamedanakano, Hakodate, Hokkaido 041-8655, Japan
[c] Department of BioEngineering, Nagaoka University of Technology, 6000 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

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ABSTRACT

We observed a three-dimensional up-and-down movement of an actin filament sliding on heavy meromyosin (HMM) molecules in an *in vitro* motility assay. The up-and-down movement occurred along the direction perpendicular to the planar glass plane on which the filament demonstrated a sliding movement. The height length of the up-and-down movement was measured by monitoring the extent of diminishing fluorescent emission from the marker attached to the filament in the evanescent field of attenuation. The height lengths whose distribution exhibits a local maximum were found around the two values, 150 nm and 90 nm, separately. This undulating three-dimensional movement of an actin filament suggests that the interactions between myosin (HMM) molecules and the actin filament may temporally be modulated during its sliding movement.

1. Introduction

There has already been accumulated a sufficient amount of investigations focusing upon the mechanisms of force generation driving muscle contraction. That is interactions between actin and myosin molecules (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954; Huxley, 1969; Huxley and Simmons, 1971; Yanagida et al., 1985; Yanagida, 1990; Harada et al., 1990; Pollack, 1996). Fluorescent microscope technique in the framework of *in vitro* motility assay enables one to directly observe the sliding movement of actin filaments on HMMs fixed on the glass surface (Yanagida et al., 1984; Honda et al., 1986; Kron and Spudich, 1986; Harada et al., 1987; deBeer et al., 1997). The movement has been supposed to smoothly be planar on the glass surface.

However, each molecular state appearing in one cycle of ATP hydrolysis has a three-dimensional structure that is stereochemical. In fact, the binding between an actin molecule and a myosin head is complementary with each other in their conformations under rigor conditions and the attachment is complete there (Rayment et al., 1993). Conformational changes in the actin–HMM complex that occur during the sliding movement could be visually monitored by quick-freeze deep-etch electron microscopy (Katayama, 1998). The results revealed that a limited number of HMM heads are in the state of rigor conformation, while most of the heads form ATP-bound crossbridges. Moreover, the direct measurements of the force generated in single actin filaments demonstrate that the interactions necessary for the force generation were implemented in a very limited number of myosin molecules (Nishizaka et al., 1995). These observations, when combined together, come to suggest that the interactions necessary for the force generation for the sliding movement may be quite sporadic between actin molecules and HMM heads. We shall then examine how intermittent spatio-temporally the interactions could be between actin and myosin for the generation of the force for driving the sliding movement of an actin filament.

For this objective, we employed the method of evaluating the exponential decrease of evanescent light-intensity to estimate the extent to which an actin filament could move in the direction perpendicular to the planar plane of the sliding movement. The vertical displacement of the filament was measured as focusing upon the fluorescent marker or as utilizing a speckled actin filament in order to gain a high spatial resolution (Honda et al., 1999).

2. Materials and methods

2.1. Proteins

Actin and myosin were extracted from rabbit skeletal muscle (Spudich and Watt, 1971; Perry, 1955). HMM was prepared by alpha-chymotryptic digestion of myosin (Okamoto and Sekine, 1985). In order to observe an actin filament, two kinds of the fluorescence-labeled actin filaments, totally labeled actin filaments and speckled actin filaments, were prepared. Totally labeled actin filament was made and treated with equal molar rhodamin-phalloidin (Sigma) in buffer A (25 mM KCl, 25 mM imidazole–HCl, 3 mM $MgCl_2$, 1 mM DTT, pH = 7.4). In order to prepare speckled actin filaments, G-actin and equal molar phalloidin (Sigma) was mixed in buffer A, and was added to the solution containing segmented fluorescence-labeled actin filaments. The mixture was placed at a temperature of 4 °C for 12 h for formation of the speckled actin filaments.

* Corresponding author. Tel.: +81 138 34 6335; fax: +81 138 34 6301.
E-mail address: sakura@fun.ac.jp (S. Sakurazawa).

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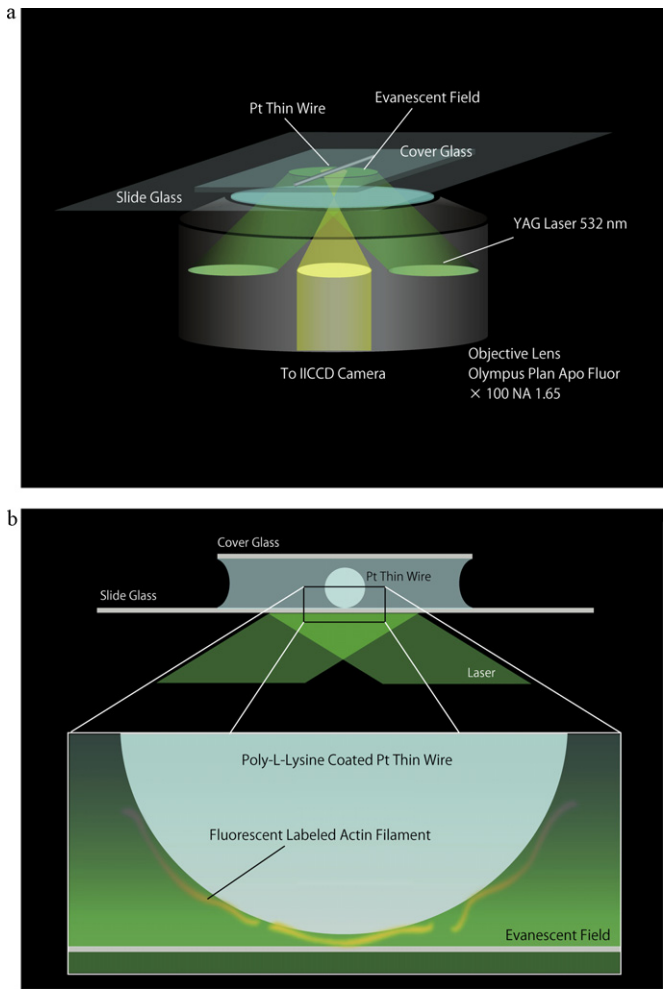

Fig. 1. (a) Schematic diagram of determination system of intensity–height transformation curve by using Pt wire, labeled actin filament and TIRFM. (b) Height estimation of actin filaments observed by TIRFM. The luminance of actin filaments adsorbed to Pt wire with a round cross section darkened exponentially with increasing the distance from glass surface based on a curvature of Pt wire.

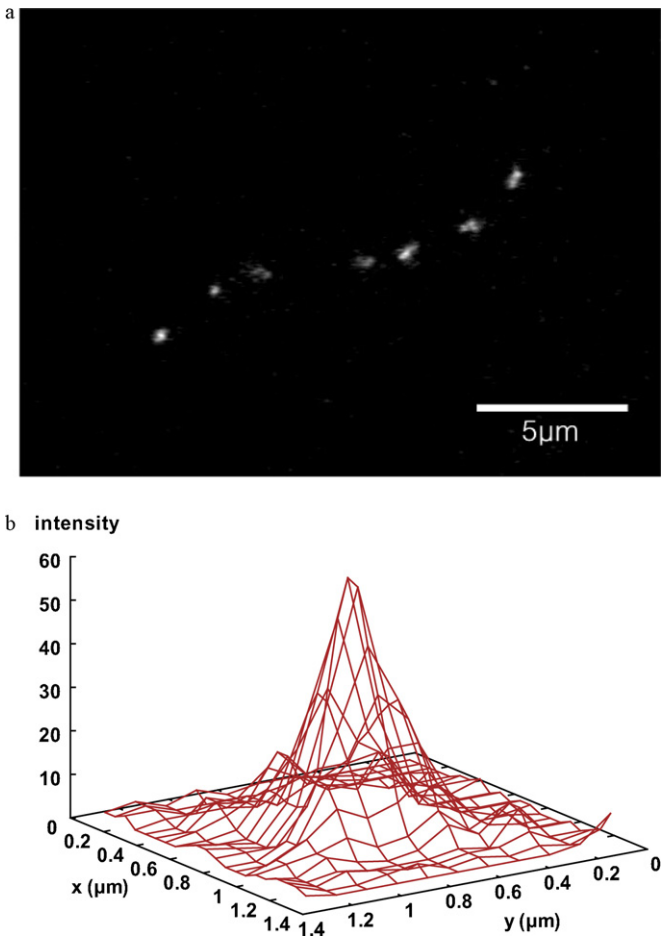

Fig. 2. (a) A typical image of a single speckled actin filament consisted of seven fluoresce-labeled spots observed with TIRFM. Each spots were sliding on surface-fixed HMMs by forming line longitudinally. (b) Intensity profile of a fluorescence-labeled spot on the actin filament. The intensity means the grayscale intensity values. The profile had 2D-Gaussian distribution. From the fitting function of the Gaussian, the position of the spot is determined as the *x*–*y* coordinate of the top.

2.2. Observations

The microscope (Olympus, IX70) attached with the objective lens (Olympus, Apo 100×, oil, NA = 1.65) was used for the fluorescence-labeled actin filaments with total reflection illumination unit (Olympus, IX2-RFAEVA). A diode pumped frequency doubled Nd:Yag laser (JDS Uniphase, Model 4611-010-0980) was used as the light source for a total internal reflection fluorescence microscopy (TRIFM). Images under the microscope were taken with an image-intensified CCD camera (Hamamatsu Photonics, C2400-97V) and recorded in a hard disk through a video capture board (I-O DATA, GV-BCTV5/PCI). Each image was captured at every 1/30 s.

In order to measure the planarity of the collodion-coated glass surface, the surface of collodion film was scanned as using a tapping mode atomic force microscopy (AFM, Veeco: Multi Mode AFM). The AFM results were recorded with software (Veeco: NanoScope Control, NanoScope Image), and analyzed with software (Image Metrology: SPIP).

2.3. Determination of intensity–height transformation curve

The height of actin filaments in the evanescent light was determined from the intensity with use of a Pt wire (see Fig. 1). The Pt wire (ϕ = 0.3 mm) were treated with poly-L-lysine (Sigma Co.). The totally labeled actin filaments adsorbed to the Pt wire were observed by TIRFM, and the images were recorded.

The relationship between the height of an actin filament and the light intensity was calculated from the curvature of the Pt wire, and estimated a laser incident angle from the theoretical values available in the following equation (Axelrod, 2003).

$$I(z) = I(0)\exp\left(\frac{-z}{d}\right)$$
$$d = \frac{1}{2k_2}\left\{\frac{1}{n}\sqrt{\sin^2\theta - n^2}\right\}^{-1} \quad (1)$$

The intensity of the evanescent field exponentially decays with the perpendicular distance z. Parameter k_2 (=$2\pi/\lambda$) here is the wavenumber of the incident laser in the solution, n is relative refractive index of the solution to the glass, and θ is the incident angle of the laser beam. An intensity–height transformation curve was determined based on theoretical values on the incident angle.

2.4. In vitro motility assay

We prepared a standardized *in vitro* motility assay (Hatori et al., 1998, 1996). The slide glass to fix HMM was hydrophobically treated with 0.1% collodion (nacalaitesque) in 3-methylbutyl acetate. The solution condition for observing the sliding movement of an actin filament was 25 mM KCl, 25 mM imidazole–HCl (pH = 7.4), 3 mM $MgCl_2$, 0–2 mM ATP, and 0.5% β-mercaptoethanol at 22 °C.

2.5. Image analysis

In order to remove the unevenness of illumination and offset of the camera, all images were corrected with the correction coefficient. The coefficient was determined by observing the images of rhodamine dispersed uniformly on the glass surface. A specimen of rhodamine was dissolved in the solution for *in vitro* motility assay.

The position of each spotted marker along an actin filament was determined as reading the light intensity distribution from the marker (see Fig. 2). The height was estimated from the intensity–height transformation curve.