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Letter

Visualization of F-actin filaments by a fluorescently labeled nucleotide analogue

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Abstract

The tightly-bound nucleotide of F-actin was replaced with $1,N^6$-etheno-adenosine ATP (ADP). An epi-fluorescence optical microscope was modified to visualize efficiently the fluorescent analogue with an excitation-maximum wavelength of 310 nm. This microscope permitted us to visualize single F-actin filaments in solution using the fluorescence of the strongly bound $1,N^6$-ethenoadenosine nucleotide. Exchange of the tightly-bound nucleotide of F-actin with a free nucleotide in solution at a high temperature was quantitatively estimated by this method, and the results showed good agreement with results from phosphate release measurements.

Keywords: Fluorescence microscopy; Visualization of F-actin; $1,N^6$-etheno-adenosine nucleotide; Nucleotide binding of actin

1. Introduction

Strongly-bound nucleotides stabilize the structure of actin in both the monomeric and filamentous states [1,2]. The polymerization/depolymerization dynamics of actin are also regulated by the bound nucleotide [3–5]. To understand the role of bound nucleotides in actin, various nucleotide analogues, including fluorescent probes, have been applied to actin [6,7]. The best way to see directly what happens in the nucleotide-binding dynamics of F-actin is to visualize single actin filaments via the tightly bound nucleotide.

Since Yanagida et al. [8] first succeeded in visualizing single filaments of actin using a fluorescence microscope by labeling actin filaments

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Abbreviations: εAT(D)P, $1,N^6$-etheno-adenosine 5′-tri(di)phosphate

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with phalloidin-rhodamine, many experiments using this technique have given a deeper insight into the function of actomyosin motility at the molecular level. This labeling technique, however, is not the best for showing the role of the nucleotide in actin, because actin filaments are visualized independently of nucleotide binding [9]. The best method for understanding the role of the strongly bound nucleotide in actin is to visualize nucleotide molecules in F-actin and directly observe in real time the binding behavior of the nucleotide to actin molecules. Oiwa et al. [10] and Funatsu et al. [11] recently developed a method for visualizing single nucleotide molecules by using the fluorescently-modified adenosine nucleotide Cy3-EDA-AT(D)P. Unfortunately, however, this molecule does not efficiently displace the tightly bound ADP of actin, presumably because of the large size of the fluorescent probe (Dr M. Anson, personal communication).

Here we report a method for visualizing single actin filaments using εATP, another fluorescently labeled adenosine nucleotide that binds to actin in a manner similar to that of native ATP [12,13]. Our method is shown to be useful for quantitative measurements of the binding characteristics of this nucleotide to actin filaments.

2. Materials and methods

2.1. Protein preparation and chemicals

Actin was extracted from acetone-dried powder which was derived from rabbit skeletal muscle, and purified by polymerization with 30 mM KCl. All chemicals except for εATP were purchased from Sigma-Aldrich Japan Inc. (Chiba, Japan) or Nakarai tesque Inc. (Kyoto, Japan). The εATP was purchased from Molecular Probes Inc. (Eugene, Oregon, USA).

2.2. Exchange of bound nucleotides on actin

For observation of single F-actin filaments (Fig. 2), actin (0.6 mg/ml) was depolymerized and polymerized repeatedly in the presence of 0.6 mM εATP. The resultant G-actin was polymerized with equimolar phalloidin and was suspended in observation solution (30 mM Na/K phosphate, 20 mM KCl, 1% β-mercaptoethanol pH 7.4, without free εATP) at a protein concentration of 20–40 nM and observed with the microscope. To observe the time course of nucleotide exchange (Fig. 3), 0.6 mM εATP was added to 0.6 mg/ml ADP-F-actin in 50 mM KCl, 10 mM TrisCl pH 8.0 and incubated for various durations at 45°C. The solution was then chilled on ice to stop incubation, and a part of the solution was used for phosphate determination. A concentration of 50 mM (final concentration) $MgCl_2$ was added to the residual aliquot to form F-actin bundles. The bundled actin filaments were subjected to microscopic observation. The same procedure was performed again starting from the addition of 0.6 mM ATP to εADP-F-actin. Phosphate was determined according to Harada et al. [14].

2.3. Optical microscope construction

An inverted optical microscope equipped with epi-fluorescence illumination optics (type TMD, Nikon Inc., Tokyo, Japan) was modified as follows: the collection lens in front of the mercury lamp and the tube lens between the dichroic mirror and the objective lens were replaced with quartz lenses (Sigma Koki Inc., Saitama, Japan) of the same focal lengths but without anti-reflection coatings. The heat absorption filter, which absorbs UV light, was taken out. Instead, a 30-mm thick aqueous solution of 1 M nickel sulfate in a quartz container was placed in front of the lamp, in order to absorb IR light and leakage light from the excitation filter at 700 nm. The excitation filter, dichroic mirror and barrier filter were specially made for this experiment (Nikon Inc. and Sigma Koki Inc., Japan) in order to pass UV light of the 310-nm region efficiently. A quartz plate was used as the base plate of the dichroic mirror to avoid background emissions caused by UV excitation. The spectral characteristics of the filters and excitation/emission spectra of εATP are shown in Fig. 1. The excitation light was chosen for a wavelength range centered at 310 nm, which includes most of the peak of the εATP fluorescence excitation spectrum. In this region the emission spectrum of the high-pressure mercury

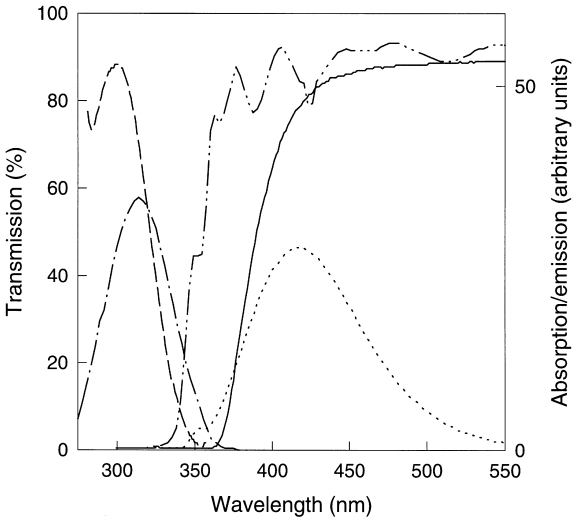

Fig. 1. Absorption/emission spectra of εATP and transmission spectra of microscope filters. Absorption (— — —) and emission (··· ···) spectra of εATP are shown in arbitrary units. — - — -, transmission spectrum of excitation filters including water path. ———, transmission spectrum of barrier filter. ———, transmission spectrum of dichroic mirror. All non-transmitted light beams are reflected by the dichroic mirror.

lamp has peaks at 297 and 313 nm. Emission light at wavelengths longer than 380 nm was passed through a barrier filter. In order not to illuminate the sample with the strong peak at 365 nm from the mercury lamp, the light leaked from the excitation filter at this peak was absorbed by the $NiSO_4$ solution and not reflected by the dichroic mirror.

To observe F-actin filaments, a quartz objective lens (Ultrafluor 100×, Carl Zeiss, Germany) was used. A sample solution was sandwiched between 0.16-mm-thick quartz cover glasses. The thickness of the sample chamber was 10–30 μm. The space between the objective lens and the nearer cover glass was filled with glycerol (Spectroscopic grade, Nakarai tesque Inc., Tokyo, Japan). The pictures were recorded with a SIT video camera (C-2400-08, Hamamatsu Photonics Co., Hamamatsu, Japan) and video recorder (SVHS, Matsushita Electric Co., Osaka, Japan). Recorded pictures were digitized and transferred to a personal computer (Centris 650, Apple Japan Inc., Tokyo, Japan) and analyzed by using NIH Image and Photoshop software.

3. Results and discussion

Under the strong illumination of 300–330-nm-wavelength excitation light, conventionally used chemicals for buffer solutions often emit background fluorescent light. As Trisma base and its analogues used for buffering pH gave strong background fluorescence, the sample solution for microscopic observation of single filaments contained a 30-mM phosphate buffer instead of other buffer chemicals. To inhibit photobleaching of the fluorescent probes, reducing reagents such as dithiothreitol or β-mercaptoethanol and enzymatic oxygen scavenger mixture [15] are commonly used. Most of these also gave high-intensity background emission and so were not added to the observation solution. Instead, oxygen dissolved in the solution was replaced with nitrogen prior to use through evacuation and nitrogen bubbling, alternately. By this replacement treatment, the decay time of the fluorescent image intensity of actin paracrystals under illumination of UV light was elongated six to 15 times. The εADP bound F-actin solution was sandwiched between quartz coverslips and the edges sealed with paraffin. The sample was observed by using the modified microscope and images of single F-actin filaments were recorded with a SIT video camera (Fig. 2a). Photobleaching of the fluorescent probes in the filaments was very fast, and the filament images mostly disappeared within 1 s. Each filamentous appearance was estimated to be a single filament of F-actin, not a bundle, because the filaments showed bending motion even within 1 s, and their bent appearance and the flexural rigidity, 3×10^{-17} dyn cm^2 ($N = 14$), were the same as those of single filaments visualized by using other methods [8,16]. Bundled F-actin appears straight and shows almost no thermal bending motion. In addition, under an accidental flow of solution in the observation chamber, actin filaments aligned to the direction of the flow with a transient bend in their shape. This could not happen in the case of bundles.

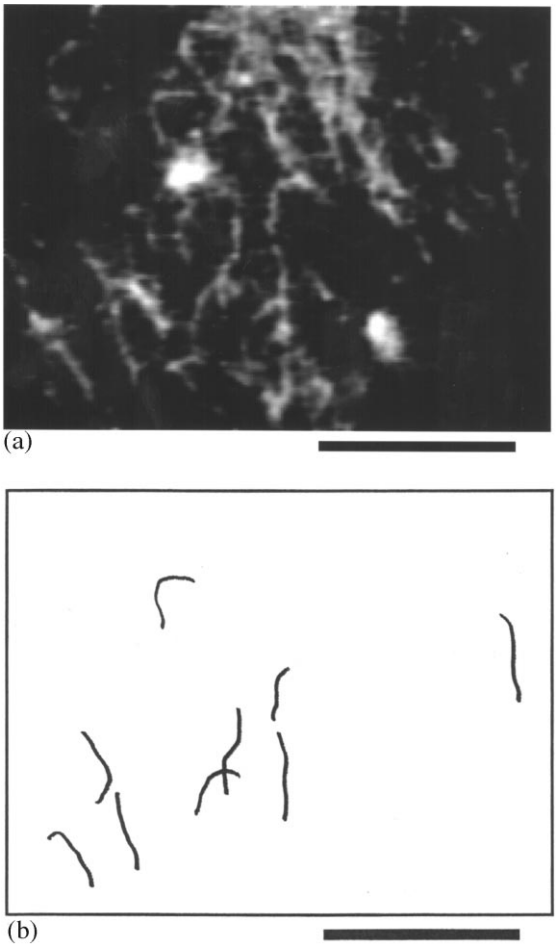

Fig. 2. Single actin filaments visualized by εADP fluorescence. (a) Microscopic picture of actin filaments. The estimated brilliance of the excitation light was 1×10^{-4} mW/μm^2. Eight successive frames from a video recording were averaged. After the subtraction of uniform background, contrast was enhanced with a gamma value of 1.2, and image noise was smoothed by using a median rank filter of NIH image software. Bar: 10 μm. (b) Representative drawing of single filaments. To indicate the single filaments in the frame (a), a lineal drawing is shown. Each line corresponds to the image of an actin filament which was estimated to be a single filament by the shapes and bending movement in (a). Bar: 10 μm.

Very fast photobleaching does not permit us to observe directly the functions of actin such as nucleotide binding or the polymerization/depolymerization process on single filaments. This is apparently because of the absence of oxygen-scavenger reagents/system in the solution. When the photobleaching of actin bound εAT(D)P was estimated using an εAT(D)P-actin solution in a cuvett as a sample, the decay time of the fluorescent intensity under the illumination of UV light was elongated 30–90 times by the addition of oxygen-scavenger reagents. Hence the observation time of a single F-actin filament could be extended to minutes if effective reducing and oxygen-scavenger reagents without serious background fluorescence emission could be developed. To estimate the possible damage to proteins caused directly by strong UV illumination, phalloidin-rhodamine-labeled F-actin with non-fluorescent nucleotides was observed by using the same excitation UV light in the presence of β-mercaptoethanol and oxygen-scavenger reagents. Rhodamine fluorescence (emission wavelength; 570–600 nm) could be effectively excited by the UV light. The appearance of and apparent length distribution of F-actin filaments did not change for more than 20–30 s under the illumination of UV light.

Part of the high-intensity background may come from residual free εAT(D)P, G-actin bound εADP and/or fluorescence from out-of-focused actin filaments. This kind of background light can be drastically decreased if the excitation is limited to the surface by using the evanescent wave illumination method [10,11].

As an example of application of the visualization method we developed, we chose the direct observation of the exchange of tightly bound nucleotides in F-actin with free nucleotides in solution. To accelerate the exchange we incubated the F-actin solution at a high temperature (45°C) and the reaction was stopped by cooling the solution on ice [17]. Estimation of the amount of exchanged nucleotides was made from the microscopic images of F-actin. Incorporation of εATP was examined by the incubation of ADP-F-actin in the presence of εATP for various time durations. For quantitative measurements of the amount of exchanged nucleotides from microscopic images, F-actin bundles were formed by the addition of 50 mM magnesium chloride prior to microscopic observation. Time-dependent changes of the average intensity of actin bundles per unit length are shown in Fig. 3a (black circles). The

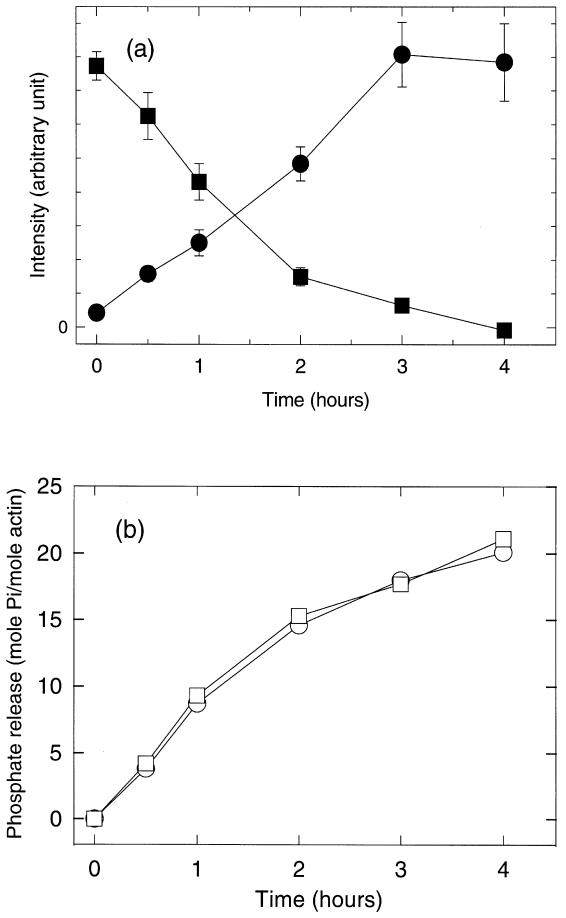

Fig. 3. (a) Exchange of strongly bound nucleotides in F-actin. ●, fluorescent intensity of ADP-bound F-actin bundle after incubation with εATP at 45°C for various durations. ■, fluorescence intensity of εADP-bound F-actin bundle after incubation with ATP at 45°C for various durations. Intensity was estimated as the average of bundle image intensity per unit length of 70–100 bundles for each point. (b) Phosphate release from F-actin as a result of 45°C incubation. Release of inorganic phosphate was estimated during the incubation of εADP-F-actin with ATP (○) and of ADP-F-actin with εATP (□). Practical aliquots of samples were subjected to phosphate determination during the image intensity estimation experiments in Fig. 3a (see Section 2).

reverse exchange of nucleotides was estimated by the incubation of εADP-F-actin with free ATP (Fig. 3a, black squares). The time course of the two cases coincides well as both decay to half at approx. 1–1.5 h of incubation and are saturated at approx. 3–4 h. This indicates that the binding constants of normal and ε-nucleotide do not differ from each other much [12,18]. During high-temperature incubation experiments, aliquots of each sample were subjected to phosphate determination and the results are shown in Fig. 3b. Upon incorporation into F-actin, actin-bound ATP(εATP) splits into ADP(εADP) and liberates phosphate [1]. Here the time course of phosphate liberation from both samples for the microscopic experiments agree well with each other and with those of reported data [17]. The results also agree with those for the nucleotide exchange estimated by microscopic observation, indicating that the quantitative estimation of bound nucleotides from microscopic observation represents the actually exchanged nucleotides well. We incubated the actin solution at 45°C to accelerate the exchange of nucleotides on F-actin. Although direct exchange of nucleotide on F-actin was shown as a result of the high-temperature incubation [17], there is a possibility that a part of the release or incorporation of the ε-nucleotide may come from continuous polymerization and deporimerization of F-actin. A clear answer to this question should come from stable observation of single εADP-F-actin filaments in the future, after this method has been further developed.

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