Single turnovers of fluorescent ATP bound to bipolar myosin filament during actin filaments sliding

Takahiro Maruta¹, Takahiro Kobatake¹, Hiroyuki Okubo¹ and Shigeru Chaen¹

¹Department of Integrated Sciences in Physics and Biology, College of Humanities and Sciences, Nihon University, Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan

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The nucleotide turnover rates of bipolar myosin thick filament along which actin filament slides were measured by the displacement of prebound fluorescent ATP analog 2′(3′)-O-[[N-[2-[(Cy3)amido]ethyl] carbamoyl]-adenosine 5′ triphosphate (Cy3-EDA-ATP) upon flash photolysis of caged ATP. The fluorescence of the thick filament where actin filament slides decayed with two exponential processes. The slower rate constant was the same as that without actin filament. Along bipolar myosin thick filament, actin filaments slide at a fast speed towards the central bare zone (forward), but more slowly away from the bare zone (backward). The displacement rate constant of fluorescent ATP from the myosin filament where actin filament moved forward was 5.0 s⁻¹, whereas the rate constant where the actin filament slid backward was 1.7 s⁻¹. These findings suggest that the slow ADP release rate is responsible for the slow backward sliding movement.

Key words: ADP release, nucleotide displacement, caged-ATP, in vitro motility assay

Muscle contraction results from the relative sliding of myosin and actin filaments, which is driven by the cyclical interaction of the myosin heads with the actin filaments during ATP hydrolysis. The myosin molecules form bipolar thick filaments, in which their heads reside on either side of a central bare zone (hereafter referred to as “center”). In striated muscle, actin filaments are generally thought to slide toward the center of the bipolar myosin filaments. More than two decades ago, an in vitro motility assay was developed to directly observe the movement of single actin filaments over a myosin-coated surface¹.

Such motility assays have shown that actin filaments move along myosin thick filament at a fast speed towards the central bare zone and at a slower speed away from the bare zone²⁻⁴. The force and displacement of myosin cross-bridges interacting with actin filament in the backward direction was smaller than in the forward direction⁵⁻⁶. Sellers and Kachar² speculated that during the backward movement, myosin heads rotate 180 degrees to face the actin in the correct configuration, and may be constrained such that the detachment of the rotated heads from actin occurs at a slower rate. Recently, we have demonstrated that the thermal activation energy of the backward movement is significantly higher than that of the forward movement⁷, suggesting that the backward movement causes the myosin heads to be constrained and increases the energy required for the ADP release step. However, there is no direct evidence for the slow ADP release rate in the backward movement.

Here, to examine whether rate of ADP release step depends on the direction of the actin movement, we measured the nucleotide turnover rates of bipolar myosin thick filament along which actin filaments slide by monitoring the displacement of prebound fluorescent ATP analog, Cy3-EDA-ATP, upon flash photolysis of caged ATP⁸⁻¹³. To perform the experiment, we constructed the apparatus shown in Figure 1, which is similar to the instrument developed by Conibear et al.¹¹. In their in vitro motility system, they used heavy meromyosin (HMM) tracks instead of the myosin filaments used here, and were only able to report the dis-
placement rate from the HMM tracks without actin filaments despite their attempts to perform the measurement with actin filaments. We found that the fluorescence of the thick filament where actin filament slides decayed with two exponential processes. The slower rate constant was the same as that without actin filament. Along bipolar myosin thick filament, actin filaments slide at a fast speed towards the central bare zone (forward sliding movement) and at a slower speed away from the bare zone (backward sliding movement). The displacement rate constant of fluorescent ATP from myosin filament where actin filament forward was 5 s$^{-1}$, whereas the rate constant where actin filaments slide backward was 1.7 s$^{-1}$. These findings suggest that the slow ADP release rate is responsible for the slow backward sliding movement.

Materials and Methods

Materials

Myosin and actin were prepared from rabbit skeletal muscle according to Perry, Spudich and Watt, respectively. Concentrations of myosin and actin were determined spectrophotometrically using extinction coefficients of 0.54 cm$^2$ mg$^{-1}$ and 1.1 cm$^2$ mg$^{-1}$ at 280 nm, respectively. Cy3-EDA-ATP (a mixture of 2’ and 3’ isomers) was synthesized as described previously. Rhodamine-conjugated phalloidin was obtained from Invitrogen (Carlsbad, CA, USA). ATTO 647N-phalloidin, ATP, bovine serum albumin, glucose oxidase and catalase were purchased from Sigma (St. Louis, MO). Caged ATP was purchased from Dojindo (Kumamoto, Japan). All other chemicals were of analytical grade.

Myosin thick filaments were prepared according to Yamada and Wakabayashi. Briefly, myosin (approx. 40 μg/ml) was dissolved in a buffer containing 0.4 M KCl, 1 mM MgCl$_2$, 1 mM EGTA, 2.5 mM DTT and 10 mM PIPES (pH 7.0) and dialyzed against the same buffer. Subsequently, the KCl concentration was reduced from 0.4 M to 0.12 M over 12 h by the slow addition of the buffer without KCl using a peristaltic pump.

Experimental setup

Figure 1 shows a schematic diagram of the apparatus used for measuring the displacement of Cy3-EDA-ATP bound to the bipolar myosin thick filaments by ATP generated from caged ATP using a xenon flash lamp apparatus (SA-200E; Eagle Shouji, Tokyo, Japan). The light guide was placed just over the coverslip of the flow chamber. The flow chamber was made of multiple small coverslips (18 mm x 18 mm, No. 1 Thickness, Matsunami, Japan) and a nitrocellulose-coated coverslip (24 mm x 36 mm, No. 1 Thickness, Matsunami, Japan). The temperature of the solution in the
flow chamber was adjusted by circulating temperature-controlled water into a brass block attached to the microscope stage and another block jacketing the objective. The chamber temperature was measured by inserting a thin thermo-probe (ST-11E; Anritsu, Tokyo, Japan) into the chamber. To protect the CCD camera from the intense flash-light, an electromagnetic shutter (F77-4; Suruga Seiki, Shizuoka, Japan) was placed between the microscope and the camera. A custom-made trigger generator controlled the timing of the flash and the shutter. The different dye-labeled myosin and actin filaments were observed with an inverted fluorescent microscope (IX71; U-ApoN 150 × oil-immersion objective lens NA=1.45, Olympus, Japan) equipped with a dichroic mirror (D2; FF560/659-Di01; Semrock), notch filter (NF1; NF03-532E-25 for 532 nm and NF2; NF03-633E-25 for 633 nm; Semrock), and dual view optics (DV2; Photometrics). Cy3-EDA-ATP bound to myosin filaments was excited by a 532 nm laser (PDL-1800D DPSS Laser, Photon R&D, Inc., Japan) and actin filaments were labeled with ATTO-647 phalloidin and excited by a He-Ne laser (MELLES GRIOT; λ=632.8 nm). The two beams were combined using a dichroic mirror (D1; Q555LP, Chroma Technology Corp.). The beams were then expanded with an expander (LBED-10, Sigma Koki, Japan), focused on the back focal plane of the objective lens by a convex lens (L), and adjusted with a mirror (M1) to produce objective-type total internal reflectance fluorescence microscopy (TIRFM).

**Experimental procedure and data analysis**

The flow chamber was first rinsed with 1 mg/ml bovine serum albumin, and after 3 min, myosin filaments were introduced. After 3 min, a solution containing 10 nM Cy3-ATP, 333 μM caged ATP and 10 nM actin filaments labeled with rhodamine-phalloidin in a motility solution (25 mM KCl, 25 mM imidazole (pH 7.5), 4 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 1% β-mercaptoethanol, 4.5 mg/ml glucose, 0.2 mg/ml glucose oxidase and 0.035 mg/ml catalase) was introduced.

Rate constants for the displacement of fluorescent nucleotides from the myosin filament upon flash photolysis of caged ATP were calculated using the method of Conibear and Bagshaw⁹. The experiment was performed at 23°C. The amount of ATP released from 333 μM caged ATP by a single flash was about 191 μM ATP, i.e., 57% of the caged ATP, which was determined by the method reported by Ishijima et al.⁵.

A video image sequence of the region around the fluorescent myosin filament along which a fluorescent actin filament was sliding was captured using NIH image and a dual base time base controlled by a customized macro program (written by C.R. Bagshaw). From the series of video images, the mean fluorescence intensity of the myosin filament was computed as a function of time. The data were analyzed to determine the displacement rate constant by nonlinear least squares fitting to an exponential function using Kaleidagraph (Synergy Software, Reading, PA).

**Results**

Figure 2 shows fluorescence intensities of myosin filament as a function of Cy3-EDA-ATP concentration. After introducing myosin filaments into a flow chamber which allows us to introduce solutions several times without changing the position of the chamber on the microscope stage¹, fluorescence images of the same myosin filament with various concentrations of Cy3-EDA-ATP (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2, 5, 10, 20, and 50 nM) were captured on the CCD camera. The Kₘ for Cy3-EDA-ATP binding was 3.8 nM.

Figure 3A shows dual images of the displacement of the Cy3-EDA nucleotide bound to a thick filament (left) and the movement of an actin filament (right) in a typical experiment. The actin filament slid towards the center (forward movement) at 0.34 ± 0.15 μm/sec (n=12). Figure 3B shows the quantitative analysis of the displacement of the Cy3-EDA nucleotide in the rectangular region of interest shown in Figure 3A (left), where the actin filament was interacting constantly. The fluorescence intensities were fitted to the sum

![Figure 2](Dependence of fluorescence intensity of a myosin filament on the concentration of Cy3-EDA-ATP. The data points were fitted to a hyperbolic curve, which gave a Kₘ of 3.8 nM for Cy3-EDA-ATP.)
of two exponential functions \( I = I_0 + A_1 \exp(-k_1t) + A_2 \exp(-k_2t) \), which gave rate constants of \( k_1 = 4.9 \text{s}^{-1} \) and \( k_2 = 0.043 \text{s}^{-1} \).

Figure 4A is similar to Figure 3A except that the actin filament is distal to the center (backward movement). The sliding velocity in this case was 0.14 \( \mu \text{m/sec} \). As shown in Figure 4B, the curve fitted to the sum of two exponential functions in the case of backward movement gave rate constants of \( k_1 = 1.5 \text{s}^{-1} \) and \( k_2 = 0.078 \text{s}^{-1} \). For both the forward and backward movement, the values of \( A_1/(A_1+A_2) \) varied from 0.05 to 0.52.

Figure 5A and Figure 5B show successive video images and the decay curve of fluorescent myosin filaments without actin filaments, respectively. The fluorescence intensities were well fitted to a single exponential function \( (1 = I_0 + A \exp(-kt)) \). The rate constant was 0.032 \( \text{s}^{-1} \).

Table 1 summarizes the results. The displacement rate constant of fluorescent ATP from myosin filaments where actin filaments slid forward was higher (5.0 \( \pm \) 0.5 \( \text{s}^{-1} \), \( n=7 \)) than the rate constant where actin filaments slide backward (1.7 \( \pm \) 0.2 \( \text{s}^{-1} \), \( n=7 \)). These findings suggest that the slow ADP release rate is responsible for the slow backward sliding movement. The rate constant of \( k_2 \) (0.040 \( \pm \) 0.022 \( \text{s}^{-1} \), \( n=7 \)) for the forward movement was similar to that of \( k_1 \) (0.063 \( \pm \) 0.017 \( \text{s}^{-1} \), \( n=7 \)) for the backward movement and \( k \) (0.037 \( \pm \) 0.021 \( \text{s}^{-1} \), \( n=10 \)) in the absence of actin filaments, suggesting that the rate is derived from the cross-bridge cycle without actin.
Discussion

It has been reported that actin filament moves along myosin thick filament at a fast speed towards the central bare zone and at a slower speed away from the bare zone. In the present experiment, we have shown that the reason for the slow sliding movement is slow ADP release rate by measuring the nucleotide turnover rates of bipolar myosin thick filament along which actin filaments slide by monitoring the displacement of prebound fluorescent ATP analog (Cy3-EDA-ATP) upon flash photolysis of caged ATP (Fig. 6).

Measurement of nucleotide turn over rates from myosin filament during actin filament sliding

In contrast to the measurement of Pi production within a flow cell, attempts to measure the ATPase activity in an in vitro motility assay in real time were made by observing the displacement of fluorescent ATP analogs with excess ATP, first for a clam thick filament in the presence of FEDA-ATP, and then for rabbit skeletal myosin filaments in the presence of REDA-ATP by using flash photolysis of caged-ATP and TIRFM. We succeeded in measuring the nucleotide turn over rates of an in vitro motility assay system in which actin filaments are sliding. To measure the myosin ATPase activity during actin filament sliding, myosin HMM tracks or myosin filaments rather than myosin monomers on the glass surface were necessary, because the contribution of myosin ATPase activity in the absence of actin in the region of interest should be as low as possible.

The displacement of fluorescent nucleotide bound to myosin filament during actin filament sliding were well fitted to the sum of two exponential functions \( I = I_0 + A_1 \exp(-k_1t) + A_2 \exp(-k_2t) \), which gave rate constants of \( k_1 = 1.5 \text{s}^{-1} \) and \( k_2 = 0.078 \text{s}^{-1} \).
face and are unable to bind Cy-3-EDA-ATP. However, the
dependence on Cy-3-EDA-ATP concentration is exponential.

**Figure 5** Typical experiment showing the displacement of Cy3-EDA nucleotide bound to a myosin filament without actin filament. (A) Successive dual images of the displacement of Cy3-EDA nucleotide bound to the myosin filament. Scale bar, 5 µm. (B) Quantitative analysis of the displacement of fluorescent nucleotide in the region of interest in (A) shown as a rectangular shape. The data points were fitted to a single exponential function \(I=I_0+A_1 \exp (-kt)\), which gave rate constants of \(k=0.032 \text{s}^{-1}\).

ADP release rate from myosin filament along which actin filaments slide away from the central bare zone

The movement of the actin filaments away from the center is thought to require myosin heads to twist by 180 degrees rather than remaining in the same orientation as in the movement towards the center as shown in Figure 6. The invoking of a 180 degrees twisting motion in myosin heads is consistent with the flexibility of the head-rod junction of myosin. It is also consistent with the fact that the actin-
myosin interface involves multiple sites, including the upper and lower 50-K domains, loops 2, 3, and 4 in myosin, and the DNase I binding loop in actin\textsuperscript{23}. The rate-limiting step of myosin Cy3-EDA-ATPase is Pi release, and the rate is 0.022 s\textsuperscript{−1}\textsuperscript{24}, which is comparable to the rate constants of the slow components in the present study. After the photolysis of caged ATP, myosin heads in the M-(Cy3)ADP-Pi state began to interact with actin, released Pi, and then released (Cy3)ADP. Either the actin binding step, the Pi release step, or the ADP release step would be slower in the backward movements than that in the forward movements. Among them, the ADP release from the actomyosin cross-bridge, which has been reported to limit the maximum shortening velocity\textsuperscript{25–27}, was shown to be sensitive to the load on the cross-bridge of smooth muscle\textsuperscript{28} and skeletal muscle myosins\textsuperscript{29} from single molecule mechanical measurements. According to the model on the strain-dependent cross-bridge cycle including the ADP release step\textsuperscript{30,31}, the release of ADP requires the relative movement of the myosin motor subdomains to open the nucleotide-binding pocket. The 180 degrees twisting motion of the myosin head to move the actin filament away from the center would increase the stiffness of the myosin to open the nucleotide-binding pocket for ADP release, so this occurs at a slower rate and thus the sliding velocity is also slow. Previous our report on the thermal activation energy suggests that the backward movement causes the myosin heads to be constrained\textsuperscript{7}. West et al.\textsuperscript{32} reported that ATP binding was not affected by showing that the K\textsubscript{m} values for ATP were not significantly different from each other during forward and backward movement. These data suggest that the twisting motion of the myosin head would cause a structural change that affects the release of the nucleotide but not its binding.

Recently, Sato et al.\textsuperscript{33} reported a possibility that slow sliding causes slow nucleotide release. They showed that the sliding velocity affects binding rate of M.ADP.Pi to actin filament. In order to examine the possibility, we are attempting to apply this method to study the phenomenon observed by Yamada & Takahashi\textsuperscript{34} of a sudden increase in the speed of an actin filament sliding backward when the leading end starts to interact with another matched myosin filament. They concluded that the mismatched slow cycling cross-bridges offered little load to the actin filament sliding at a fast speed on matched crossbridges. The method described here may clarify whether the mismatched cross-bridges remain in a slow cycling state but offer little load, or change to a fast cycling state to drive actin filaments at a fast speed.

In summary, this study shows that the slow sliding of actin filament away from the center is due to slow ADP release and displacement of a fluorescent nucleotide using caged-ATP reveals kinetics in \textit{in vitro} motility system.

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References

1. Kron, S. J. & Spudich, J. A. Fluorescent actin filaments move on myosin fixed to a glass surface. Proc. Natl. Acad. Sci. USA 83, 6272–6276 (1986).

2. Sellers, J. R. & Kachar, B. Polarity and velocity of sliding filaments: control of direction by actin and of speed by myosin. Science 249, 406–408 (1990).

3. Yamada, A., Ishii, N. & Takahashi, K. Direction and speed of actin filaments moving along thick filaments isolated from molluscan smooth muscle. J. Biochem. 108, 341–343 (1990).

4. Yamada, A. & Wakabayashi, T. Movement of actin away from the center of reconstituted rabbit skeletal myosin filament is slower than in the opposite direction. Biophys. J. 64, 565–569 (1993).

5. Ishijima, A., Kojima, H., Higuchi, H., Harada, Y., Funatsu, T., & Yanagida, T. Multiple- and single-molecule analysis of the actomyosin motor by nanometer-piconewton manipulation with a microneedle: unitary steps and forces. Biophys. J. 70, 383–400 (1996).

6. Tanaka, H., Ishijima, A., Honda, M., Saito, K. & Yanagida, T. Orientation dependence of displacements by a single one-headed myosin relative to the actin filament. Biophys. J. 75, 1886–1894 (1998).

7. Okubo, H., Iwai, M., Iwai, S. & Chao, S. Thermal activation energy for bidirectional movement of actin along bipolar tracks of myosin filaments. Biochem. Biophys. Res. Commun. 396, 539–542 (2010).

8. Sowerby, A. J., Seehra, C. K., Lee, M. & Bagshaw, C. R. Turnover of fluorescent nucleoside triphosphates by isolated immobilized myosin filaments. Transient kinetics on the pico- to nanosecond scale. J. Mol. Biol. 234, 114–123 (1993).

9. Conibear, P. B. & Bagshaw, C. R. Measurement of nucleotide exchange kinetics with isolated synthetic myosin filaments using flash photolysis. FEBS Lett. 380, 13–16 (1996).

10. Chao, H., Shirakawa, I., Bagshaw, C. R. & Sugii, H. Measurement of nucleotide release kinetics in single skeletal muscle myofibrils during isometric and isovelocity contractions using fluorescence microscopy. Biophys. J. 73, 2033–2042 (1997).

11. Conibear, P. B., Kuhlman, P. A. & Bagshaw, C. R. Measurement of ATPase activities of myosin at the level of tracks and single molecules. Adv. Exp. Med. Biol. 453, 15–26 (1998).

12. Bagshaw, C. R. in Current methods in muscle physiology: advantages, problems and limitations. (Sugi, H. ed.) Chapter 4, pp. 91–132 (Oxford University Press, USA 1998).

13. Shirakawa, I., Chao, S., Bagshaw, C. R. & Sugii, H. Measurement of nucleotide exchange rate constants in single rabbit skeletal myofibrils during shortening and lengthening using a fluorescent ATP analog. Biophys. J. 78, 918–926 (2000).

14. Perry, S. V. Methods in enzymology volume 2, 582–588 (Elsevier, 1955).

15. Spudich, J. A. & Watts, S. The regulation of rabbit skeletal muscle contraction. J. Biol. Chem. 246, 4866–4871 (1971).

16. Harada, Y., Sakurada, Y., Aoki, T., Thomas, D. D. & Yanagida, T. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. J. Mol. Biol. 216, 49–68 (1990).

17. Kron, S. J., Toyoshima, Y. Y., Uyeda, T. Q. P. & Spudich, J. A. Assays for actin sliding movement over myosin-coated surfaces. Methods Enzymol. 196, 399–416 (1991).

18. Huxley, H. E. The mechanism of muscular contraction. Science 164, 1356–1366 (1969).

19. Eccleston, J. F., Oiwa, K., Ferenczi, M. A., Anson, M., Corrie, J. E. T., Yamada, A., Nakayama, H. & Trentham, D. R. Ribose-linked sul-foindocyanine conjugates of ATP: Cy3-EDA-ATP and Cy5-EDA-ATP. Biophys. J. 70, A159 (1996).

20. Thirwell, H., Corrie, J. E. T., Reid, G. P., Trentham, D. R. & Ferenczi, M. A. Kinetics of relaxation from rigor of permeabilized fast-twitch skeletal fibers from the rabbit using a novel caged ATP and apyrase. Biophys. J. 67, 2436–2447 (1994).

21. Winklemann, D. A. & Lowey, S. J. Probing myosin head structure with monoclonal antibodies. J. Mol. Biol. 188, 595–612 (1986).

22. Reedy, M. C., Beall, C. & Fyrberg, E. Formation of reverse rigor chevrons by myosin heads. Nature 339, 481–483 (1989).

23. Lorenz, M. & Homes, K. C. The actin-myosin interface. Proc. Natl. Acad. Sci. USA 107, 12529–12534 (2010).

24. Oiwa, K., Eccleston, J. F., Anson, M., Kikumoto, M., Davis, C. T., Reid, G. P., Ferenczi, M. A., Corrie, J. E., Yamada, A., Nakayama, H. and Trentham, D. R. Comparative single-molecule and ensemble myosin enzymology: sulfoindocyanine ATP and ADP derivatives. Biophys. J. 78, 3048–3071 (2000).

25. Siemankowski, R. F. & White, H. D. Kinetics of the interaction between actin, ADP and cardiac myosin-S1. J. Biol. Chem. 259, 5045–5053 (1984).

26. Pereira, J. S., Pavlov, D., Nili, M., Greaser, M., Homsher, E. & Moss, R. L. Kinetic differences in cardiac myosins with identical loop 1 sequences. J. Biol. Chem. 276, 4409–4415 (2001).

27. Weiss, S., Rossi, R., Pellegrino, M. A., Bottinelli, R. & Gpees, M. A. Differing ADP release rates from myosin heavy chain isoforms define the shortening velocity of skeletal muscle fibers. J. Biol. Chem. 276, 45902–45908 (2001).

28. Veigel, C., Molloy, J. E., Schmitz, S. & Kendrick-Jones, J. Load-dependent kinetics of force production by smooth muscle myosin measured with optical tweezers. Nat. Cell Biol. 11, 980–986 (2003).

29. Capitanio, M., Canepari, M., Cacciafesta, P., Lombardi, V., Cicchì, R., Maffei, M., Pavone, F. S. & Bottinelli, R. Two independent mechanical events in the interaction cycle of skeletal muscle myosin with actin. Proc. Natl. Acad. Sci. USA 103, 87–92 (2006).

30. Smith, D. A. & Gpees, M. A. Strain-dependent cross-bridge cycle for muscle. Biophys. J. 69, 524–537 (1995).

31. Nyitrai, M. & Gpees, M. A. Adenosine diphosphate and strain sensitivity in myosin motors. Philos. Trans. R. Soc. B. Biol. Sci. 359, 1867–1877 (2004).

32. West, J. M., Higuchi, H., Ishijima, A. & Yanagida, T. Modification of the bi-directional sliding movement of actin filaments along native thick filaments isolated from a clam. J. Muscle Res.Cell Motil. 17, 637–646 (1996).

33. Sato, M. K., Ishihara, T., Tanaka, H., Ishijima, A. & Inoue, Y. Velocity-dependent actomyosin ATPase cycle revealed by in vitro motility assay with kinetic analysis. Biophys. J. 103, 711–718 (2012).

34. Yamada, A. & Takahashi, K. Sudden increase in speed of an actin filament moving on myosin cross-bridges of “ mismatched” polarity observed when its leading end begins to interact with cross-bridges of “matched” polarity. J. Biochem. 111, 676–680 (1992).