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Biochimica et Biophysica Acta 1185 (1994) 346–349

BBA Biochimica et Biophysica Acta

In vitro ATP-dependent F-actin sliding on myosin is not influenced by substitution or removal of bound nucleotide

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(Received 9 November 1993)

Abstract

To examine possible role of F-actin-bound nucleotide in ATP-dependent actin-myosin sliding, we prepared various actin filaments with different nucleotide contents and compared their sliding velocities on heavy mero-myosin in the presence of 2'-deoxyadenosine 5'-triphosphate (dATP) to exclude possible exchange of external ATP with the actin-bound nucleotide. Neither the sliding velocity nor the length of the actin filaments was significantly influenced by substitution or removal of actin-bound nucleotide, indicating that actin-bound nucleotide may not play a significant role in the sliding between actin and myosin.

Key words: Actin-bound nucleotide; Nucleotide depletion; Muscle contraction; Motility assay, in vitro; Actin, F-

1. Introduction

Actin monomer (G-actin) is known to bind one molecule of adenosine nucleotide, which rapidly exchanges with unbound nucleotides in the external medium [1]. When G-actin with bound ATP polymerizes into filamentous actin (F-actin), the bound ATP is dephosphorylated to ADP [2], which is essentially non-exchangeable [1]. Since muscle contraction results from relative sliding between the thin filament consisting of F-actin and the thick filament consisting of myosin, possible role of the actin-bound nucleotide in muscle contraction has attracted the attention of investigators in the field of muscle research. Until now, however, little information has been available concerning the role of the actin-bound nucleotide in the ATP-dependent actin–myosin interaction. The ability of F-actin to cause superprecipitation with myosin in the presence of ATP and to activate myosin ATPase has been reported to be unaffected by substitution of the F-actin-bound ADP with other nucleotides or its removal from F-actin [3–6], except for one report that F-actin-bound 8-bromoadenosine 5'-diphosphate (BrADP) affects myosin ATPase activation by F-actin to some extent [7].

Recent development of in vitro motility assay systems has made it possible to study the ATP-dependent sliding between actin and myosin under microscopic observation [8–10]. The present experiments were undertaken to study the role of the actin-bound nucleotide in the actin-myosin sliding responsible for muscle contraction, using a motility assay system in which fluorescently labeled F-actin was made to slide on myosin fixed to a glass surface in the presence of ATP [10]. To exclude possible exchange of the actin-bound nucleotide with ATP in the external medium [3,11], the ATP in the eternal medium was replaced by 2'-deoxyadenosine 5'-triphosphate (dATP), which is not readily exchangeable with the actin-bound nucleotide even in the presence of myosin [4,12]. It is shown that dATP is equally potent substrate for the actin-myosin sliding to ATP, and that the rate of the actin-myosin sliding remains unchanged by substitution of the F-actin-bound ADP with other nucleotides or its removal from F-actin, indicating that the actin-bound nucleotide is not essential for muscle contraction. Preliminary results of this work have been presented in the form of abstracts [13,14].

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SSDI 0005-2728(94)00014-V

2. Materials and methods

Preparation of G-ATP-actin and G-ADP-actin

G-actin with bound ATP(G-ATP-actin) was purified from rabbit skeletal muscle according to the method of Pardee and Spudich [15]. G-actin with bound ADP(G-ADP-actin) was prepared as follows. G-ATP-actin was treated with AG 1-X8 (Bio-Rad Laboratories) for 2 min to remove free ATP, polymerized by adding KCl (25 mM) for 30 min at 4°C, and centrifuged for 90 min at 150000 × *g* (Beckman, TLA 100.2). The pellet containing F-actin was sonicated with an ultrasonic generator (Branson, B-12) at 50 W for 30 s on ice. Residual F-actin was removed through a Millipore filter (Nihon Millipore, HAWP) [16], to obtain G-ADP-actin.

Preparation of F-actins

F-actin was prepared (1) by polymerization of G-ATP-actin (12.5 μM) in 5 mM Tris-HCl (pH 8.0)/100 mM KCl/0.1 mM $MgCl_2$ (buffer A) containing 2.5 mM ATP overnight at 4°C; (2) by polymerization of G-ADP-actin in buffer A overnight at 4°C (F-actin B) or (3) by the treatment of 1 unit · ml^{-1} hexokinase (Sigma, H-5875) on G-ATP-actin in 2 mM Tris-HCl (pH 8.0)/2 mM $MgCl_2$/0.2 mM $CaCl_2$/0.2 mM ATP/0.5 mM glucose for 2 h at 25°C (F-actin C) [17].

Preparation of nucleotide-substituted F-actins

Nucleotide-substituted F-actins were prepared by polymerization of G-ADP-actin (12.5 μM) in buffer A (without ATP) in the presence of 2.5 mM adenylyl imidodiphosphate (Ado*PP*[NH]*P*) (F-NS-actin A), 8-bromoadenosine 5′-triphosphate (F-NS-actin B), or inosine triphosphate (F-NS-actin C).

Preparation of nucleotide-depleted F-actins

Nucleotide-depleted F-actins (F-NR-actins) were prepared in two ways. F-NR-actin A was prepared essentially according to the method of Kasai et al. [18]. This method is based on the principle that the chelation of the actin-bound divalent cations releases the bound ATP. G-ATP-actin (75 μM) in 1.8 M sucrose/1.5 M urea was mixed with a 10^{-3} vol. of 0.2 M disodium ethylenediamine tetraacetate (EDTA, 2Na), and gently stirred to remove bound Ca^{2+} for 3 min at 4°C, and the released ATP was trapped by the addition of 100 mg · ml^{-1} AG 1-X8 (Bio-Rad Laboratories). After further stirring for 15 min, the mixture was filtered through Tetron mesh to remove resin. These treatments were repeated five times, and the nucleotide-depleted G-actin obtained was polymerized into F-NR-actin A in 50 mM KCl/1.8 M sucrose overnight at 4°C.

F-NR-actin B was prepared by apyrase based on the principle that the affinity of AMP to actin is low [19]. G-ADP-actin (12.5 μM) in 4 mM Pipes (pH 7.0)/100 mM KCl, 0.625 mM $MgCl_2$/5 unit · ml^{-1} apyrase (Sigma, A-6160) was sonicated (50 W) for 3 min at 25°C, and stood for 20 h at 4°C to obtain F-NR-actin B.

Preparation of myosins

Myosin was purified from rabbit skeletal muscle according to Perry [20] and stored in 50% glycerol at −20°C. Heavy mero-myosin (HMM) was prepared according to Okamoto and Sekine [21], and used within 3 days.

Determination of protein and nucleotide concentrations

Protein concentrations were determined by ultraviolet absorption using coefficients of $26.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 290 nm for G-actin [22], $2.3 \cdot 10^6$ $M^{-1} \cdot cm^{-1}$ at 280 nm for HMM [23], or by the method of Bradford [24]. Nucleotide concentrations of F-actins were determined as follows. F-actins were first spun down by centrifugation for 90 min at 150000 × *g* (Beckman, TLA 100.2), and dissolved in deionized water. Quickly after the addition of ammonia to the final concentration of 2 M, the mixture was neutralized with acetic acid and again centrifuged (for 5 min at 3000 × *g*). The supernatant was evaporated and dissolved in 20 mM sodium phosphate buffer (pH 7.0), and aliquot was applied to an ion-exchange column (Shimadzu, Shimpak WAX-1). Nucleotides were eluted with a gradient of 20–480 mM sodium phosphate (pH 7.0) and quantified by high-performance liquid chromatography (Shimadzu, LC-6B).

Fluorescent labeling of F-actins

All F-actins used in the present study were polymerized from G-actins in the presence of equimolar rhodamine-phalloidin (Molecular Probes). The fluorescently labeled F-actins were diluted to a final concentration of 12.5 nM in 25 mM imidazole-Cl (pH 7.5)/25 mM KCl/5 mM $MgCl_2$/2 mM dithiothreitol/1 mM ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid (buffer B).

In vitro motility assay

HMM (80 μM in buffer B) was spread on a nitrocellulose-coated cover glass constituting the bottom of a flow cell (24 × 9 × 0.2 mm deep) to fix HMM onto the bottom of the cell, free HMM being removed by washing with buffer B containing 15 μM bovine serum albumin (Sigma, A-7906). Then, the fluorescently labeled F-actin (12.5 μM in buffer B) was introduced into the cell. ATP-dependent F-actin sliding on HMM was induced by exchanging buffer B with ATP or dATP solution, prepared by adding 2 mM ATP (Sigma, A-5395) or 2 mM dATP (Yamasa) to buffer B. The movement of F-actin was observed with an inverted microscope equipped with epifluorescence optics

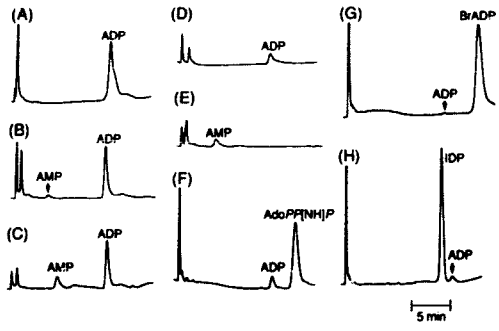

Fig. 1. Chromatograms of F-actin samples showing the composition of nucleotides in each sample. Nucleotides were loaded on a anion-exchange column, eluted with a gradient of 20–480 mM sodium phosphate (pH 7.0), and monitored by absorbance at 259 nm. (A) F-actin A; (B) F-actin B; (C) F-actin C; (D) F-NR-actin A; (E) F-NR-actin B; (F) F-NS-actin A; (G) F-NS-actin B; (H) F-NS-actin C.

(Olympus, IMT-2 with an oil immersion objective ×100, numerical aperture = 1.30) at 30°C, and recorded on videotape with a high-sensitivity video camera (Ikegami, CTC-9000) and a video cassette recorder (Sony, VO-9600). Analysis of the movement was performed on replay from the video cassette recorder.

3. Results

Contents of bound nucleotides in the F-actin samples

Chromatograms showing bound nucleotides in the F-actin samples are presented in Fig. 1. F-actin A contained only ADP, while F-actins B and C contained both ADP and AMP. F-NS-actins A, B and C contained Ado*PP*[NH]*P* and ADP, BrADP and ADP, and inosine diphosphate (IDP) and ADP respectively. F-NR-actin A and B contained only a small amount of ADP and AMP respectively. Table 1 shows bound nucleotide contents (expressed relatively to G-actin) in the F-actin samples. The ADP content was maximum in F-actin A, while it was much smaller in F-actins B and C, indicating that the ADP content depends on the method of preparation of F-actin. The ADP content of F-actin B (35%) was in accord with the result of Higashi and Oosawa [25]. The ADP content of F-actin A (83% < 100%) might result partly from denatured actins and partly from limit of accuracy in determining the protein and nucleotide concentrations.

On the other hand, the bound nucleotides in F-NS-actins A, B and C were mostly Ado*PP*[NH]*P*, BrADP and IDP respectively, indicating fairly effective nucleotide substitution. F-NR-actins A and B contained only a small amount of ADP and AMP respectively, indicating effective removal of bound nucleotides.

Table 1
Bound nucleotide contents of F-actin samples (mol/ mol of actin monomer)

	ADP	AMP	Ado*PP*[NH]*P*	BrADP	IDP
F-actin A	0.83	0.00	–	–	–
F-actin B	0.35	0.02	–	–	–
F-actin C	0.24	0.05	–	–	–
F-NS-actin A	0.04	0.00	0.62	–	–
F-NS-actin B	0.01	0.00	–	0.69	–
F-NS-actin C	0.03	0.00	–	–	0.61
F-NR-actin A	0.08	0.00	–	–	–
F-NR-actin B	0.00	0.06	–	–	–

Table 2
Sliding velocities of F-actin filaments on HMM in the presence of 2 mM dATP and F-actin filament length (mean ± S.D., $n = 30–40$)

	Sliding velocity ($\mu m \cdot s^{-1}$)	Filament length (μm)
F-actin A	8.3 ± 1.5	2.7 ± 1.5
F-actin B	8.9 ± 1.4	2.7 ± 0.9
F-actin C	7.3 ± 1.0	3.2 ± 1.0
F-NS-actin A	7.8 ± 1.1	2.8 ± 1.4
F-NS-actin B	8.2 ± 1.0	2.2 ± 1.3
F-NS-actin C	8.2 ± 1.4	3.4 ± 1.9
F-NR-actin A	7.9 ± 0.9	2.7 ± 1.5
F-NR-actin B	9.0 ± 1.3	3.6 ± 2.2

Motility assay experiment

First, we examined the ability of dATP in producing sliding of F-actins on HMM by comparing the sliding velocity of F-actin A in the presence of 2 mM ATP with that in the presence of 2 mM dATP. The value was 7.7 ± 1.4 $\mu m \cdot s^{-1}$ (mean ± S.D., $n = 40$) for ATP, and 8.3 ± 1.5 $\mu m \cdot s^{-1}$ ($n = 40$) for dATP. Since the two values did not differ significantly ($P > 0.05$), we concluded that dATP was equally potent to ATP in producing F-actin sliding on HMM. Further experiments were therefore performed only with dATP.

The results obtained are summarized in Table 2, in which average sliding velocities of the F-actin filaments on HMM in the presence of 2 mM dATP are listed together with the corresponding average filament lengths.

No significant differences in the sliding velocity were observed between F-actin A, which may serve as control F-actin, and all other kinds of F-actins. In addition, the filament length of F-actin A did not differ significantly from that of all other F-actins.

4. Discussion

In the present study, we examined possible role of actin-bound nucleotide in the ATP-dependent actin-myosin sliding by preparing various F-actins with different nucleotide contents and comparing their sliding velocities on HMM using a motility assay system. The possible exchange of actin-bound nucleotide with external ATP could be successfully avoided by use of dATP which was shown to be equally potent to ATP in producing F-actin filament sliding on HMM.

The results obtained were very simple; the ATP-dependent sliding of F-actins was not significantly influenced neither substitution nor removal of actin-bound nucleotide. The present study also showed that the filament length of F-actins was not significantly affected by the nucleotide contents of F-actins. It follows from the present results that, although substitution or removal of actin-bound nucleotide is expected to change the F-actin structures to some extent, such structural changes may not have any appreciable effect on the sliding velocity of F-actin on HMM and also on the filament length of F-actin.

Although it has been reported that, in solution systems, the ability of F-actin to cause superprecipitation with myosin and to activate myosin ATPase is unaffected by the removal of actin-bound nucleotide, the nucleotide-depleted F-actin still contains ADP up to 5% [4] or 13% [5]. In this case, the possibility can not be excluded that the residual ADP in the nucleotide-depleted F-actin is sufficient to cause superprecipitation and myosin ATPase activation at the normal rate. In the present study, however, such a possibility is completely excluded by the result that F-NR-actin B, which does not contain ADP, slides on HMM at the velocity not significantly different from that of other F-actins.

It should be noted that, in the motility assay system used in the present study, F-actin filaments slide on HMM only under unloaded conditions. It remains to be investigated whether the ATP-dependent F-actin sliding velocity is influenced by substitution or removal of actin-bound nucleotide under an externally applied load.

Acknowledgments

The authors wish to thank Drs. S. Suda, A. Muraoka, K. Oiwa and S. Chaen for their help in setting up the movement assay system. This work was supported in part by grants-in-aid for Scientific Research (No. 01740434) from the Ministry of Education, Science and Culture of Japan.

References

[1] Martonosi, A., Gouvea, M.A. and Gergely, J. (1960) J. Biol. Chem. 235, 1700–1703.
[2] Straub, F.B. and Feuer, G. (1950) Biochim. Biophys. Acta 4, 455–470.
[3] Bárány, M. and Finkelman, F. (1963) Biochim. Biophys. Acta 78, 175–193.
[4] Bárány, M., Tucci, A.F. and Conover, T.E. (1966) J. Mol. Biol. 19, 483–502.
[5] Tokiwa, T., Shimada, T. and Tonomura, Y. (1967) J. Biochem. 61, 108–122.
[6] Estes, J.E. and Moos, C. (1969) Arch. Biochem. Biophys. 132, 388–396.
[7] Hegyi, G., Szilagyi, L. and Belagyi, J. (1988) Eur. J. Biochem. 175, 271–274.
[8] Sheetz, M.P. and Spudich, J.A. (1983) Nature, 303, 31–35.
[9] Shimmen, T. and Yano, M. (1984) Protoplasma, 121, 132–137.
[10] Kron, S.J. and Spudich, J.A. (1986) Proc. Natl. Acad. Sci. USA, 83, 6272–6276.
[11] Szent-Györgyi, A.G. and Prior, G. (1966) J. Mol. Biol. 15, 515–538.
[12] Martonosi, A. (1962) Biochim. Biophys. Acta 57, 163–165.
[13] Oishi, N. and Sugi, H. (1989) J. Muscle Res. Cell Motil. 10, 257.
[14] Oishi, N. and Sugi, H. (1991) J. Muscle Res. Cell Motil. 12, 310.
[15] Pardee, J.D. and Spudich, J.A. (1982) Methods Enzymol. 85, 164–181.
[16] Grazi, E. and Magri, E. (1981) FEBS Lett. 123, 193–194.
[17] Pollard, T.D. (1984) J. Cell Biol. 99, 769–777.
[18] Kasai, M., Nakano, E. and Oosawa, F. (1965) Biochim. Biophys. Acta 94, 494–503.
[19] Cooke, R. and Murdoch, L. (1973) Biochemistry 12, 3927–3932.
[20] Perry, S.V. (1955) Methods Enzymol. 2, 582–588.
[21] Okamoto, Y. and Sekine, T. (1985) J. Biochem. 98, 1143–1145.
[22] Houk, T.W. and Ue, K. (1974) Anal. Biochem. 62, 66–74.
[23] Young, D.M., Himmelfarb, S. and Harrington, W.F. (1964) J. Biol. Chem. 239, 2822–2829.
[24] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[25] Higashi, S. and Oosawa, F. (1965) J. Mol. Biol. 12, 843–865.