EVIDENCE FOR BIASED BIDIRECTIONAL POLYMERIZATION OF ACTIN FILAMENTS USING HEAVY MEROMYOSIN PREPARED BY AN IMPROVED METHOD

DIANE T. WOODRUM, STEVEN A. RICH, and THOMAS D. POLLARD. From the Physiology Course, Marine Biological Laboratory, Woods Hole, Massachusetts 02543. The affiliating institutions of the investigators while taking this course were Northwestern University, Evanston, Illinois, State of New York Department of Health, Albany, New York, and Harvard Medical School, Boston, Massachusetts. D. Woodrum's present address is the Anatomy Department, Harvard Medical School, Boston, Massachusetts 02115.

The ubiquitous protein actin polymerizes to form 6-nm wide filaments. In muscle, thin filaments composed of actin with other proteins play a central role in the generation and transmission of contractile force (7). Indirect evidence suggests that actin filaments perform similar functions in nonmuscle cells (9).

The process of actin filament growth is potentially important for understanding the mechanisms of myofibril assembly and cell movement. Consequently, we designed experiments to determine the directionality of actin polymerization. Our results using homogeneous muscle actin indicate that actin filaments can grow bidirectionally, although the addition of actin monomers to the ends of actin filaments “decorated” with heavy meromyosin (HMM) is strongly biased toward the end attached to the Z line in striated muscle. In the course of presenting preliminary accounts of this work (12, 8), we learned that other investigators (footnote 2 and reference 1) independently have carried out experiments similar to some of those described here. In addition, we report on an improved method for preparing HMM which minimizes the number of peptide bonds cleaved in the myosin heavy chain.

MATERIALS AND METHODS

Preparation of Proteins

HEAVY MEROMYOSIN: HMM was prepared by limited digestion of rabbit skeletal muscle myosin with trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK-trypsin) (Worthington Biochemical Corp., Freehold, N. J.). Myosin purified from rabbit back and leg muscles (5) was dissolved at a concentration of 14.4 mg/ml in 0.5 M KCl, 0.1 M phosphate buffer, pH 7, and digested with 0.1 or 0.01 vol of a TPCK-trypsin solution (0.5 mg/ml in 0.001 M HCl, activity toward p-toluenesulfonyl-L-arginine methyl ester of 177 U/mg at 25°C for 5 min at 25°C with constant stirring. The reaction was terminated by the addition of 0.1 vol of a soybean trypsin inhibitor solution (Worthington; 1 mg/ml in deionized water). After dialysis at 4°C against 20 mM imidazole pH 6.6, insoluble material, including undigested myosin and light meromyosin, was removed by centrifugation at 100,000 g for 1 h. The yields of HMM were 3.5 mg/ml (40% of theoretical maximum yield) with 0.1 vol of TPCK-trypsin and 1.3 mg/ml (14% of the theoretical maximum yield) with 0.01 vol of TPCK-trypsin. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of these HMM preparations showed that both the heavy chains and light chains were remarkably intact in the preparation made with the lower concentration of TPCK-trypsin, while there was somewhat more wide-spread digestion with the higher concen-

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1 Abbreviations used in this paper: DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; G-ATP-actin, monomeric actin with bound ATP; HMM, heavy meromyosin; TPCK-trypsin, trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone to inhibit contaminant chymotryptic activity.

2 Personal communications: Spudich, J. and R. Cooke, 1974; Kondo, H. transmitted by S. Ebashi and F. Oosawa, 1974.
tration of TPCK-trypsin (Fig. 1). Each type of HMM had ATPase activity in 0.5 M KCl, 2 mM EDTA, 10 mM imidazole, pH 7 at 25°C of 1.5-2.0 µmol/min per mg and each bound to actin filaments in the same way, judging by electron microscopy.

ACTIN: Actin was extracted from an acetone powder of the muscle residue remaining after the myosin extraction and was purified by either of two methods: (a) by two cycles of polymerization and sedimentation from 0.1 M KCl; or (b) by one cycle of polymerization and sedimentation from 0.8 M KCl as suggested by Spudich and Watt (10). By gel electrophoresis in sodium dodecyl sulfate, the actin purified by both methods was free of tropomyosin and troponin and was more than 99% pure. Filaments of purified actin were depolymerized by dialysis against 0.1 mM CaCl₂, 0.2 mM ATP, 2 mM Tris-Cl, pH 7.8, to yield monomeric actin with a bound ATP nucleotide (G-ATP-actin). The experiments described here were carried out with this monomeric actin. Identical results were obtained with actin stored as a lyophilized powder and reconstituted with deionized water.

Experimental Design

It is believed that actin polymerization is a cooperative process initiated by the formation of a nucleus of three or four monomers (7). Preformed actin filaments can also serve as nuclei for filament growth (4). To determine the direction of polymer growth, we added actin monomers to preformed nuclei consisting of actin filaments decorated with HMM. We will refer to these preformed

The size and proportions of the proteolytic peptides formed from myosin in these experiments suggest that TPCK-trypsin initially attacks myosin in a very discrete region of the tail of the molecule. The HMM thus formed consists largely of a 130,000 dalton fragment of the heavy chain without internal cleavages. Secondly, the TPCK-trypsin appears to attack another discrete region of the heavy chain, roughly in the middle of the 130,000 dalton fragment, yielding the 72,000 and 66,000 dalton fragments. When the low concentration of TPCK-trypsin is used, this second cleavage takes place in about 20% of the HMM heavy chains. When the higher concentration of TPCK-trypsin is used, most of the heavy chain is cleaved at the second site as well. Our view that the 130,000 dalton fragment represents intact HMM heavy chain is supported by our isolation from these myosin digests of light meromyosin with a subunit molecular weight of 70,000 daltons. Together the 70,000 dalton light meromyosin peptide and the 130,000 dalton HMM peptide account for the mass of the whole myosin heavy chain (200,000 daltons). The light chains of myosin appear to be retained quantitatively in the TPCK-trypsin HMM, although the light chain of intermediate electrophoretic mobility is reduced slightly in molecular weight during the digestion (Fig. 1).

HMM-actin filaments as decorated nuclei. The periodic arrowhead configuration distinguished the decorated nuclei from new growth and indicated the polarity of the filaments.

It was necessary to use actin monomer solutions devoid of free ATP. Free ATP would have dissociated the HMM from the decorated nuclei and allowed the HMM to redistribute onto other actin filaments after the ATP was hydrolyzed. We used gel filtration to remove unbound ATP from the actin solution. 3 ml of G-ATP-actin were passed through 0.9 × 25-cm column of Sephadex G-25, fine equilibrated, and eluted with 0.1 mM CaCl₂, 1 mM dithiothreitol (DTT), 2 mM Tris-Cl, pH 7.8. The resulting G-ATP-actin in the ATP-free buffer appeared to polymerize normally for several days.

Decorated nuclei and monomeric G-ATP-actin were mixed in various proportions. The final concentration of decorated nuclei was varied between 0.03 and 0.25 mg/ml and the final concentration of added monomers was varied between 0.05 and 0.5 mg/ml. Immediately after the addition of monomer to the nuclei, the concentration of KCl was readjusted to 0.1 M with a concentrated KCl solution to promote optimal polymerization. In addition to variations in the concentrations of nuclei and monomer, we tested the effects of temperature (0 or 25°C), the presence or absence of 1 mM MgCl₂, and pH (6.0, 7.0, and 8.0). Samples of these solutions were diluted to about 0.1 mg/ml actin with 0.1 M KCl and immediately applied to carbon-over-Formvar grids rendered hydrophilic by glow discharge. After 20 s, excess sample was removed and the grid was stained with 1% uranyl acetate. The extent and polarity of polymerization was determined by electron microscopy using a Philips EM-201 electron microscope (generously loaned to the Marine Biological Laboratory Physiology Course by Philips Electronic Instruments, Mount Vernon, N. Y.).

RESULTS

The decorated actin filament nuclei used in our experiments were prepared by mixing HMM and actin filaments in a molar ratio of HMM to actin of about 1:2. Under these conditions nearly all of the filaments were decorated from end to end with HMM arrowheads (Fig. 2 a). A small proportion of the filaments was completely bare and the proportion of the bare filaments increased with lower ratios of HMM to actin. No partially decorated filaments were observed when HMM was mixed with preformed actin filaments. These findings indicate that there was no free HMM in the solutions of decorated nuclei. The all or none binding of HMM to each actin filament might be interpreted to mean that the binding process is cooperative.

Addition of actin monomers to decorated nuclei was observed to result in the growth of the

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decorated nuclei and the formation of additional actin filaments. The newly polymerized actin was bare and easily distinguished from the decorated nuclei by electron microscopy.

**Low Monomer Concentrations**

When a low concentration (0.05–0.1 mg/ml) of monomeric G-ATP-actin was added to decorated nuclei, about half of the newly formed (bare) actin filaments were attached to decorated nuclei and about half were completely bare (Fig. 2 and Table I). Thus, the assembly of about half of the filaments was initiated on decorated nuclei while the rest was initiated on other nuclei. Under the conditions used in the experiments in Fig. 2 and Table I, 60–80% of the decorated nuclei had bare actin extensions from the barbed end. Of hundreds of filaments observed, only one was found with a bare extension from the pointed end of a decorated segment and none had bare actin extensions at both ends. At these low monomer concentrations, neither variation of the pH between 6 and 8 (Table I), variation of the temperature (0° or 25°C; data not shown), nor the presence or absence of 1 mM MgCl₂ (Table I) had a large influence on the extent or direction of nucleated actin polymerization. These and similar experiments indicate that at low monomer concentrations, the polymerization of actin onto nuclei decorated with HMM is limited to the barbed end of the filament.

**High Monomer Concentrations**

When a high concentration (0.5 mg/ml) of monomeric actin was added to the decorated nuclei in 0.1 M KCl at pH 7 and 25°C, nearly all of the nuclei had bare actin extensions from both ends (Figure 3 a-c). In addition, more than 90% of the actin filaments were completely bare, indicating
FIGURE 2 Electron micrographs of actin filaments negatively stained with 1% uranyl acetate. (a) An actin filament decorated with HMM, typical of the decorated nuclei used in these experiments. (b and c) The result of mixing decorated nuclei (0.05 mg/ml) with a low concentration (0.1 mg/ml) of monomeric G-ATP-actin at pH 7 in 0.1 M KCl. Note the bare extensions on the barbed end of most of the decorated segments. Bars are 0.1 μm. (a) × 135,000; (b) × 100,000; (c) × 75,000.
TABLE I

| Conditions | Barbed end | Pointed end | Both ends | Neither end |
|------------|------------|-------------|-----------|------------|
| pH 6.0     | 39         | 0           | 0         | 15         | 46         |
| pH 7.0     | 53         | 0           | 0         | 19         | 28         |
| pH 8.0     | 43         | 1           | 0         | 20         | 36         |
| pH 7.0     | 47         | 0           | 0         | 10         | 43         |
| +1 mM MgCl₂|            |             |           |            |            |

In each experiment, approximately 100 filaments were scored and put into one of the following categories: a decorated nucleus with a bare extension at the barbed end, at the pointed end, at both ends or at neither end; or a completely undecorated filament.

* A low concentration (0.1 mg/ml) of monomers was added to the same concentration of decorated nuclei, and the KCl concentration adjusted to 0.1 M. The pH and MgCl₂ concentrations were varied as indicated. Calcium added with the protein samples gave a final CaCl₂ concentration of about 6 μm.

In considering the functional meaning of these experiments, it is most interesting that, in striated muscle, actin filaments are attached to the Z line at their barbed ends (2) and that at least some cytoplasmic actin filaments appear to be attached to membranes at their barbed ends (3, 6, 8, 11). In these cases, we presume that only the unattached, pointed ends of the filaments are available for growth. The limited ability of the pointed ends of that many nucleating sites besides the decorated nuclei were active in initiating assembly at these high actin concentrations. In the experiment in Fig. 3, and in similar experiments, the length of the bare actin extension at the barbed end of a decorated segment was always four to eight times longer than the bare extension at the pointed end. Thus, actin filaments can grow at both ends, but the extent of growth is always greater at the barbed end of the nucleus.

DISCUSSION

The experiments show that actin filaments can grow bidirectionally, although under the conditions examined actin monomers add preferentially to one end of nuclei consisting of actin filaments decorated with HMM. Thus, in addition to the structural polarity of actin filaments revealed by the pattern of myosin binding (2), actin filaments have an assembly polarity. The addition of monomers to the two ends is clearly not equivalent, so the mechanisms of actin filament elongation may be fundamentally different at the two oppositely polarized ends. The reasons that growth at the "barbed" end is favored are not revealed by these qualitative experiments, but we suspect that it reflects a change in the bonding properties of actin upon incorporation into the filament.

A complicating factor in the interpretation of these experiments is the presence of HMM bound to the nuclei. We do not know whether the biased growth of decorated nuclei is a property of the actin itself or imposed upon it by the HMM. Because there is bidirectional growth at high monomer concentrations, the unidirectional growth at low monomer concentrations may not be due to steric blockage of the pointed end by HMM. Rather we suspect that the results are due to a very strong concentration dependence for polymerization at the pointed end and a higher rate of polymerization at the barbed end.

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4 The idea that there is change in the actin-actin binding sites upon the polymerization of actin arises from the following reasoning: If each actin molecule has a polarity, B-P (barbed and pointed ends respectively), the actin molecules constituting a filament will be aligned BP~BP~BP etc., with axial P~B bonds holding them together. Of course, there also will be lateral bonds with the molecules in the parallel chain of the double helix. At the barbed end of the filament a B binding site will be open, while at the pointed end a P binding site will be open. All free monomers will have both B and P binding sites open. If the open B binding site at the barbed end of a filament were equivalent to a B binding site on a free monomer and if the same were true for the P binding sites, one would expect that the addition of monomers to both ends of the filament would be equally favored. Since this is not found, and since all of the monomer B and P sites should be equivalent, one must postulate an alteration in the open P or B sites at the ends of the filaments. Our results could be explained if an open B site at the barbed end of a filament were more active than a B site on a free monomer in forming P--B bonds, or if an open P site at the pointed end of the filament were less active than a P site of a monomer in forming P--B bonds. This difference might be due to a conformational change of the B or P sites upon the incorporation of an actin molecule in a filament. Alternatively, it might arise because the actin molecules at one end of a filament have a bound ADP while the actin molecules at the other end and the free actin monomers have a bound ATP.
actin filaments to nucleate polymerization might thus contribute to the stability of these attached actin filaments, and might allow the cells to maintain a relatively high concentration of free monomers in equilibrium with the filaments.

The rapid polymerization of actin filaments in the Thyone sperm acrosomal process presents a special problem (11). It is thought that these actin filaments polymerize at a rate of about 9 μm/s from their proximal (pointed) ends. The cause of this rapid growth from the pointed end might be a special property of the sperm actin filaments, or it might be due to the exceptionally high concentration of unpolymerized actin in the acrosomal vesicle adjacent to the growing actin filaments, or might be related to the presence of additional proteins.

SUMMARY
Isolated actin filaments decorated with HMM can grow by addition of actin monomers to either end, although there is a bias toward addition at the end which is normally attached to the Z line in striated muscle.

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