Proc. Nat. Acad. Sci. USA
Vol. 68, No. 11, pp. 2703–2707, November 1971

Isolation and Characterization of Myosin and Two Myosin Fragments from Human Blood Platelets

(agarose filtration/SDS–acrylamide gel electrophoresis/actin binding/electron micrographs)

ROBERT S. ADELSTEIN, THOMAS D. POLLARD, AND W. MICHAEL KUEHL*

Laboratory of Biochemistry, National Heart and Lung Institute, Bethesda, Maryland 20014

Communicated by C. B. Anfinsen, August 26, 1971

ABSTRACT **Platelet myosin (thrombosthenin M) and two additional proteins corresponding to the head and rod portion of the myosin molecule have been prepared from human blood platelets. Characterization of these proteins by SDS–polyacrylamide gel electrophoresis, actin binding studies, assay of enzymic ATPase activity, and electron microscopy has shown that the platelet contractile proteins closely resemble the corresponding muscle proteins. Platelet myosin and platelet myosin-head bind to both muscle and platelet actin and have an EDTA + K-stimulated ATPase activity, which is suppressed by Mg^{2+} in high salt concentration, whereas platelet rod does not possess either of these properties; platelet myosin and platelet myosin rod aggregate to form thick filaments at low ionic strength. Both intact platelet myosin and myosin head form typical arrowhead-shaped complexes with either platelet or muscle F-actin.**

Clot retraction, which is important for hemostasis in higher organisms, is mediated by the contraction of blood platelets. This contraction of platelets is thought to be caused by thrombosthenin, a complex of contractile proteins that shares many important properties with muscle actomyosin (1). Thromobosthenin has been separated into two fractions that have the properties of actin (thrombosthenin A) and myosin (thromobosthenin M), and that form functional hybrids with muscle actin or myosin (1). Several attempts have been made to purify the myosin-like component of thrombostenin, but the resulting enzyme has had extremely low specific activity compared with muscle myosin and has not been tested for the functional properties that characterize myosin: ability to bind to actin and to interact enzymically with actin (2, 3).

We have developed methods for fractionating human thrombosthenin, and have identified and partially characterized intact platelet myosin, platelet actin, and two additional proteins, which correspond to the head and rod portions of the platelet myosin. Characterization of these proteins by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, ATPase assay (ATP phosphohydrolase EC 3.6.1.3), actin binding studies, and electron microscopy has shown that the platelet contractile proteins closely resemble the corresponding muscle proteins in many of their properties.

MATERIALS AND METHODS

Platelets. Human platelet concentrates 1- to 5-days-old were obtained from the NIH Blood Bank, pooled, washed three times in 10 volumes of 0.9% NaCl–0.3% sodium citrate, 3 mM dithiothreitol (DTT), and 1 mM EDTA, and sedimented each time at 27,000 × *g*. After the third centrifugation, the pellets of platelets were stored at −15°C for up to 6 weeks before use. In one case, the platelets were stored in 50% glycerol at −15°C, without any significant difference in the results obtained. The washed platelets were essentially free from leucocyte and erythrocyte contamination.

Purification procedures

All steps were performed at 2–4°C; deionized water was used throughout the investigation.

1. Preparation of Thrombosthenin. 100–200 g of wet platelets were mixed with two volumes of 0.6 M KCl–15 mM Tris·HCl (pH 7.5, hereafter referred to as Buffer A), 10 mM sodium pyrophosphate, 5 mM $MgCl_2$, and 10 mM DTT; the cells were lysed by making the solution 3% in *n*-butyl alcohol. After extraction for 16 hr, the lysate was centrifuged for 1 hr at 27,000 × *g*, yielding the extract supernatant (see Table 1). Thrombosthenin was precipitated from this supernatant by dilution with 2 volumes of 2 mM $MgSO_4$ and adjustment of the pH to 6.4 (4). The precipitate was collected by centrifugation at 27,000 × *g* for 30 min, and then dissolved in Buffer A and 5 mM DTT. The precipitation was repeated three times to yield a cloudy suspension (in Buffer A and 5 mM DTT) with the ATPase activity and ATP-sensitive viscosity changes characteristic of thrombosthenin (1).

2. Fractionation of Thrombosthenin. The final thrombosthenin precipitate was taken up in Buffer A–5 mM $MgCl_2$–5 mM ATP–5 mM DTT, and centrifuged for 2 hr in a Spinco no. 30 head at 30,000 rpm, yielding 106,000 × *g* at the tip of the tube. This Mg–ATP treated 100,000 × *g* supernatant was then fractionated with saturated ammonium sulfate containing 10 mM EDTA (pH 7.0). The fraction containing the platelet myosin, usually the 35–60% cut, was dissolved in Buffer A–5 mM $MgCl_2$–5 mM ATP, and chromatographed on a 2.5 × 90 cm column of Sepharose 4B, equilibrated and eluted with Buffer A–3 mM DTT–0.5 mM $MgCl_2$, and either 0.5 mM ATP or 0.5 mM sodium pyrophosphate. The resulting fractions were assayed for ATPase activity, and pooled for further study.

Biochemical Assays. ATPase: the assay mixture contained 10 mM imidazole·HCl (pH 7.0), 2 mM ATP, 0.6 M KCl, and either 2 mM EDTA, 10 mM $CaCl_2$ or 5 mM $MgCl_2$. Samples were incubated at 37°C and 0.9-ml aliquots were

Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

* Present address: Albert Einstein College of Medicine, Department of Cell Biology, 1300 Morris Park Ave., Bronx, N.Y. 10461.

TABLE 1. *Platelet myosin: purification and recovery of enzyme activity and protein*

	Protein (total mg)	Total EDTA*-ATPase	Specific activity†		
			EDTA-ATPase	Ca-ATPase	Mg-ATPase
Extract supernatant	3150	125	0.04	0.05	0.01
Mg-ATP 100,000 × *g* supernatant	110	35	0.32	0.31	0.01
Ammonium sulfate (35–60%)	65	19	0.29	0.31	0.01
Sepharose 4B					
Fraction 1	6.8	3.7	0.55	0.44	<0.01
2	7.2	5.0	0.70	0.50	<0.01
3	7.0	6.0	0.85	0.54	<0.01

* Total EDTA-ATPase is expressed in μmoles of Pi released/min.

† Specific activity is expressed in μmoles of Pi released/mg of protein per min. Conditions of assay are outlined in text.

removed for the assay of inorganic phosphate production by a modification of the method of Martin and Doty (5). Phosphate production was linear with time during the period used for the assay (15–90 min). Protein concentration was estimated by the method of Lowry *et al.* (6) after Cl_3CCOOH precipitation; bovine serum albumin was used as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis was performed by a modification of the method of Weber and Osborn (7). 6 × 0.5 cm gels were prepared from 5% (or 10%) acrylamide–0.1% SDS–20 mM sodium phosphate (pH 7.0), and prerun at 5 mA/gel for 30 min. Protein samples in 8 M urea were reduced with DTT, and alkylated with iodoacetamide (8). After dialysis against water, they were dried and then dissolved in 1% SDS–1 mM sodium phosphate (pH 7.0)–10% glycerol. 10–100 μl samples of protein were electrophoresed for about 60 min at 6 mA/gel. The gels were stained with 1% aniline blue-black in 40% ethanol–7% acetic acid, and then destained in 40% ethanol–7% acetic acid, in the presence of a small amount of Bio-Rad AG1-X2.

Binding to F-Actin. Rabbit skeletal-muscle actin was mixed with equal (milligram) quantities of platelet myosin in 0.6 M KCl–25 mM imidazole·HCl (pH 7.0), and either 5 mM $MgCl_2$, 5 mM ATP, or an equal volume of water. The samples were centrifuged for 90 min in a Spinco no. 40 rotor at 40,000 rpm, yielding 143,000 × *g* at the tip of the tube. The supernatants were assayed for ATPase activity and electrophoresed as described above.

Materials

Rabbit skeletal-muscle myosin, a gift of W. W. Kielley, was prepared by the method of Kielley and Bradley (9). Rabbit skeletal-muscle actin was prepared as outlined previously (10). Urea solutions were prepared freshly with SBR ultra pure biological-grade urea from Schwarz. Iodoacetamide was

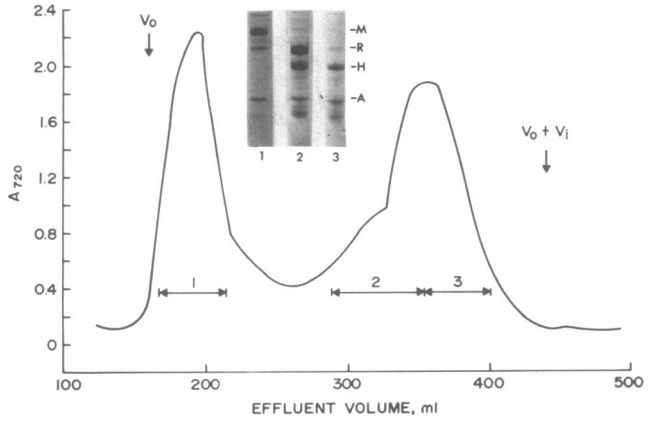

FIG. 1. Profile of Sepharose 4B agarose filtration of the 35–60% ammonium sulfate fraction. A 2.5 × 90 cm column equilibrated with Buffer A–3 mM DTT–0.5 mM $MgCl_2$–0.5 mM pyrophosphate was used. A 32-mg sample was applied in 9 ml of Buffer A–5 mM DTT. The column was eluted with the equilibration buffer at 30 ml/hr. 10-ml fractions were collected, of which 0.2 ml was used for ATPase assay. The abscissa indicates A_{720} of the ATPase assay. The void and salt boundary are indicated by V_o and $V_o + V_i$. The photographs are of 0.1% SDS–5% acrylamide gels from each of the indicated pooled fractions. Migration is from top to bottom. *M*, *R*, *H*, and *A* are identified in the text.

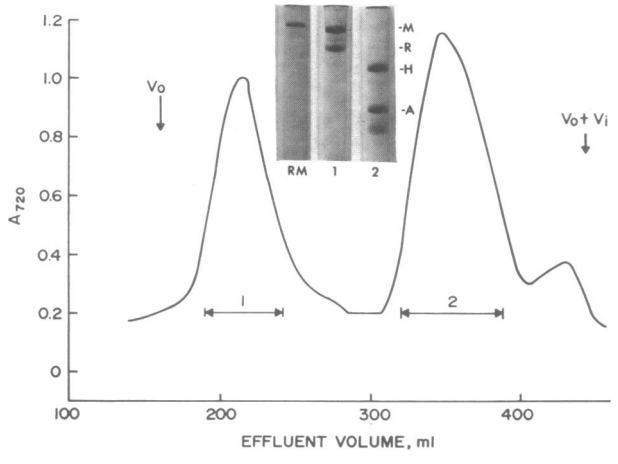

FIG. 2. Profile of Sepharose 4B agarose filtration of the 35–60% ammonium sulfate fraction after addition of 5 mM $MgCl_2$–5 mM ATP, and centrifugation (see text for details). The equilibration buffer used was Buffer A–3 mM DTT–0.5 mM $MgCl_2$–0.5 mM ATP. An 11-mg sample was applied in 11 ml of Buffer A–3 mM DTT–5 mM ATP–5 mM $MgCl_2$. The column was eluted with the equilibration buffer and 0.5 ml was used for ATPase assay. The small peak at the salt boundary is from the ATP in the applied sample. The photographs are of 0.1% SDS–5% acrylamide gels of the two pooled fractions and of rabbit skeletal myosin (*RM*).

recrystallized from ethyl acetate and washed with cold anhydrous ether.

RESULTS

The yields of protein and ATPase activity, as well as the specific activities obtained in the various fractions, during a typical purification of platelet myosin are given in Table 1. The 35–60% ammonium sulfate fraction was chromatographed on a column of Sepharose 4B and the resulting fractions were assayed for ATPase activity (Fig. 1). The protein was then pooled into the three fractions indicated in Fig. 1. Each of these fractions was concentrated with an Amicon Ultrafiltration Cell, assayed for the ATPase specific-activities shown in Table 1, and analyzed by the SDS–polyacrylamide gel electrophoresis shown in the upper part of Fig. 1.

Fraction 1 contained three bands of protein. The heaviest band, *M* (for intact myosin), corresponds to a molecular weight of 200,000; the next band, *R* (for rod), corresponds to a molecular weight of 130,000; and the lightest band, *A* (for actin), corresponds to a molecular weight of 46,000. Band *A* could be eliminated from this fraction by the addition of 5 mM Mg–ATP to the sample and sedimentation of the actin at 143,000 × *g* for 1 hr before chromatography of the supernatant. (See Fig. 2).

Fraction 2 contained equal amounts of *R* and another band, *H* (for head), with a molecular weight of 100,000. Fraction 3 had more *H* than *R*, and always contained some actin. The broad band below actin corresponds to a molecular weight of 30,000; it is probably platelet tropomyosin (11).

Fig. 2 shows a second Sepharose 4B chromatogram. In this case, the ammonium sulfate fraction (35–60%) was made 5 mM with respect to Mg–ATP, sedimented at 143,000 × *g* to remove actin, and applied to a column equilibrated with ATP rather than pyrophosphate. The proteins were pooled as indicated and electrophoresed as shown in the upper part of Fig. 2. A gel with rabbit skeletal-muscle myosin is included for comparison to show the position of the 200,000-molecular-weight band. The first peak contains both *M* and *R*, and the second peak *H*, actin, and some tropomyosin.

Fig. 3 is a molecular weight calibration curve of the proteins isolated from Sepharose. The muscle proteins and bovine serum albumin (*open circles*) were used as standards.

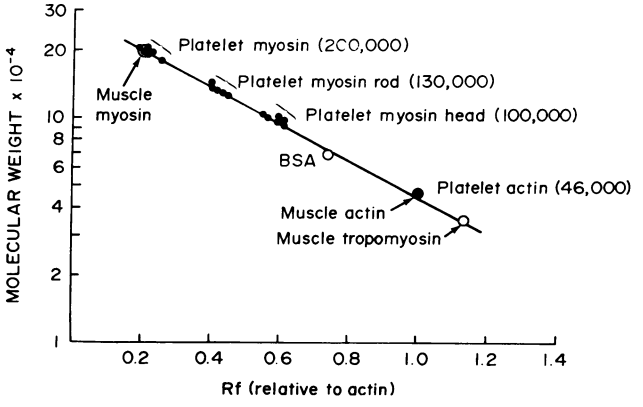

FIG. 3. Molecular weight calibration curve for 0.1% SDS–5% acrylamide gels. The standards are rabbit muscle myosin (molecular weight, 200,000), tropomyosin (34,500), and bovine serum albumin (BSA; 69,000). The mobilities of all proteins are relative to rabbit skeletal actin (46,000).

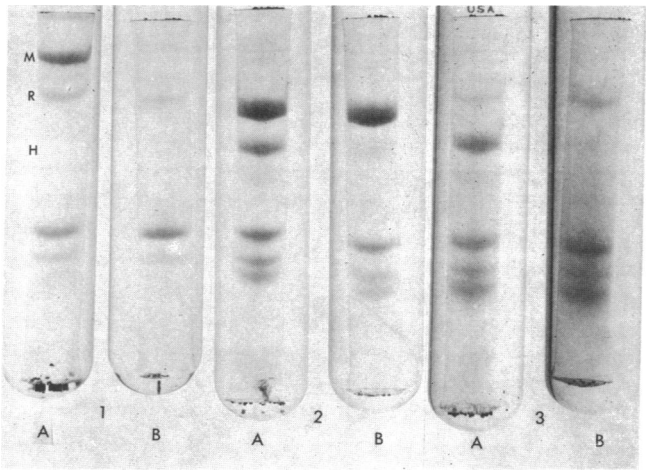

FIG. 4. Supernatant fractions from the binding of rabbit skeletal actin to platelet myosin. Two 0.1% SDS–5% acrylamide gels are shown for each of the three fractions shown originally in Fig. 1. In each case, the *A* fraction had 5 mM $MgCl_2$–5 mM ATP added, as well as actin. The *B* fraction had actin alone added. *M*, *R*, and *H* are identified in the text. A small amount of Bio-Rad AGl-X2, used for destaining, can be seen at the top and bottom of the gels.

These results show that partially purified thrombosthenin can be divided into four distinct proteins. These four proteins have been identified as platelet myosin, myosin rod, myosin head, and actin in the following experiments.

First the Sepharose fractions shown in Fig. 1 were tested for actin binding (see Fig. 4). After centrifugation of the samples with muscle F-actin in the presence or absence of Mg–ATP, the supernatants were analyzed by electrophoresis and ATPase assay to determine if any of the proteins bound to actin and sedimented with it. By comparing the supernatants from tubes with Mg–ATP (*A*), and those without Mg–ATP (*B*), one can determine which protein bands bind to muscle F-actin and are dissociated from it by Mg–ATP. It is clear that both *M* and *H* bind to F-actin in the absence, but not in the presence, of Mg–ATP, and that *R* does not bind to actin in either case. As expected, ATPase activity disappeared from those fractions where *M* or *H* bound to and sedimented with F-actin. This suggested that *M*, which had ATPase activity, and an actin-binding site, and coelectrophoresed with muscle myosin in SDS–acrylamide gels was intact platelet myosin, and that *H*, which had ATPase activity and an actin-binding site might be a fragment of the myosin retaining these functions. *R*, on the other hand, did not bind to actin and had little or no ATPase activity.

Electron microscopy of these proteins confirmed the identification of *M* and *H* and provided evidence that *R* corresponded to the rod portion of the platelet myosin.

At low ionic strength, *M* aggregated to form bipolar thick filaments up to 700 nm long, characterized by a bare central portion, tapered ends, and lateral projections (especially prominent at the ends) (Fig. 5*A*). When mixed with actin filaments from either muscle or platelets, these lateral projections bound to the actin filaments in a series of repeating arrowhead-shaped complexes (Fig. 5*B*). The spacing and appearance of these complexes and the dissociation by Mg–ATP was typical of the complex of muscle actin and myosin.

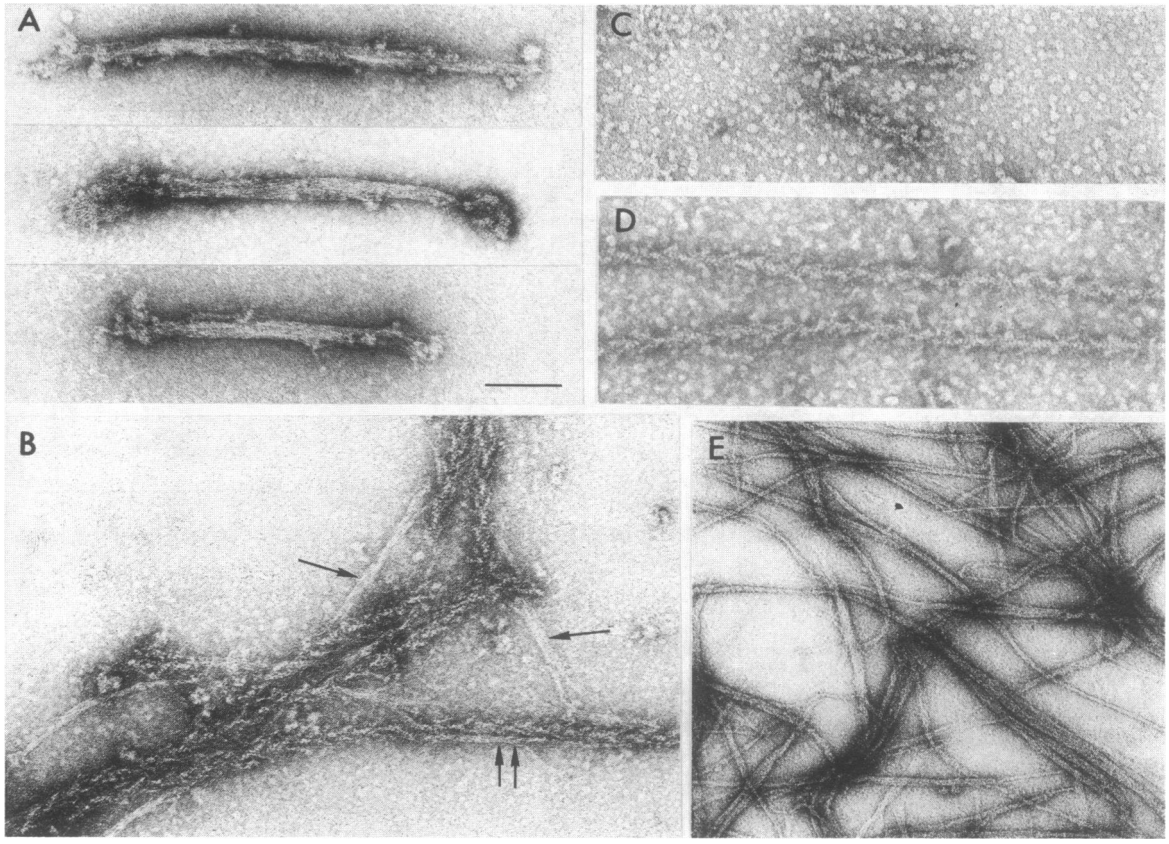

FIG. 5. Electron micrographs of purified platelet contractile proteins, negatively stained with 1% uranyl acetate. All samples were dialyzed against 0.1 M KCl–10 mM imidazole–HCl (pH 7.0). Bar represents 100 nm, magnification ×100,000. (*A*) Platelet myosin thick filaments. Note the filamentous substructure of the bare central region and the tapered ends with lateral projections. These bipolar filaments disappear when the concentration of KCl is raised to 0.5 M. (*B*) Platelet myosin and F-actin. The side arms on the thick filaments bind to the F-actin to form repeating arrowhead-shaped complexes along the thin filament. The bare central region of the myosin filament is seen bridging two actin filaments (*arrow*) or lying lateral to an actin filament (*double arrows*). (*C*) Platelet myosin head with platelet F-actin. Regular arrowhead complexes form along the actin filament. (Viewing the micrograph from the side enhances the arrowhead pattern.) No tails extend from the myosin head attached to the actin. This complex is dissociated by Mg–ATP. (*D*) Hybrid complex of platelet myosin head with rabbit F-actin. The appearance of the complex is indistinguishable from the homologous complex shown in *C*. (*E*) Platelet myosin rod. The rods form bare thick filaments with filamentous substructure. Like the myosin filaments, these thick filaments disappear at high ionic strength.

These morphological characteristics of platelet myosin (*M*) are identical to those of muscle myosin (12).

Fractions enriched in *H* contained no thick filaments at either high or low ionic strength, but did contain a protein that formed arrowhead complexes with either platelet or muscle F-actin (Fig. 5*C* and *D*), that were indistinguishable from the complex of F-actin with muscle *H*-meromyosin or Subfragment-1 (12, 13). These complexes were dissociated by Mg–ATP.

In an attempt to separate the various myosin components, a more discrete ammonium sulfate fractionation was performed. Whereas the 30–40% and 40–50% fractions contained all of the described proteins, the 50–60% ammonium sulfate fraction consisted primarily of pure *R* (rod). Electron micrographs of this fraction showed that *R* aggregated at low ionic strength to form smooth, tapered thick filaments, which lacked lateral projections (Fig. 5*E*). Unlike the thick filaments of *M*, these *R* filaments continued to grow in length and width until some of these paracrystalline aggregates were many micrometers long. These aggregates rapidly dissolved at high ionic strength. Low ATPase activity and the ability to form paracrystals at low ionic strength suggested that *R* was the rod portion of the platelet myosin molecule, from which the head (*H*) containing the ATPase and actin-binding sites has been cleaved.

Since the 50–60% ammonium sulfate fraction contains mostly rod, we now routinely fractionate the 30–50% fraction on Sepharose 4B and use the 50–70% fraction as a source of the rod.

Band *A* has tentatively been identified as actin because its presence correlated exactly with the observation of 6-nm wide filaments that formed arrowhead complexes with either *M* or *H* in electron micrographs of these fractions. Moreover, this protein has since been purified and shown to contain, similar to skeletal actin, 1 mol of 3-methylhistidine per 46,000 grams of protein.

In order to determine the possible effect of platelet age (1–5 days) and/or frozen storage on the production of rod and head, fresh platelets were used in one preparation. The entire procedure through the Mg–ATP dissociation was performed in 24 hr, the partially purified material having specific activities similar to those shown in Table 1 (Mg–ATP, 100,000 × *g* supernatant). SDS–gel electrophoresis of this

material showed the presence of rod and head in quantities similar to preparations made from stored platelets.

DISCUSSION

We have developed a new method for the fractionation of thrombosthenin and have found that platelet myosin is remarkably similar to muscle myosin in enzymic activity, ability to bind to platelet and rabbit muscle actin (being dissociated by Mg–ATP), size of its major polypeptide chain, and appearance by electron microscopy. Furthermore, both rod and head show properties similar to their respective proteolytic fragments that are isolated from muscle myosin, in that the head contains both the ATPase and actin-binding site and the rod aggregates to form thick filaments at low ionic strength (14).

Platelet myosin with a low ATPase specific activity (0.01–0.02 μmol Pi/mg per min) has been isolated by Cohen *et al.* and Booyse *et al.* (2, 3). Differences in assay conditions cannot entirely account for the much higher specific activity that we have observed (0.4–0.6 μmol Pi/mg per min). Moreover, we have found the EDTA+K-stimulated ATPase activity to be higher than the Ca^{2+}-stimulated activity, provided that DTT is used throughout the preparation. Similar to muscle myosin, the Ca^{2+} activation increases and the EDTA+K activation falls with age, possibly secondary to the oxidation of a unique-SH group (15). Since the intact molecule has not been isolated completely free of rod (see Fig. 2, peak 1), the true specific activity of platelet myosin is probably somewhat higher, rod being devoid of activity.

Although Table 1 shows no increase in specific activity after ammonium sulfate fractionation, this step was included because in most preparations it did result in an increase in specific activity and it afforded a simple method of concentrating the protein.

The graph shown in Fig. 3 indicates that the major polypeptide chains of both rabbit skeletal and platelet myosin have similar molecular weights (200,000) in SDS–polyacrylamide gels. The molecular weight obtained for *R* (130,000) in SDS–polyacrylamide gels is substantially higher than the expected (dissociated) molecular weight for light meromyosin (75,000), and closer to that determined for the rod isolated from muscle myosin (110,000) (14).

The presence of the rod and head portion of the myosin molecule, as well as intact platelet myosin, suggests that proteolytic cleavage has taken place. Since fresh platelets also showed the presence of rod and head, it is likely that cleavage was initiated *in vivo*, during the preparation of the platelet concentrates, or during the 24 hr required for preparation of the myosin.

The appearance of only head and rod (as opposed to meromyosins) in our preparation may be explained by one or more of the following: (*a*) Digestion occurred only when the platelet myosin was in the fibrous state and not after it was solubilized; (*b*) platelet myosin is cleaved both in its soluble and fibrous form into rod and head that are resistant to further digestion; (*c*) there is a specific enzyme, e.g., thrombin, which performs this specific cleavage in platelet myosin. Cohen *et al.* (2) suggested that thrombin may digest thrombosthenin *M* when it is still bound to the platelet membrane and Baenziger *et al.* (16) described a thrombin-sensitive protein in platelet membranes with a subunit molecular weight of 190,000. Further careful work will be necessary before deciding if thrombin can digest platelet myosin, either on or off the membrane, and what the physiological significance of such a reaction might be. Regardless of the mechanism by which the platelet myosin molecule is split, the occurrence of this cleavage has been helpful in the elucidation of the platelet myosin structure.

The presence in human blood platelets of a myosin molecule markedly similar in structure and function to muscle myosin, along with both actin and tropomyosin (11), strongly suggests that the contractile process in platelets is similar at a molecular level to the contractile process in muscle.

We thank Miss Mary Anne Conti for her outstanding technical assistance and Dr. W. W. Kielley for many helpful discussions. We are indebted to Mrs. Elise Yanchulis for her help in preparing the platelet concentrates.

1. Bettex-Galland, M., and E. F. Lüscher, *Advances in Protein Chemistry* (Academic Press, New York and London, 1965), Vol. 20, pp. 1–35.
2. Cohen, I., Z. Bohak, A. deVries, and E. Katchalski, *Eur. J. Biochem.*, **10**, 388 (1969).
3. Booyse, F. M., T. P. Hoveke, D. Zschocke, and M. E. Rafelson, Jr., *J. Biol. Chem.*, **246**, 4291 (1971).
4. Grette, K., *Acta Physiol. Scand. Suppl.*, **56**, 46 (1962).
5. Martin, J. B., and D. M. Doty, *Anal. Chem.*, **21**, 965 (1949).
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7. Weber, K., and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
8. Craven, G. R., E. Steers, Jr., and C. B. Anfinsen, *J. Biol. Chem.*, **240**, 2468 (1965).
9. Kielley, W. W., and L. B. Bradley, *J. Biol. Chem.*, **218**, 653 (1956).
10. Adelstein, R. S., and W. M. Kuehl, *Biochemistry*, **9**, 1355 (1970).
11. Cohen, I., and C. Cohen, *Fed. Proc.*, **30**, 1309 Abs. (1971).
12. Huxley, H. E., *J. Mol. Biol.*, **7**, 281 (1963).
13. Moore, P. B., H. E. Huxley, and D. J. DeRosier, *J. Mol. Biol.*, **50**, 279 (1970).
14. Lowey, S., H. S. Slayter, A. G. Weeds, and H. Baker, *J. Mol. Biol.*, **42**, 1 (1969).
15. Sekine, T., L. M. Barnett, and W. W. Kielley, *J. Biol. Chem.*, **237**, 2769 (1962).
16. Baenziger, N. L., G. N. Brodie, and P. W. Majerus, *Proc. Nat. Acad. Sci. USA*, **68**, 240 (1971).