Characterization of Cytoplasmic Actin Isolated from Acanthamoeba castellanii by a New Method

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Cytoplasmic actin has been isolated from Acanthamoeba castellanii by a new method, employing chromatography on DEAE-cellulose, that improves the yield by more than 20-fold over the previously reported method. This procedure should be particularly useful for isolating actin from cells in which it is present in relatively low concentration because the method does not depend initially on the polymerization of actin or its interaction with myosin. Systematic comparison of the properties of purified Acanthamoeba actin and rabbit skeletal muscle actin shows them to be similar in many ways: viscosity of F-actin, stoichiometry of bound nucleotide, stoichiometry of binding of muscle heavy meromyosin and myosin subfragment 1 in the absence of ATP, and ability to inhibit the KATPase activity of heavy meromyosin. The amino acid compositions of Acanthamoeba and muscle actin are also quite similar, but significant differences, especially the presence of ε-N-methyllysines in Acanthamoeba actin, have been confirmed. In addition to this structural difference, we find that Acanthamoeba actin is only one-third as effective as muscle actin as an activator of the MgATPase of muscle heavy meromyosin and subfragment 1. For Acanthamoeba actin, as for muscle actin, this activation exhibits hyperbolic dependence on actin concentration; i.e. the double reciprocal plot of ATPase activation versus actin concentration is linear. From these plots we find that the two actins give the same extrapolated ATPase activity at infinite actin concentration (Vmax) but differ by a factor of three in the concentration of actin needed to produce half-maximal activation (Kapp).

Eukaryotic cells contain cytoplasmic microfilaments, 5 to 7 nm wide and of indeterminate length, that, in about 50 types of mammalian, protozoal, and plant cells, have been identified as actin by their ability to bind HMM under specific conditions to form decorated filaments indistinguishable from those formed by the interaction of HMM with muscle actin (1, 2). A protein resembling muscle actin has been isolated from about 10 of these non-muscle cells (1-12). A protein with myosin-like ATPase activity has also been shown to be present in most of these cases. It is reasonable to assume that these proteins are the primary molecular constituents of systems involved in ameboid movement, phagocytosis, cell division, and other essential motile processes. However, if we are to understand the physiological events of cell motility in molecular terms, in the way that muscle contraction can be understood from the properties of the molecular interactions of actin and myosin, it is necessary to investigate the biochemistry of cytoplasmic actins and myosin in great detail. In this paper we report the first results of a systematic comparison of the properties of Acanthamoeba actin and rabbit skeletal muscle actin.

Efforts to isolate actin from other non-muscle cells have generally been aimed at demonstrating its existence in these cells, rather than at detailed biochemical characterization. The isolation procedures, most of which are based on the ability of actin to form a complex with endogenous or exogenous myosin in a crude cell extract, give very low yields; 1% from rabbit macrophages (10) and 2.5% from Dictyostelium discoideum (8) are two well documented examples. Biochemical comparison of these cytoplasmic actins with muscle actin by conditions for polymerization, viscosity of the polymeric form, bound nucleotide, methylhistidine content, and ability to bind HMM and S-1 and to activate their MgATPase has demonstrated qualitative similarities, but quantitative differences. All actins depolymerize at low ionic strength and polymerize in 0.1 M KCl. For some cytoplasmic actins Mg2+ was reported to induce polymeric forms which are quite unlike muscle F-actin (4, 9, 12); in two of these three cases, however, the occurrence of these unusual polymers was eliminated by further purification of the actin (9, 11). The viscosities of most preparations of cytoplasmic F-actins are less than half that of muscle F-actin (4, 9, 12); in two of these three cases, however, the occurrence of these unusual polymers was eliminated by further purification of the actin (9, 11). The viscosities of most preparations of cytoplasmic F-actins are less than half that of muscle F-actin, but actin of comparable viscosity to muscle actin has been isolated from human platelets (7) and Physa- ranum polycephalum (3). Bound nucleotide contents of 0.7 to 1.0 mol/mol have been reported for a few cytoplasmic actins (1, 3, 10). Like muscle actin, all cytoplasmic actins which have been analyzed contain approximately 1 mol/mol of 3-methylhistidine (1). Binding of HMM or S-1 has been demonstrated by...
electron microscopy for nearly all cytoplasmic actins, but no one has quantitated this binding. Many actins have been shown to activate the MgATPase of HMM or S-l (1, 5, 7, 8, 10), but in most cases this activation was weak compared to that of muscle actin. However, none of these kinetic measurements has been made at high actin concentrations, where maximal activation is approached.

In 1971, Weihing and Korn (5) isolated actin from Acanthamoeba castellanii by a modification of the method used by Hatano and Oosawa (3) for the isolation of actin from T. polycephalum. The basis of this method was the precipitation of a hybrid actomyosin by the addition of muscle myosin to a crude ameba extract. The purified actin accounted for about 0.06% of the total cell protein and less than 1% of the estimated actin content of Acanthamoeba. Although clearly similar to muscle actin, these preparations of Acanthamoeba F-actin had less than 50% of the viscosity of muscle F-actin and were only 20% as effective in activating the MgATPase of muscle HMM.

In 1976, after completion of the present research, Pollard (12) reported a totally different procedure for isolating Acanthamoeba actin in much higher yield. The biochemical characterization of this actin is incomplete, but its viscosity is reported to be about half that of muscle actin.

For the present biochemical studies, a new procedure for isolating actin using chromatography on DEAE-cellulose was introduced. This procedure offered three important advantages. First, the yield is 15 to 20% of the total cytoplasmic actin. This is not only because it enabled us to obtain sufficient actin for detailed kinetic studies, but because it eliminated the possibility that the actin we isolated might be a minor species differing significantly from the bulk of the cytoplasmic actin pool. Second, the procedure is mild and reproducible so that differences between Acanthamoeba and muscle actin are not likely to be artifacts of the purification procedure. Finally, the procedure does not require that actin polymerize or form actomyosin complexes in a crude extract where other proteins present may prevent this behavior. This unique feature probably accounts for the high yield we obtained for Acanthamoeba actin and may be particularly useful for isolating actin from cells in which it is only a minor component.

Highly purified Acanthamoeba actin was readily obtained in 75 to 100 mg quantities by this procedure. We have been able to demonstrate a significant functional difference between this actin and muscle actin.

MATERIALS AND METHODS

Culture of Acanthamoeba castellanii—Amebae were grown in 1-liter aerated carboys at 28°C as described by Pollard and Korn (13). One carboy grown to a density of 1.2 x 10^6 cells/ml provided about 100 g wet weight of ameba.

Buffers—Buffer G contained 3 mM imidazole base/0.1 mM CaCl_2/0.5 mM ATP/0.75 mM β-mercaptoethanol, pH 7.5. In this buffer, actin assumes the monomeric globular or "G" form. Buffer F contained 5 mM imidazole chloride/2 mM MgCl_2/0.5 mM ATP, pH 7.0. In this buffer, actin assumes the polymeric filamentous or "F" form. Buffer D, the buffer for DEAE-cellulose chromatography, was the same as Buffer G, except that the imidazole concentration was 10 mM; pH was adjusted to 7.5 with HCl.

Muscle Proteins—Actin, HMM, and S-l were prepared from rabbit back and leg muscle. Actin was extracted at low ionic strength from an acetone powder of muscle and purified by two extractions with 0.8 M KCl (14, 15). Myosin was isolated from fresh muscle by the method of Kielley and Harrington (16). HMM was prepared by tryptic digestion of myosin (17). S-l was prepared by papain digestion of myosin (18).

DEAE-cellulose Chromatography—It is known for muscle actin that ATP and a divalent cation are necessary to maintain G-actin in its native form. Since DEAE-cellulose is an anion exchanger which binds ATP strongly, it is necessary to saturate it with ATP in order to obtain a detectable level of ATP in the eluate. Muscle actin was more than 50% denatured by DEAE-cellulose chromatography when this was not done. There was no denaturation of actin, however, in ATP-saturated DEAE-cellulose columns prepared freshly for each actin preparation, as follows.

Approximately 300 ml (wet volume) of Whatman DE52 was washed with 1 x NaOH, 1 x HCl, and water, then suspended in about 200 ml of Buffer G containing 0.1 mM ATP. ATP was added until its concentration (calculated from the absorbance at 280 nm) in the supernatant was 0.5 ± 0.1 mM at equilibrium; the pH was readjusted to 7.5 with KOH before and after ATP was added. It was found empirically that 1.5 g of ATP were sufficient to achieve saturation under these conditions and that equilibration was complete within 15 min. The ATP-saturated DEAE-cellulose was then packed into a column (2.5 x 45 cm) and equilibrated by flowing through 500 ml of 0.1 M KCl in Buffer D. Other details are given in the legend of Fig. 2.

ATPase Assays—All of the assays reported in this paper were carried out with an automatic pH-stat apparatus (17) at 25°C with the pH maintained at 7.0. Reactions were started by the addition of either HMM or S-l. The volume of 60 mM KOH titrant automatically added to neutralize the H+ generated by hydrolysis of ATP was recorded continuously. Hydrolysis of ATP was linear with time up to 50 to 70% of the substrate was exhausted. ATPase activity was calculated from the slope of the early linear portion of such records.

Protein Assay—Protein concentration was determined by the method of Lowry et al. (19) or, for purified proteins, by the absorbance at 280 nm. The A_280 at concentrations of 1 mg/ml and light paths of 1 cm were assumed to be 1.15 for actin (17), 0.675 for HMM (20), and 0.770 for S-l (21). All absorbances at 280 nm were corrected for scattering by subtracting the absorbance at 290 nm, which was always less than 5% of the A_280 for the purified proteins.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—In all cases 11% acrylamide gels were used in the Laemmli buffer system (22). Protein samples were boiled for 30 min at concentrations no greater than 2 mg/ml in 2% sodium dodecyl sulfate/0.06 M Tris chloride (pH 6.5)/5% sucrose/5% β-mercaptoethanol/0.0025% bromphenol blue. After electrophoresis the gels were fixed and stained according to the methods of Fairbanks et al. (23).

Viscosity—Viscosities were measured at 25°C in Ostwald viscometers with capacities of 3 ml and flow times for Buffer F of about 50 s. The buffer for all viscometer runs was Buffer F and samples were diluted from concentrated stock solutions at least 24 hours before the measurements. Relative viscosities, η_r, were determined as the ratio of the flow time of samples to that of buffer; specific viscosity, η_η, equals η_r - 1. Reduced viscosity is defined as specific viscosity divided by protein concentration in grams per ml.

NADH Oxidase—NADH was diluted to a concentration of 1.0 to 1.5 mg/ml and free ATP was removed by adsorption of Dowex 1-X4-C1 in three batches of 0.15 g of resin/ml of actin solution. The actin was then divided into two portions. Water (0.1 ml/ml) was added to one portion and its protein concentration determined by its A_280. To the second portion, 70% HClO_4 (0.1 ml/ml) was added to precipitate the actin and release the bound nucleotide. The concentration of the released ADP was determined from the A_280 of the supernatant solution using a molar extinction coefficient of 15.0 x 10^3 M^-1 cm^-1.

Amino Acid Analysis—Protein samples were incubated for 2 hours at 95°C in 5 N NaOH/5 N HCl/15 mM dithiothreitol/0.2 M NaOH, and then cooled to 22°C, made 0.2 M in iodoacetic acid (recrystallized and preadjusted to pH 8.0) and incubated for 15 min. β-Mercaptoethanol was added to 2.0 M. The reduced, alkylated protein was then dialyzed against several changes of distilled water and lyophilized. Two milligrams of the lyophilized sample was dissolved in 1 ml of 6 N HCl and hydrolyzed for 18 hours at 100°C. The hydrolysate was lyophilized, redissolved in water, adjusted to pH 9 with NaOH, lyophilized again, and finally dissolved in 2 ml of 0.01 M HCl and adjusted to pH 2.

Neutral and acidic amino acids were determined by the method of Spackman et al. (24). Basic amino acids were analyzed by the method of Adelstein and Kuehl (25) as modified by Weihing and Korn (6), which resolves lysine from its mono-, di-, and trimethyl derivatives as well as histidine and 3-methylhistidine. All amino acid analyses were carried out on a Beckman 121 amino acid analyzer with automatic...
Purification and Characterization of Acanthamoeba Actin

RESULTS

Purification of Acanthamoeba actin—Acanthamoeba actin was usually prepared from about 2 × 10⁷ amebae obtained from one 15-liter carboy. All purification steps were carried out at 0-4°C unless otherwise noted. Cells were harvested by low speed centrifugation and washed three times in 600 to 800 ml of 10 mM imidazole chloride, pH 7.5. The cells were then suspended in Buffer G, 2 ml/g of cells, and ruptured by release from a Parr bomb equilibrated with nitrogen at 400 p.s.i. The homogenate was centrifuged at 100,000 × g for 90 min and the pellet was discarded. The low ionic strength extract thus obtained generally consisted of 200 to 300 ml of a variably turbid (depending on the lipid content), amber-colored liquid containing 12 to 16 mg/ml of protein of which about 15% was actin (45,000-dalton band) by dodecyl sulfate gel electrophoresis (Fig. 1).

The ameba extract was fractionated on DEAE-cellulose as described in the legend to Fig. 2, which shows a typical elution pattern. Eluted fractions were monitored for the presence of actin filaments by observing through crossed polarizers to detect flow birefringence. As long as these fractions were kept cold, no flow birefringence developed. However, when they were warmed to 25°C and MgCl₂ was added to 2 mM, fractions eluting between 0.19 and 0.24 M KCl rapidly developed flow birefringence. Dodecyl sulfate gel electrophoresis of selected fractions throughout the gradient confirmed the presence of actin as the major component in these fractions, the absence of actin in fractions eluting before 0.17 M KCl, and the gradual decrease of actin content in fractions eluting beyond 0.24 M KCl (Fig. 3). The material eluting between 0.19 and 0.24 M KCl, about 15% of the protein applied to the column, was pooled as the DEAE-actin fraction (Table I). Its actin content was 40% by gel electrophoresis (Fig. 1).

The pooled polymerized actin was centrifuged at 100,000 × g for 3.5 hours at 20°C. The clear gelatinous pellets were combined and homogenized in 20 to 30 ml of Buffer G containing 0.02% NaN₃ to retard growth of bacteria. The suspension was then dialyzed against two to three changes of this buffer for 60 to 80 hours to allow complete depolymerization of the actin. Residual material that had not depolymerized was removed by centrifugation at 100,000 × g for 90 min. The depolymerized supernatant solution contained about 5% of the protein of the original extract (Table I); its actin content by gel electrophoresis was about 70% (Fig. 1).

The depolymerized actin was applied to a column containing Sephadex G-150 and eluted with Buffer G containing 0.02% NaN₃ (Fig. 4). Actin was eluted as an asymmetric peak with a slowly rising ascending limb and a sharply declining descending limb similar to the pattern reported for muscle actin (26). Contaminating protein was removed in a voided fraction and in material eluting between the voided and included (actin) fraction. The eluted actin was concentrated to 5 to 8 mg/ml by ultrafiltration on an Amicon PM-10 membrane, polymerized by adjusting the solution to 0.1 M KCl and 2 mM MgCl₂, and dialyzed for 40 hours against two to three changes of Buffer F to remove KCl. The purified actin accounted for about 3% of the protein of the original extract and was 95% pure according to the scans of electrophoretic gels (Fig. 1). The gel scans indicate that 15 and 20% of the actin was recovered from the original extract.

Specific Activity of Actin—Each of the fractions obtained during the purification procedure was also assayed for its ability to activate the MgATPase of HMM. Because of the high intrinsic ATPase activity of the crude extract it was impossible to detect activation of HMM MgATPase by this fraction. An increase in the specific activity of actin at each of the three following purification steps was consistently observed (Table I). There are two apparent discrepancies between the specific

![Graph](http://www.jbc.org/)

**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Acanthamoeba actin at different stages of the purification procedure. Gels were fixed and stained with Coomassie blue as described under “Materials and Methods” and scanned at 550 nm in a Beckman Acta III spectrophotometer. The areas under the peaks are directly proportional to the amount of protein applied to the gels. In addition to a sample of whole ameba homogenate, the fractions listed in Table I were analyzed: the crude extract, fraction eluted from DEAE-cellulose between 0.19 to 0.24 M KCl, polymerized-depolymerized actin, and purified actin from the included peak on Sephadex G-150. These scans were used to obtain the data in Table I.
activities and the dodecyl sulfate gel electrophoretic data. First, the increase in specific activity at each step of the purification far exceeds the increase in per cent actin by electrophoretic analysis; in the final two steps specific activity increased 7-fold, while per cent actin increased only by a factor of 2.4. Second, the recovery of actin activity from the Sephadex column was more than 150%. While the first observation can be explained by removal of denatured actin (or removal of a different 45,000-dalton polypeptide which co-purifies with actin on DEAE-cellulose), the second observation strongly suggests that an inhibitor is removed in the Sephadex step. Such an inhibitor might act either directly by blocking the interaction of F-actin with HMM or indirectly by interfering with G-actin so that polymerization produces F-actin which activates HMM only weakly. It cannot act by preventing polymerization in the partially purified Acanthamoeba actin fractions, because these fractions show flow birefringence and because most of their actin sediments at 100,000 g under polymerizing conditions. Experiments discussed later suggest that if an inhibitor is removed during the purification of Acanthamoeba actin, it must be of the indirect type, which interacts only with G-actin. A search for such an inhibitor has begun.

**Table I**

**Purification of Acanthamoeba actin**

The purification procedure is described in the text and in the legends to Figs. 2 and 4. Total protein in each fraction was determined by the method of Lowry et al. (14). The actin concentration was calculated from the total protein and the percentage of 45,000-dalton component in the gel scans shown in Fig. 1. Activation of the MgATPase of HMM was measured at 25° in 2.0 mM ATP/2.5 mM MgCl₂/10 mM imidazole chloride, pH 7.0. The concentration of HMM was 0.2 μM and the concentration of protein from the actin fractions varied between 0.1 and 1.0 mg/ml. Under these conditions, activation is proportional to the amount of protein added and to HMM concentration. The units for specific activity are micromoles of P<sub>i</sub>/(s)(μM HMM head)(mg of protein). The value for muscle actin under these assay conditions was 35.1. Total activity is the product of the specific activity and the total protein in the fraction.

| Fraction       | Protein (mg) | Actin (%) | HMM activity (specific total) |
|----------------|--------------|-----------|-------------------------------|
| Extract        | 4080         | 15        | 612                           |
| DEAE-Sephadex  | 616          | 40        | 246                           |
| Polymerized-depolym. | 189   | 70       | 118                           |
| Sephadex G-150 | 120          | 95        | 114                           |

**Fig. 2.** DEAE-cellulose chromatography of the crude Acanthamoeba extract. The low ionic strength ameba extract (200 to 300 ml, 12 to 16 mg of protein/ml) was applied to a DEAE-cellulose column (2.5 x 45 cm) previously equilibrated with 0.1 M KCl in Buffer D as described under "Materials and Methods." The extract was applied as soon as possible because after 4 to 6 hours it became increasingly turbid, eventually forming a precipitate. Fifty milliliters of Buffer G were applied immediately before and after the sample; this procedure insured that ameba actin would not be exposed to 0.1 M KCl until after it was adsorbed to the DEAE-cellulose. The column was then eluted with 200 to 300 ml of 0.1 M KCl Buffer D and 2 liters of a linear gradient from 0.1 to 0.5 M KCl Buffer D. The flow rate was maintained at about 50 ml/hour under gravity. Protein in the eluate was monitored by A<sub>280</sub>, at which wavelength the contribution of ATP is diminished but not eliminated. The absorbance of ATP cannot be balanced by a reference sample because the concentration of ATP in the eluate changes with the KCl concentration. The concentration of KCl was monitored by measuring conductance at 0°. The conductance of 0.1 M KCl in Buffer D is 4.6 mmho; that of 0.5 M KCl is 21 mmho. The times of appearance in the eluant of Buffer G, 0.1 M KCl, and the beginning of the KCl gradient are indicated by arrows. The position of the eluted actin, determined by the development of flow birefringence at 25° after the addition of 2 mM MgCl₂, and confirmed by gel electrophoresis (Fig. 3), is indicated by the shaded area.

**Fig. 3.** Sodium dodecyl sulfate gel electrophoresis of the extract applied to DEAE-cellulose and selected eluted fractions from Fig. 2. The gel on the extreme left is the original extract. The next 15 gels from left to right are aliquots from material eluting at the following volumes (milliliters): (2) 294, (3) 367, (4) 435, (5) 538, (6) 686, (7) 689, (8) 665, (9) 916, (10) 1056, (11) 1105, (12) 1225, (13) 1271, (14) 1454, (15) 1488, (16) 1593. Actin is the major band of the initial extract and is otherwise present only in Gels 12 to 15, which correspond to the action peak in Fig. 2, and in Gel 16. Approximately equal amounts of total protein were applied to each gel.
Purification and Characterization of \textit{Acanthamoeba} Actin

The specific activity of purified \textit{Acanthamoeba} actin as an activator of the MgATPase of HMM was found to be 12.2 \textmu mol of P$_i$/s (\textmu M HMM head) (mg of protein). The specific activity of muscle actin under identical conditions was 35.1 \textmu mol of P$_i$/s (\textmu M HMM head) (mg of protein), or approximately 3 times as great. The ratio of specific activities of \textit{Acanthamoeba} and muscle actin was never less than 0.31 or greater than 0.38 for any \textit{Acanthamoeba} actin preparation. Extraction with 1 M urea (29) or 0.8 M KCl (9), rechromatography on DEAE-cellulose, and an additional cycle of polymerization and depolymerization each failed to produce any further increase in specific activity of purified \textit{Acanthamoeba} actin. These observations suggest that the difference in specific activity of the two actins is not due to a residual impurity in the \textit{Acanthamoeba} actin. In order to understand this consistent difference in specific activity, an extensive and quantitative biochemical comparison of the two actins was undertaken.

\textbf{Amino Acid Analysis—}The amino acid composition of muscle actin is remarkable except for the presence of 1 mol of 3-methylhistidine/mol, \textit{Acanthamoeba} actin, like many other cytoplasmic actins, also contains 3 methylhistidine residue according to Weihing and Korn (5). In addition, they detected about 1 mol/mol of \(\epsilon\)-N-methyllysines, of which about 75% was dimethyllysine and 25% was monomethyllysine. Since methyllysines are not present in muscle actin, it was desirable to confirm their presence in \textit{Acanthamoeba} actin prepared in much greater yield by a different method.

The amino acid compositions of \textit{Acanthamoeba} and muscle actin are listed in Table II. The presence of 1 mol of 3-methylhistidine and 1 mol of methyllysines in \textit{Acanthamoeba} actin is confirmed. Dimethyllysine is always the major methylated lysine but the percentage of mono- and trimethyllysine varies. Other than the presence of methyllysines in \textit{Acanthamoeba} actin, the two proteins are generally very similar in composition.

\textbf{Viscosity—}One important characteristic of actin is its ability to form long filamentous polymers under appropriate ionic conditions (e.g., 0.1 M KCl or 2 M MgCl$_2$) in the presence of ATP. The extent of polymerization of purified \textit{Acanthamoeba} actin was evaluated by comparing its reduced viscosity at 25° in Buffer F (2 M MgCl$_2$/0.5 M ATP/5 M imidazole chloride, pH 7) to muscle actin in the same buffer at protein concentrations between 0.5 and 1.0 mg/ml (Table III).

Although there was significant variability in the viscosity of different preparations of \textit{Acanthamoeba} and muscle actin there was no systematic difference in viscosity between the two types of actin. Variations in viscosity between different preparations of actin may reflect differences in average filament length caused by imprecisely controlled factors, such as temperature and protein concentration at the time of polymerization.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Amino acid} & \textbf{Acanthamoeba} & \textbf{Muscle} \\
\hline
Lys & 21 & 20 & 20 & 21 & 21 \\
Lys(Me)$_2$ (total) & 0.6 & 0.9 & 0.8 & 0 & 0 \\
Lys(Me)$_1$ & Tr.$^a$ & Tr. & 0.13 \\
Lys(Me)$_0$ & 0.6 & 0.9 & 0.6 \\
\hline
His & 9 & 9 & 9 & 10 & 9 \\
His(r-Me) & 1.4 & 1.3 & 1.3 & 1.1 & 1.1 \\
\hline
Arg & 22 & 21 & 21 & 20 & 21 \\
Asp & 32 & 31 & 32 & 32 & 34 \\
Thr & 28 & 27 & 25 & 27 & 27 \\
Ser & 25 & 24 & 22 & 25 & 23 \\
Glu & 43 & 41 & 43 & 41 & 40 \\
Pro & 15 & 14 & 14 & 20 & 18 \\
Gly & 32 & 31 & 32 & 29 & 28 \\
Ala & 31 & 30 & 30 & 29 & 30 \\
Cys & 4 & 4 & 4 & 5 & 4 \\
Val & 22 & 21 & 21 & 18 & 18 \\
Met & 13 & 12 & 11 & 12 & 13 \\
Ile & 22 & 21 & 23 & 29 & 23 \\
Leu & 32 & 31 & 31 & 26 & 26 \\
Tyr & 18 & 17 & 15 & 18 & 18 \\
Phe & 15 & 14 & 14 & 13 & 13 \\
Trp & N.D. & N.D. \\
\hline
\end{tabular}
\caption{Amino acid compositions of \textit{Acanthamoeba} and muscle actin}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Properties} & \textbf{Acanthamoeba} & \textbf{Muscle} \\
\hline
Viscosity, dl/g at 25° & 7.9 & 6.7 \\
& 4.9 & 8.0 \\
& 8.0 & 7.5 \\
Bound nucleotide, mol/mol & 0.93 & 0.91 \\
& 1.03 & 0.91 \\
HMM heads bound, mol/mol & 1.08 & 1.00 \\
& 0.96 & 1.05 \\
Activation of HMM MgATPase & 12 & 12 \\
& 22 & 8 \\
Activation of 1 MgATPase & 17 & 17 \\
& 24 & 9 \\
\hline
\end{tabular}
\caption{Comparison of properties of \textit{Acanthamoeba} and muscle actin}
\end{table}
That the variability in viscosity is not due to residual unpolymerized G-actin can be seen from the experiment reported in Fig. 5. For both Acanthamoeba and muscle actin the plots of viscosity as a function of actin concentration are linear and pass through, or very close to, the origin. The intercept of such a plot indicates the critical concentration of G-actin (which has very low viscosity) required for polymerization. It was below the limits of detection (<0.05 mg/ml) for both Acanthamoeba and muscle actin. Acanthamoeba actin, like muscle actin, is virtually all filamentous in 2 mM MgCl₂ at 25°.

Bound Nucleotide—Muscle F-actin in its native state contains 1 mol of bound ADP/mol (28). The molar ratio of bound nucleotide was measured for two preparations of Acanthamoeba actin and one preparation of muscle actin (Table III). Like muscle actin, Acanthamoeba actin was found to contain 1 mol of bound nucleotide/mol of actin monomer.

Stoichiometry of Binding of HMM and S-1—In the absence of ATP, muscle F-actin binds S-1 in a molar ratio of 1:1 (S-1/actin monomer) and HMM (which has two ATPase heads per molecule) in a molar ratio of 1:2 or 1 mol of actin monomer/mol of ATPase heads (29). The stoichiometry of binding of HMM and S-1 to Acanthamoeba actin and muscle actin was determined (Table III) by incubating actin (0.5 mg/ml) with S-1 (3 mg/ml) in the presence of 2 mM MgCl₂. The tubes were incubated for 30 min at 25° to allow hydrolysis of the ATP which had been added with the actin as a constituent of Buffer F. The tubes were centrifuged at 100,000 x g for 3.5 hours at 0° to pellet the acto-HMM or acto-S-1 complexes, and the supernatant solutions were assayed for ATPase activity in the presence of 0.5 mM KCl/2 mM EDTA. The amount of actin-bound HMM or S-1 in the pellet was calculated from the decrease in soluble KATPase compared to samples of HMM and S-1 incubated and centrifuged in the absence of actin. The molar ratios were calculated assuming molecular weights of 350,000 for HMM (175,000 per ATPase head), 190,000 for S-1, and 45,000 for Acanthamoeba and muscle actin. The results in Table III show that Acanthamoeba actin, like muscle actin, binds 1 mol of S-1 or 1 mol of HMM heads/mol of actin monomer.

Kinetics of Activation of MgATPase of HMM and S-1—As stated above, the ratio of specific activities of Acanthamoeba and muscle actin as activators of HMM MgATPase was always found to be close to ½. Although the individual ATPase values for the two actins were not the same for S-1 as for HMM and varied slightly from one preparation to another, their ratio was remarkably constant and was not affected by assay conditions that significantly altered the MgATPase of acto-HMM, i.e. increased ionic strength (Table IV). Between 0 and 40 mM KCl the specific activities of Acanthamoeba and muscle actin decreased almost by a factor of 10, yet the ratio of their specific activities remained essentially constant.

To determine the origin of this difference in specific activity, a more extensive characterization of the kinetics of the activation of the MgATPase of HMM and S-1 by Acanthamoeba actin was essential. For muscle actin this activation exhibits a hyperbolic dependence on actin concentration; i.e. a plot of the reciprocal of the increment in ATPase activity versus the reciprocal of the actin concentration is linear (30, 31). The two kinetic parameters Vₘₐₓ (the extrapolated ATPase activity at infinite actin) and Kₚₐₜ (the concentration of actin required to give half-maximal activation) can be derived from such a double reciprocal plot; the y intercept is 1/Vₘₐₓ and the x intercept is -1/Kₚₐₜ. These intercepts are most accurately determined when actin concentration is varied over a 10-fold range with Kₚₜ near the middle of this range. Only at low ionic strength is Kₚₜ sufficiently small that these ideal conditions can be realized at experimentally attainable concentrations of Acanthamoeba and muscle actin.

Double reciprocal plots were obtained for the activation of the MgATPase of HMM (Fig. 6) and S-1 (Fig. 7) by purified Acanthamoeba actin. Corresponding plots of muscle actin (with the same HMM and S-1 on the same day under identical conditions) are shown for comparison in each case. The plots for Acanthamoeba actin, like those for muscle actin, are linear. In each figure the lines for Acanthamoeba and muscle actin intersect the y axis at a common point; i.e. they have the same Vₘₐₓ (within experimental error of about 10%). However, in each figure the lines for the two actins deviate in x intercept by a

![Graph](image-url)
Agreed with the values predicted for mixtures of two actins of concentrations, and the simplifying assumption that most of NY+, inhibition does not approach 100% at infinite actin concentration.

The kinetic data just described show a factor of about 3; i.e. $K_{\text{app}}$ for Acanthamoeba actin is 3 times that of muscle actin. The values of $V_{\text{max}}$ and $K_{\text{app}}$ derived from Figs. 6 and 7 (and listed in Table III) are typical of the results for four separate Acanthamoeba actin preparations. Clearly, the difference in specific activity between Acanthamoeba and muscle actin is due to their 3-fold difference in $K_{\text{app}}$. The observation that S-1 has a higher $V_{\text{max}}$ and higher $K_{\text{app}}$ than HMM for both actins was also reproducible, but its meaning is not known.

**Effect of Acanthamoeba Actin Fractions on Activation of MgATPase of HMM and S-1 by Muscle Actin and Purified Acanthamoeba Actin**—The kinetic data just described show a significant functional difference between Acanthamoeba and muscle actin. Despite its high degree of purity by gel electrophoresis and the reproducibility of its specific activity from one preparation to the next, we wished to test directly the possibility that purified Acanthamoeba actin might contain an inhibitor which accounted for its lower specific activity. The possibility of a direct inhibitor of the interaction of F-actin and myosin was tested by measuring the effect of Acanthamoeba actin fractions on the MgATPase of muscle acto-S-1 (Table V, Part A) and on the MgATPase of Acanthamoeba acto-HMM (Table V, Part B). In all cases the observed ATPase values agreed with the values predicted for mixtures of two actins of differing $K_{\text{app}}$; in no case was the presence of an inhibitor detected. Furthermore, similar experiments failed to detect such an inhibitor even in the fractions discarded in the last two steps of purification, where one might have expected to find it in excess.

**Actin Inhibition of KATPase of HMM**—An established but poorly understood property of muscle actin is its ability at very low concentrations to inhibit the KATPase activity of HMM. The MgATPase of HMM was varied between 0.25 and 1.0 $\mu$M heads to attain hydrolysis rates between 0.1 and 0.3 $\mu$M P$_i$/min. The ATPase activity of HMM (16.5 $\mu$M) in the absence of actin was 0.11 $\mu$M P$_i$/($\mu$M HMM head); this value was subtracted from the observed activities in the presence of actin.

**Fig. 6. Activation of the MgATPase of heavy meromyosin by Acanthamoeba and muscle actin. Double reciprocal plots are shown for activation as a function of actin concentration at 24° in 2.5 mM MgCl$_2$, 2.0 mM ATP, 2.4 mM imidazole chloride, pH 7.0.**

**Fig. 7. Activation of the MgATPase of subfragment 1 by Acanthamoeba and muscle actin. Double reciprocal plots are shown for activation as a function of actin concentration at 24° in 2.5 mM MgCl$_2$, 2.0 mM ATP, 2.4 mM imidazole chloride, pH 7.0.**

**DISCUSSION**

The first goal of these investigations has been realized. Acanthamoeba actin has been purified in almost 20% yield (on the conservative assumption that actin is the only 45,000-dalton protein in crude extracts) by mild procedures that do not require the addition of muscle proteins. This yield of actin is very much higher than those previously obtained for cytoplasmic actins, except for the recent report of Pollard (12). The introduction of chromatography on DEAE-cellulose may be particularly useful for the isolation of cytoplasmic actin when it accounts for only a small percentage of the total cell protein.

Because Abramowitz et al. (33) have suggested the presence of two kinds of cytoplasmic actin in human platelets, it is worthwhile to consider whether there may be other forms of actin in Acanthamoeba castellanii. The initial extract contains essentially all of the actin of the whole amebae according to gel electrophoresis. Reference to Table I shows that most of the loss of Acanthamoeba actin during its purification occurs at two steps, DEAE-cellulose chromatography and the subsequent polymerization and depolymerization. The loss of DEAE-cellulose is a consequence of discarding the leading and trailing portions of the single broad peak that contains all of the actin (Figs. 2 and 3), not to the separation of a second peak of actin. Loss at the next purification step results from discarding some actin that does not polymerize and some that does not depolymerize, as well as a sizable handling loss.
Thus, one need not postulate a second form of actin to account for losses of Acanthamoeba actin during its purification.

HMM and S-l at infinite actin concentrations and the stoichiometry of binding to HMM and S-l measured separately for the myosin fragment used, and $V_{\text{max}}$ is the velocity at infinite actin concentration.

The expected ATPase rate for a mixture of actins (a and b) is given by

$$V = V_{\text{max}}/(1 + (A/K_a + A/K_b)^{-1})$$

where $A$ is the actin concentration, $K_a$ and $K_b$ are the $K_{\text{app}}$ for actins a and b measured separately for the myosin fragment used, and $V_{\text{max}}$ is the velocity at infinite actin concentration.

### Table V

Effect of Acanthamoeba actin fractions on activation of MgATPase of S-1 and HMM by muscle actin and purified Acanthamoeba actin

| Experiment | Actin mixture | MgATPase |
|------------|---------------|----------|
|            | Observed      | Expected |
|            | $\mu$P/min    | ($\mu$S-1 or HMM head) |
| A. 1.      | Muscle, 5 $\mu$m | 5.44     | 4.42  |
| 2.         | Acanthamoeba DEAE, 5 $\mu$m | 0.09     | 4.39  |
| 3.         | Acanthamoeba, 1.25 $\mu$m + muscle, | 2.37     | 4.41  |
| 4.         | Acanthamoeba poly-depoly, 5 $\mu$m | 0.24     | 4.45  |
| 5.         | Acanthamoeba Sephadex, 5 $\mu$m | 2.03     | 4.93  |
| B. 1.      | Acanthamoeba Sephadex | 2.12     | 4.06  |
| 2.         | Acanthamoeba DEAE, 5 $\mu$m | 0.10     | 3.93  |
| 3.         | Acanthamoeba Sephadex, 5 $\mu$m | 0.27     | 4.93  |

* Acanthamoeba DEAE, poly-depoly (polymerized-depolymerized), and Sephadex refer to the fractions of increasingly purifying Acanthamoeba actin obtained during the isolation procedure (Table I). Acanthamoeba Sephadex actin is the purified material used for Tables II to IV and Figs. 5 to 8.

Although the $V_{\text{max}}$ for Acanthamoeba acto-HMM and acto-S-1 are the same, respectively, as for muscle acto-HMM and acto-S-1, the specific activity of Acanthamoeba actin is only one-third as great as for muscle actin because of a 3-fold difference in $K_{\text{app}}$. The reproducibility of this result, the electrophoretic purity of Acanthamoeba actin, and the data in Table V argue strongly against the possibility that this is due to a contaminating inhibitor. It is conceivable that purified Acanthamoeba actin might contain an inhibitor that is already saturated with actin and, therefore, unable to inhibit further when mixed with muscle actin, but this is much less likely to be true for the partially purified and discarded fractions of Acanthamoeba actin, which also do not inhibit the activity of added muscle actin or added purified Acanthamoeba actin.

The hypothesis that the 3-fold decrease in activity might be explained by the presence of two-thirds denatured actin is also untenable for several reasons. First, the conditions for polymerization and depolymerization and the viscosity of the polymerized form are the same for Acanthamoeba and muscle actin; denatured muscle actin often has altered polymerization properties. Second, the data show equimolar binding of nucleotide, HMM and S-1 to Acanthamoeba F-actin; thus each of the actin monomers is native in these respects. It seems probable that this would not be so for denatured actin. Third, the ability of Acanthamoeba actin to inhibit the KATPase of HMM is...
greater than would be expected if two-thirds of the actin were denatured. Finally, the ability of muscle actin to activate HMM and S-1 MgATPase is unaffected when it is subjected to DEAE-cellulose chromatography or any of the other steps used to purify Acanthamoeba actin. Similarly, recycling of purified Acanthamoeba actin through the purification procedure did not affect its activity.

Available data also argue against the possibility that Acanthamoeba actin is not polymerized under the assay conditions. The viscosity data show Acanthamoeba actin to be at least as highly polymerized as muscle actin at all concentrations. Furthermore, if Acanthamoeba actin depolymerized under the assay conditions of acto-HMM and acto-S-1, a linear double reciprocal plot would not be obtained, because at low actin concentrations depolymerization would be relatively favored and the concentrations of F-actin would be less than assumed.

Thus, we conclude that Acanthamoeba actin is one-third as effective as rabbit skeletal muscle actin as an activator of the MgATPase of HMM and S-1 because of a real difference in the interaction of the actin monomers with the myosin ATPase heads. Since the interaction of actin and myosin is central to their roles in muscle contraction and, by inference, to the roles of cytoplasmic actin and myosin in cell motility, this is an important functional difference.

Experiments with rabbit skeletal muscle tropomyosin provide further evidence that Acanthamoeba actin differs functionally from muscle actin (35). In the presence of excess Mg2+, tropomyosin strongly inhibits the MgATPase of muscle acto-HMM, but has no effect on Acanthamoeba acto-HMM. Preliminary experiments in this laboratory indicate that Acanthamoeba actin is unable to bind to tropomyosin under these conditions.

The only other difference thus far detected between Acanthamoeba and muscle actin is in their generally very similar amino acid compositions. In addition to small variations in five amino acids only Acanthamoeba actin contains ε-N-methyllysines, as was previously reported (3). The significance of this compositional difference and its relationship to the difference in actin activity is unknown. The studies reported in this paper are only the beginning of the characterization of the molecular properties of one cytoplasmic actin. Such studies must ultimately be extended to cytoplasmic myosin and other associated proteins if we are to unravel the complexities of cell motility.

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