ACTOMYOSIN CONTENT OF *PHYSARUM* PLASMODIA AND DETECTION OF IMMUNOLOGICAL CROSS-REACTIONS WITH MYOSINS FROM RELATED SPECIES

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ABSTRACT

The content of myosin in plasmodia of the myxomycete *Physarum polycephalum* was measured by an immunological technique, quantitative microcomplement (C′) fixation. Migrating plasmodia (starved after growth on rolled oats) contained 0.60 ± 0.08 (SD) mg myosin per g fresh plasmodia. Myosin comprised 0.77% ± 0.05 (SD) of the total plasmodial protein. When total plasmodial proteins were separated by electrophoresis on SDS-polyacrylamide gels, a large amount of protein appeared in a band comigrating with muscle actin. Densitometry performed after Coomassie blue staining indicated that as much as 15–25% of the total protein in the plasmodium could be actin. This gives an actin/myosin ratio by weight in the myxomycete plasmodium as high as 19–33, a very "actin-rich" actomyosin compared with rabbit skeletal muscle actomyosin with an actin/myosin ratio of 0.6. Starvation stimulates rapid migration and is correlated with a higher percent of both myosin and actin in the total protein of the plasmodium compared with normally growing cultures. Immunological cross-reaction of myosins from a variety of species was measured by C′ fixation using an antiserum produced against purified native myosin from *P. polycephalum*. Although myxomycete and vertebrate striated muscle myosins have very similar morphological and biochemical properties, and apparently possess similar binding properties to F-actin, only myosins from myxomycetes in the order *Physarales*, rather closely related to *P. polycephalum*, gave detectable cross-reactions. This finding suggests that many amino acid sequences in myosin have been variable during evolution.

Plasmodia of the slime mold *Physarum polycephalum* exhibit vigorous shuttle streaming of the protoplasm within endoplasmic channels; the ectoplasm remains stationary and forms the walls of the channels (21). The proteins actin and myosin have been detected in plasmodia by ultrastructural and biochemical methods. The interaction of these proteins is believed to produce the force required for protoplasmic streaming (for a review, see [27]).

Physarum actin has been isolated in pure form and has been shown to have properties quite

similar to those of vertebrate muscle actin with regard to molecular weight, polymerization to F-actin filaments, and interaction of the filaments with muscle myosin to form arrowhead complexes (1, 14, 16, 37). *Physarum* myosin has been purified by several investigators (2, 13, 17, 34, 35). Ultrastructural studies have shown that the size and shape of myosin monomers, each with a long rodlike tail and a globular head region, are very similar to those of skeletal muscle myosin (15). *Physarum* myosin forms arrowhead complexes of similar appearance with F-actin from both rabbit skeletal muscle and *Physarum* plasmodia (17, 34, 38).

The similar properties of proteins from such different sources as slime mold plasmodia and vertebrate muscle suggests that these actomyosins are members of a family of proteins related in an evolutionary manner. In particular, the appearance of arrowhead complexes in which *Physarum* actin and muscle myosin are used, and vice versa, implies that the binding sites interacting to produce this complex on both actin and myosin are conserved in evolution. Studies in which amino acid sequences of actin from another eucaryotic protist, *Acanthamoeba castellanii*, and of rabbit muscle actin are compared show that many amino acid sequences are shared in the two proteins (50). Comparison of tryptic peptides from *Physarum* actin and vertebrate muscle actin also demonstrates extensive similarity (19). The amino acid sequences of myosins from eucaryotic protists, however, have not been investigated. In this study we have examined the immunological cross-reaction of myosins from several myxomycetes and from other species including vertebrates, using a quantitative immunological technique, micro complement (C′) fixation (30), with an antiserum produced against purified *P. polycephalum* myosin. The extent of immunological cross-reaction can give an indication of the amount of change in amino acid sequence among these proteins related in an evolutionary manner.

To develop a functional model for protoplasmic streaming in *Physarum* in which the roles of the relevant molecules are correctly specified, the concentration of actomyosin in the plasmodium must be known. Accurate information has not been available until now because most calculations of *Physarum* actomyosin content have been based on yields of the purified proteins, necessarily involving loss of some material during isolation (2, 16). In this study we have used the C′ fixation technique as an accurate immunological measure of the amount of myosin in crude plasmodial homogenates of *Physarum* plasmodia. The actin content in plasmodia has been calculated by densitometric measurement of the proportion of the total plasmodial protein which migrates in the same position as muscle actin during electrophoresis on SDS-polyacrylamide gels. We have found the plasmodial myosin content to be low, but the actin content to be surprisingly high, making the actomyosin of *Physarum* plasmodia very actin-rich compared with muscle actomyosin. The myosin content that we have measured in the plasmodium is sufficient to generate the force, calculated by Kamiya (22), produced during isometric contraction of thin plasmodial strands of *Physarum*, assuming a mechanism of force production similar to that found in vertebrate muscle.

MATERIALS AND METHODS

Culture Methods

Plasmodia of the myxomycete *P. polycephalum* originating from an isolate obtained from Dr. H. P. Rusch, McArdle Laboratory, University of Wisconsin, Madison, Wisc., were cultured by several methods for this study. Stock cultures of microplasmodia were grown axenically in shake flasks as previously described (8, 39). Plasmodial surface cultures were grown, using a sterile technique, according to the method of Guttes and Guttes (11) in petri plates on filter paper supported by glass beads. These axenic plasmodial surface cultures were starved by taking plasmodia on filter paper grown for 24 h from the growth medium and placing them on beads in petri plates containing a starvation medium composed of inorganic salts and citrate buffer (10). The plasmodia migrate extensively after 24 h on the starvation medium and are somewhat reduced in size. For purification of *Physarum* myosin, larger surface cultures of plasmodia were grown in trays with old-fashioned rolled oats (39).

Plasmodia of *Physarum flavicomum*, *P. melleum* (obtained from Dr. H. C. Aldrich, University of Florida, Gainesville, Fla.), and two other isolates of *P. polycephalum* (I-Turtox, Turtox Products, General Biological Supply House, Inc., Chicago, Ill. and II-Iowa State) (obtained from Dr. O. R. Collins, University of California, Berkeley, Calif.), were grown on 2% agar with rolled oat flakes added occasionally. Plasmodia of *Didymium iridis* (obtained from Dr. N. S. Kerr, University of Minnesota, Minneapolis, Minn.) were grown on *Aerobacter aerogenes* spread on agar in petri plates (23). *Fuligo septica*, *Ceratiomyxa fruticulosa*, and a species of *Stemonitis* were collected as plasmodia just before

sporulation, from rotting stumps on the Haverford College campus and then frozen.

Preparation of Antigens

Plasmodia cultured on the semidefined growth medium and plasmodia growing on agar with bacteria were washed several times in a low salt buffer (50 mM KCl, 50 mM imidazole, and 0.1 mM dithiothreitol, pH 7.0) and collected by centrifugation at 900 *g* for 4 min before homogenization.

To obtain crude preparations of myosin from the various myxomycetes for measurement of concentration or cross-reaction by the quantitative C′ fixation technique, 1 vol of plasmodium was homogenized at 4°C with 1 vol. 1 M KCl, 1 vol 0.03 M Ethyleneglycolbis[β-aminoethyl·ether]*N*,*N*′-tetraacetic acid (EGTA), pH 8.2, and 0.15 vol of 20 mM dithiothreitol (16, 36). Several methods of homogenization were employed to ensure maximum extraction of myosin from the plasmodia into solution. Some plasmodia were homogenized with a tissue grinder, using a Teflon pestle and a glass vessel (Arthur H. Thomas Co., Philadelphia, Pa.), 2–10 strokes by hand. Other plasmodia were homogenized by a tissue disintegrator (Sorvall Omnimixer, Dupont Instruments, Sorvall Operations, Newtown, Conn.) run at 60% full current for 30 s in 3-s pulses, or by sonication for 2 s at 35 W (Sonifier Cell Disruptor model W185D, Heat Systems, Ultrasonics, Inc., Mt. Vernon, N.Y.). In one experiment, Mg-ATP was added to a final concentration of 3 mM just before homogenization, and the equivalent amount added again to the homogenate just before preparing the antigen dilutions for C′ fixation analysis.

After homogenization, the suspensions of disrupted plasmodia in homogenization medium were readjusted to pH 8.2 and continuously stirred for 1–2 h at 4°C. Microscope examinations were made to verify cell disruption, using a Carl Zeiss model GFL microscope at a magnification of 300 with phase-contrast optics. In some experiments these suspensions, referred to as crude homogenates, were diluted appropriately without further treatment for C′ fixation analysis of myosin. In other experiments the large particulate material in the crude homogenates including nuclei, mitochondria, and pigment vesicles were removed by centrifugation at 43,500 *g* for 30 min. The resulting clear solutions, referred to as crude clear supernates, were then diluted as appropriate for myosin analysis by C′ fixation. The plasmodial homogenates from other species of myxomycetes examined for cross-reaction against antiserum KR3 were all clarified in this manner, as were some of the preparations of *P. polycephalum* plasmodia used for analysis of myosin concentration.

Purified Physarum Myosin

Purified myosin from *P. polycephalum* was used as a standard in the C′ fixation assay to determine the antigen concentration required for maximum percent C′ fixation using antiserum KR3. Starting with plasmodia of *P. polycephalum* grown on rolled oat flakes in trays, the myosin was purified by gel filtration, using prior potassium iodide treatment to depolymerize contaminating actin (36).

Protein Concentration

The protein concentration in crude plasmodial homogenates, supernates, and column fractions collected during the purification of *Physarum* myosin were measured by the method of Lowry et al. (31), using bovine serum albumin as a standard.

C′ Fixation

The quantitative immunologic technique, microcomplement C′ fixation, has been previously described (25, 30, 49). In another paper (39), we have established that antiserum KR3 used in this study is directed against the myosin of *P. polycephalum*.

Each sample of plasmodial crude homogenate, crude supernate, or purified myosin to be examined for myosin content was diluted to produce a series of antigen dilutions. When 1 ml of each dilution in the series was analyzed by C′ fixation with a fixed amount of KR3 antiserum and guinea pig complement C′, a curve was produced with a peak percent C′ fixation occurring at that dilution in which the antigen-antibody ratio was favorable for the formation of the complex which fixed or removed C′ most effectively. The remaining C′ was assayed by its ability to lyse sensitized sheep erythrocytes.

SDS-Polyacrylamide Gel Electrophoresis

Plasmodia from oat tray surface cultures or washed microplasmodia from shake flasks were lyophilized. 50 mg of the powder was resuspended per milliliter of 8 M guanidine-HCl containing 2% β-mercaptoethanol, heated at 100°C for 3 min, and centrifuged for several minutes in a clinical centrifuge to sediment any insoluble material. The supernate was dialyzed against 0.064 M Tris-HCl, pH 6.8, to remove the guanidine-HCl. The heavy protein precipitate was dissolved by adding the following reagents to their respective final concentrations: 8 M urea, 2% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol, then resolubilized by boiling for 1 or 2 min, and run on gels composed of 12% polyacrylamide (28). Densitometry was performed on Coomassie blue-stained gels with a Canalco model K instrument (Canal Industrial Corp., Rockville, Md.). To calculate the proportion of actin and myosin in the total plasmodial protein, the areas under the densitometer tracing on the recorder paper corresponding to the actin, the myosin heavy chain and the total protein were cut out, weighed, and compared. Since the large actin band was not completely resolved in the tracing, a maximum value

for actin was estimated by measuring a standard curve drawn over the peak of the actin band and extending to the baseline, and a minimum value by measuring the smaller curve produced by drawing vertical parallel lines from the actin peak to the baseline (Fig. 4). Myosin heavy chain polypeptide was assumed to make up 84% of native myosin (36, 48). Protein from glycerinated rabbit muscle was solubilized and subjected to electrophoresis in the same manner to allow identification of the protein chains associated with the thick or thin filaments.

RESULTS

C' Fixation, Purified Physarum Myosin

When fractions from a gel filtration column for the KI purification of *Physarum* myosin were each diluted from 1/10 to 1/7290 and then assayed for percent C' fixation using the antiserum KR3, a series of curves were produced. For the curve corresponding to each column fraction, there was a particular dilution value for which the percent C' fixation was maximum. This dilution value in each curve may be used as an estimate of the relative amount of antigen in the column fractions.

In Fig. 1, the dilution series from several column fractions has been analyzed. Column fraction no. 93 must be diluted to about 1/1000 its original concentration in order to obtain maximum percent C' fixation. This dilution is more than that required by any other fraction. Hence, fraction 93 contains more antigen specific to the antiserum than any other column fraction.

In Fig. 2, the relative antigen concentration in each column fraction is compared with the protein concentration (absorbance at wavelength 280 nm) and the ATPase activity in each fraction. The relative antigen concentration was calculated as percent of fraction 93. The greatest antigen concentration corresponds with a protein peak which contains the maximum ATPase activity in the column fractions. This correlation is an indication that *Physarum* myosin is the antigen toward which our antiserum is directed.

We have found that the concentration of purified *Physarum* myosin giving the maximum percent C' fixation averages 0.215 μg myosin/ml. This value has been used in determining the concentration of myosin in samples which contain a variety of proteins in addition to myosin.

C' Fixation, Myosin Content of Plasmodia

To measure the myosin content of slightly starved plasmodia migrating from tray cultures after growth on rolled oats, we analyzed the complement fixation curves to find the protein concentration of crude homogenates and clear supernates at the percent C' fixation peak for each sample (Table I). Knowing that the antiserum is reacting specifically only with myosin (39), although there are many other proteins present, we know then that the concentration of myosin giving the maximum percent C' fixation for each curve must be 0.215 μg myosin/ml as determined with

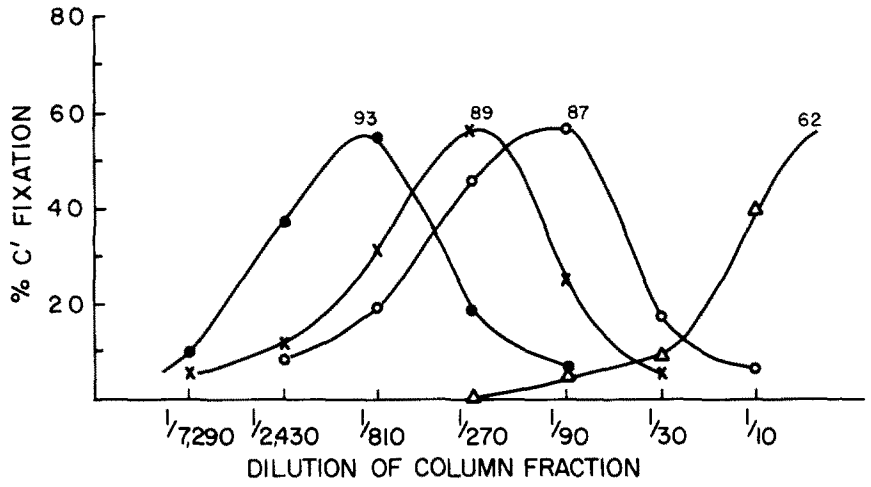

FIGURE 1 Fixation of C' in dilutions of fractions 93, 89, 87, and 62 from a gel filtration column for the purification of *Physarum* myosin using antiserum KR3. Fraction 93 required the most dilution in order to obtain maximum percent C' fixation and hence contains the highest concentration of antigen.

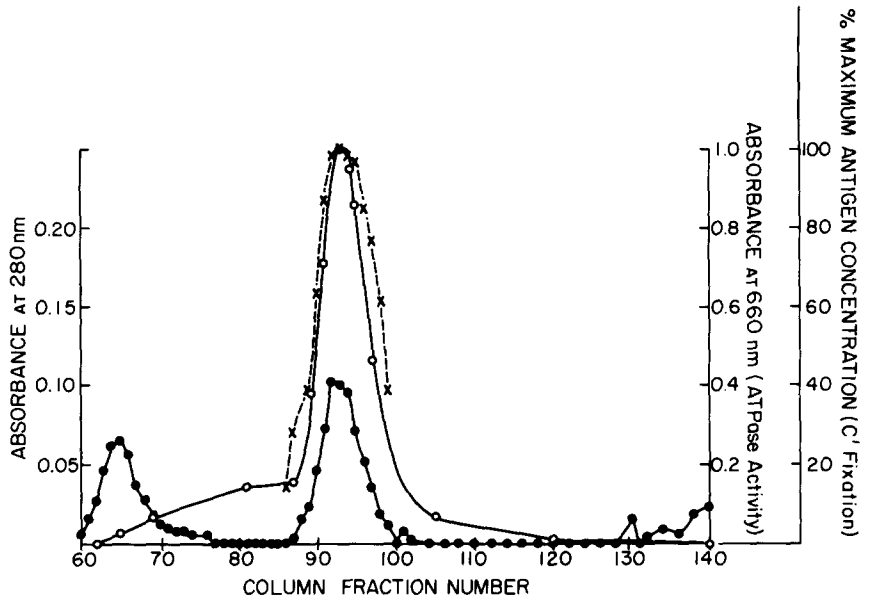

FIGURE 2 Comparison of protein concentration (●——●, absorbance at 280 nm), ATPase activity (×- - -×, colorimetric assay for inorganic phosphate measuring absorbance at 660 nm), and antigen concentration (O——O), percent of maximum antigen concentration as measured by C′ fixation) in fractions from a gel filtration column for the purification of *Physarum* myosin. Chromatographic procedures and assay conditions for determination of protein concentration and ATPase activity are identical to previously published methods (34).

TABLE I

Determination of Myosin by C′ Fixation (C′F) in Crude Homogenates and Supernates of Plasmodia of P. polycephalum Harvested during Migration from Tray Cultures after Growth on Rolled Oats

Exp no. and description of antigen	Protein concn of Ag at C′F peak	Myosin in total protein of Ag	Plasmodium (wet wt)	Ag	Factor by which Ag must be diluted to obtain max % C′F	Myosin concn as mg myosin per g plasmodium
	μg/ml	%	*g*	*ml*		
Crude homogenate obtained by:						
1. Teflon-glass homogenizer	27.0	0.80	3.65	10.95	1,033	0.67
2. Sonicator	30.0	0.72	3.45	10.35	960	0.62
3. Omnimixer	29.0	0.74	10.2	30.6	1,055	0.68
4. Teflon-glass homogenizer	29.5	0.73	5.0	15.75	705	0.48
5. Teflon-glass homogenizer	25.5	0.84	5.0	15.75	805	0.55
Mean ± SD		0.77 ± 0.05				0.60 ± 0.08
Crude supernate obtained by:						
1. Teflon-glass homogenizer	15.5	1.39	3.65	8.2	1,174	0.57
2. Sonicator	19.0	1.13	3.45	7.8	1,132	0.55
3. Omnimixer	15.5	1.39	10.2	23.0	1,413	0.69
Mean ± SD		1.30 ± 0.15				0.60 ± 0.08

purified *Physarum* myosin. From this information, the ratio of myosin to total protein in the plasmodium, or the percent myosin in the total protein, was found to average 0.77%. We have also calculated from the data on Table I that the myosin concentration is about 0.6 mg/g fresh plasmodium from these oat tray cultures. Since similar results were found with three different methods of homogenization, and since identical values of myosin content per gram of plasmodium were found in crude homogenates and clear supernates, and since the presence of Mg-ATP in the homogenate did not alter the amount of myosin detected, we conclude that the myosin is quantitatively extracted from the cytoplasm by our methods. Because our homogenization usually does not disrupt the nuclei, we have actually measured the amount of myosin in the cytoplasm rather than the total amount of myosin in all parts of the plasmodium. Recent reports (20, 29) indicate that myosin is also present in nuclei of the plasmodium. We have not yet made quantitative measurements of nuclear myosin.

In Table II we have recorded the results of similar calculations of myosin content for microplasmodia growing exponentially on semidefined medium in shake flasks. Migrating plasmodia have 1.5 times more myosin in the total protein than microplasmodia growing in shake flasks. The shake flask microplasmodia also have a significantly lower myosin concentration calculated as milligram myosin per gram fresh plasmodium than the actively migrating plasmodia. Since this latter comparison might be influenced by the dehydration of the oat tray plasmodia while migrating on a dry surface, we compared the myosin contents of actively growing and starving plasmodia in axenic culture on moist filter paper in petri plates (Table II). The migrating, starving plasmodia have 1.5 times more myosin in the total plasmodial protein and 1.7 times the concentration of myosin than the actively growing plasmodia in otherwise similar culture conditions. These results indicate that starvation does increase the proportion of myosin in the total protein, and the concentration of myosin in the cytoplasm of the plasmodium.

Actin and Myosin Content by Densitometric Analysis of SDS-Polyacrylamide Gel Electrophoresis Bands

When plasmodia of *Physarum polycephalum* are lyophilized, and the protein in the powder is solubilized, then separated into bands by electrophoresis in 12% polyacrylamide gels with SDS (Fig. 3), the percent of the total protein which is actin and myosin may be estimated by densitometric measurement of the relative staining of the bands (Fig. 4). The most intensely stained band on the gels after electrophoresis of plasmodial total protein corresponds to the molecular weight of actin. The myosin heavy chain band is very faint by comparison and is therefore subject to some error in measurement. By densitometry, the actin content in oat tray cultures is calculated to be 15.0–25.4% of the total protein, and in shake flask microplasmodia to be 9.6–16.2% of the total protein (Table III). These very large values for

TABLE II

Determination of Myosin by C' Fixation in Crude Homogenates of P. polycephalum Plasmodia from a Variety of Culture Conditions

Description of plasmodia and no. of Exp	Myosin in total protein of plasmodium	Myosin concn as mg myosin per g plasmodium (wet wt)
	%	
Migrating plasmodia grown on trays with rolled oats (five exp, from Table I)	0.77 ± 0.05*	0.60 ± 0.08*
Microplasmodia growing in shake flasks in semidefined medium (six exp)	0.52 ± 0.03*	0.14 ± 0.01*
Ratio, migrating plasmodia/shake flask microplasmodia	1.5	4.3
Growing plasmodia in petri plates on semidefined medium (one exp)	0.42	0.18
Starving plasmodia in petri plates on salts medium (one exp)	0.61	0.30
Ratio, starving/growing	1.5	1.7

* Mean ± SD.

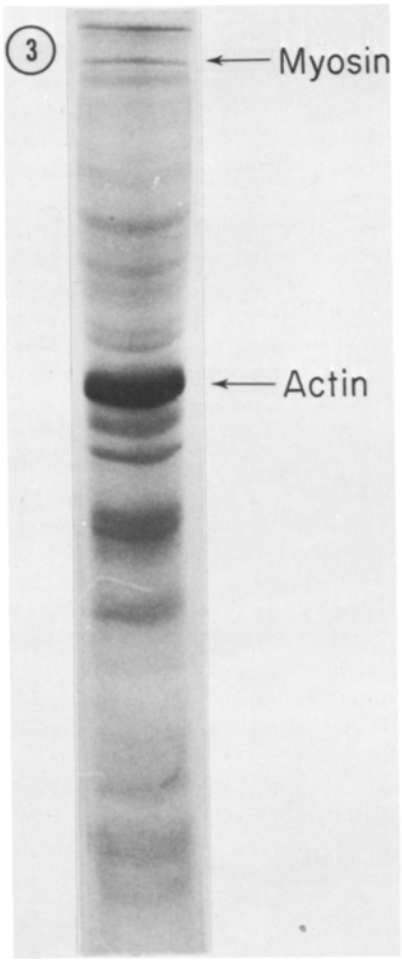

FIGURE 3 SDS-acrylamide gel (9%) of a total protein extract of *P. polycephalum* microplasmodia grown in shake flasks. The myosin band was identified by comparing its mobility with that of rabbit skeletal muscle myosin, the former being somewhat heavier than the latter (34). Slime mold actin has the same mobility as rabbit skeletal muscle actin.

actin must be considered as upper estimates of the plasmodial actin content since undetected contamination by an unrelated protein of molecular weight identical to that of actin could increase the band density in the actin region. For example, very conservative minimum values for actin may be obtained, 5.0% and 7.8% of the total protein in shake flask microplasmodia and oat tray cultures, respectively, by measuring only that portion of the actin peak which rises above the background in Fig. 4. The assumption is made here that all the proteins in the lyophilized plasmodial samples are solubilized by the preliminary boiling step in 8 M guanidine-HCl in 2% mercaptoethanol, followed by dialysis and resolubilization by boiling in 8 M urea, 2% SDS, and 2% mercaptoethanol before

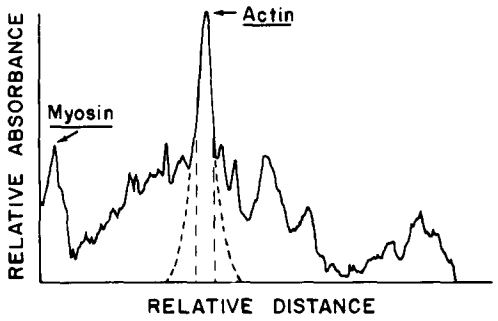

FIGURE 4 Densitometer scan of the SDS-acrylamide gel in Fig. 3. Both the actin and the myosin bands are imbedded in regions containing other material. The concentration of the myosin band was estimated by drawing a rectangle of the proper width as determined by direct inspection of the gel. Actin concentration was estimated by measuring the normal curve (maximum value) and the more narrow curve (minimum value) drawn in the region of the actin band as shown in the dashed lines on the figure.

TABLE III

Actin Content of P. polycephalum Plasmodia Calculated from Densitometric Analysis of Protein Bands after SDS-Polyacrylamide Gel Electrophoresis

Culture conditions	Actin as % of total protein*	
	Min value	Max value
Plasmodia migrating from oat tray cultures		
Exp 1	14.4	24.4
Exp 2	15.5	26.3
Mean	15.0	25.4
Microplasmodia from growing shake flask cultures		
Exp 3	10.3	17.4
Exp 4	8.9	15.0
Mean	9.6	16.2

* Max and min values for actin calculated from two curves drawn over the trace of the actin peak as indicated in Fig. 4.

electrophoresis. This assumption is not unreasonable since the average value in two experiments for myosin in the total protein of oat tray plasmodia by electrophoresis is 0.66%, which corresponds fairly well with the more accurate measurement done by C′ fixation (Table I), a completely unrelated technique. In doing these calculations, we have assumed that Coomassie blue does not stain actin or myosin preferentially as compared with other proteins in the polyacrylamide gels.

C′ Fixation, Species Comparison

When a sample containing the homologous antigen *P. polycephalum* myosin and a sample containing myosin from another species are compared by C′ fixation, the curve corresponding to dilutions of the homologous antigen exhibit a higher maximum percent C′ fixation than the curve corresponding to dilutions of the cross-reacting antigen. In other words, a vertical shift downward in the curve is seen when maximum percent C′ fixation values of the cross-reacting antigens are compared with that of the homologous antigen (30). In the case of a very weakly cross-reacting myosin, no fixation is detected at all unless the antiserum concentration is increased. The amount of cross-reaction among different myosins may be quantitatively compared by calculating the index of dissimilarity (I.D.), i.e., the factor by which the antiserum concentration must be raised in order that a particular myosin gives maximum C′ fixation equal to that given by the homologous *P. polycephalum* myosin (25, 46, 52).

In Fig. 5, the crude supernate from the homogenates of *P. polycephalum* (McArdle isolate), *P. flavicomum*, and *P. melleum* have been examined by C′ fixation. The curve for *P. flavicomum* does not appear to be significantly different from that of *P. polycephalum*. However, the homogenate from *P. melleum* gives a detectable reaction only when the antiserum concentration is raised considerably more than that required for maximum C′ fixation with *P. polycephalum*. The I.D.'s for these three species and for four other myxomycetes are given in Table IV.

The positions of these species within a taxonomic system for the class *Myxomycetes* may be seen in Table V (33). A comparison of the C′ fixation data with the taxonomic scheme permits the conclusion that myosin is often significantly different, but cross-reaction is still detectable, in plasmodia from species in the genera of the order *Physarales*. However, for myxomycetes outside the order *Physarales*, the myosin molecule no longer cross-reacts with our antiserum KR3. No cross-reactions have yet been detected for myosins from organisms outside the class *Myxomycetes* (Table IV). Among the myosins examined have been those from the skeletal muscle of two vertebrates, chicken and rabbit, and that from the primitive colonial protist, *Dictyostelium discoideum*. Although the latter organism, a member of the *Acrasiales*, is referred to as a cellular slime mold, its taxonomic affinity to the myxomycetes or plasmodial slime molds is very doubtful (6, 33).

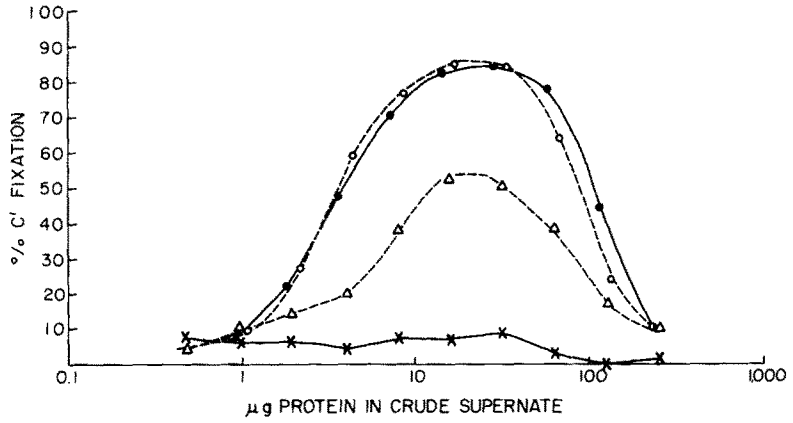

FIGURE 5 C′ fixation with dilutions of crude supernates as antigens from plasmodia of (●——●) *P. polycephalum* (McArdle isolate), (○- -○) *P. flavicomum*, and (×——×) *P. melleum* using antiserum KR3 at 1/3,500 dilution, and (Δ- -Δ) *P. melleum* with KR3 at 1/1,000 dilution.

TABLE IV

Index of Dissimilarity (Relative Antiserum Concn Required for 100% C' Fixation) of Myosins from Various Species with Antiserum to Purified Physarum polycephalum Myosin (KR3)

Species	Index of Dissimilarity (I.D.)
Myxomycetes	
Physarum polycephalum, McArdle isolate	1.0 ± 0.3*
Physarum polycephalum I, Iowa State isolate	0.9
Physarum polycephalum II, Turtox isolate	1.3
Physarum flavicomum	1.1
Fuligo septica	2.7
Physarum melleum	3.4
Didymium iridis	8.2
Stemonitis species	NR‡
Ceratiomyxa fruticulosa	NR‡
Others	
Dictyostelium discoideum myosin	NR‡
Chicken skeletal muscle myosin	NR‡
Rabbit skeletal muscle myosin	NR‡

* Mean of 20 exp with SD.

‡ No reaction within the limits of sensitivity of the technique, i.e., >8.2 I.D.

DISCUSSION

Myosin in Growing/Starving Plasmodia

In this study we have found that in otherwise equivalent culture conditions, the cytoplasmic myosin concentration increases about 1.7 times upon starvation, and the percent of myosin in the total protein increases by about the same amount (Table II). The amount of actin also increases upon starvation (Table III). White and Lascalles (51) have reported that although extensive degradation of total protein occurs during starvation in the plasmodium, myosin synthesis continues. An increase in the myosin concentration may contribute to more rapid migration. Upon starvation, plasmodia change their morphologic pattern and begin to migrate extensively, presumably increasing the likelihood of discovering a new food source. Plasmodia migrating from oat tray cultures are in a starved condition compared with microplasmodia growing in shake flasks, and can be expected to contain a greater amount of actomyosin.

Actin/Myosin Ratio

In Table VI, the actin and myosin contents of a number of different cell types are compared. In rabbit skeletal muscle the myosin concentration is about 120 times that of *Physarum* plasmodia grown on rolled oats. Myosin makes up 38% of the total protein in the muscle fibers compared with 0.77% in the plasmodia. In muscle, the actin concentration can be as high as 43 mg/g of muscle, or 23% of the total muscle protein. This gives an actin/myosin ratio by weight of 0.6, or a molar ratio of seven molecules of G-actin for every myosin monomer in rabbit skeletal muscle (44). The actin/myosin ratio in *Physarum* plasmodia has been difficult to obtain because an accurate measurement of the actin concentration has not been reported. We have estimated the plasmodial actin content by densitometric measurement of that band comigrating with muscle actin after the proteins in the whole plasmodium are subjected to SDS-polyacrylamide gel electrophoresis. By this method, actin comprises about 15–25% of the total protein of plasmodia migrating from oat tray

TABLE V

Taxonomic Classification of the Myxomycetes Examined in this Study (Abbreviated from Martin and Alexopoulos [33])

Class Myxomycetes
- Subclass Myxogastromycetidae
  - Order Physarales
    - Family Physaraceae
      - Genus *Physarum*
        - *P. polycephalum*
        - *P. melleum*
        - *P. flavicomum*
      - Genus *Fuligo*
        - *F. septica*
    - Family Didymiaceae
      - Genus *Didymium*
        - *D. iridis*
  - Order Stemonitales
    - Family Stemonitaceae
      - Genus *Stemonitis*
        - *S.* species
- Subclass Ceratiomyxomycetidae
  - Order Ceratiomyxales
    - Family Ceratiomyxaceae
      - Genus *Ceratiomyxa*
        - *C. fruticulosa*

TABLE VI

Comparison of Actin and Myosin Contents in Cells from a Variety of Organisms

Species and tissue	Protein	As % of total protein (wt)	Concn as mg per g fresh tissue	Actin/ myosin (wt)	Actin/ myosin (molar)*
Vertebrate skeletal muscle					
Rabbit psoas	Myosin	38‡	72‡	0.6§	7§
	Actin	23	43	or	
				0.56‖	
Vertebrate smooth muscle					
Bovine carotid artery	Myosin		3¶	3.0	34
	Actin		9¶		
Guinea pig *Taenia coli*				2.0**	20
				or	or
				3.45‖	39
Eucaryotic protists					
Acanthamoeba castellanii	Myosin	0.2‡‡		40	172
	Actin	10‡‡			
Dictyostelium discoideum	Myosin	0.5§§	0.35§§	14	157
	Actin	7	5§§		
Physarum polycephalum					
Shake flask microplasmodia	Myosin	0.52‖‖	0.14‖‖	18–31	202–347
	Actin	9.6–16.2¶¶	2.6–4.4		
Oat tray plasmodia	Myosin	0.77‖‖	0.60‖‖	19–33	213–370
	Actin	15.0–25.4¶¶	11.7–19.8		

* Ratio of myosin/actin molecular weights taken to be 11.2 except for *Acanthamoeba* where ratio is 4.3 (43, 44). References for data given on Table VI: ‡ (18), § (44), ‖ (48), ¶ (12), ** (47), ‡‡ (43), §§ (7), ‖‖ (Table II), ¶¶ (Table III). Other values on Table VI calculated from these data.

cultures, and 10–16% of the total protein in growing shake flask microplasmodia (Table III). As seen in Table II, the myosin measured by C′ fixation in plasmodia from these two culture conditions varies in the same proportion as the densitometric estimates of actin, so that the actin/myosin ratio by weight is about the same in the two types of plasmodia, equaling about 18–33. This gives an estimated molar actin/myosin ratio of 200–370 molecules of G-actin for every monomer of myosin in the plasmodium. This is a very high actin/myosin ratio compared with that in vertebrate skeletal muscle, or even in vertebrate smooth muscle (Table VI). However, for the soil amoeba *Acanthamoeba castellanii* (43) and for the cellular slime mold *Dictyostelium discoideum* (7), actin/myosin ratios are tentatively estimated to be 160–170 (molar), also much higher than the molar ratio of seven for rabbit skeletal muscle. A more accurate method of measuring actin content must be devised to verify these preliminary findings that the amoeboid-like eucaryotic protists possess actomyosin which is highly actin-rich compared with actomyosin from vertebrate skeletal muscle.

Myosin Filaments in Physarum Plasmodia

We have found that the concentration of myosin in migrating plasmodia grown on rolled oats is 0.6 mg/g plasmodium (Table I), or approx. 7.8×10^{14} monomers of myosin per milliliter. If such a plasmodium were fixed, embedded in plastic, and sectioned for subsequent electron microscope observation, a section 100 nm thick and 0.1 mm on the sides (about the size of an individual square on a 200-mesh grid) would have a volume of about 10^{-6} mm^3. If the myosin were evenly distributed in the plasmodium, each section would contain about 7.8×10^5 myosin monomers. However, this myosin might be polymerized into thick filaments containing approx. 250 molecules per filament as in striated muscle (44) or into smaller filaments containing about 10 times fewer monomers as in platelets (40). If all the myosin were polymerized into filaments within this size range, then from

3,000 to 30,000 myosin filaments would be present in each section of *Physarum* plasmodium. Although all these filaments might not be easily observed with the electron microscope due to poor filament orientation, one would still expect to see a considerable number of myosin-containing filaments in every thin section.

In fact, thick filaments have not been observed in the cytoplasm of *Physarum* plasmodia treated with a wide variety of fixatives unless the plasmodia are damaged by glycerination before fixation or subjected to slow fixation which results in convulsive contractions (3, 24, 27). This suggests that myosin is normally present in the plasmodium as monomers and oligomers too small to be detected with the electron microscope. Alternatively, the fixatives currently employed may inadequately preserve thick filaments which might occur normally in the plasmodium. In any case. it appears that the aggregation of myosin is quite transitory. Whether or not the equilibrium between monomer and polymer is an important physiological mechanism for protoplasmic streaming cannot be determined at present.

Comparison of Force Production in Physarum with Vertebrate Striated Muscle

Although the force for protoplasmic streaming in myxomycete plasmodia is believed to be generated by the interaction of actin and myosin in a manner similar to muscle contraction (26), direct evidence has been difficult to obtain. Can the efficiency of *Physarum* actomyosin in force production in vivo be compared with the efficiency of vertebrate actomyosin during contraction of a striated muscle? This comparison is not easily made since the actin and myosin filaments are arranged differently in the two contractile systems. The parallel thick and thin filaments in striated muscles slide together systematically in linearly arranged sarcomeres to apply force in one direction only (18). Maximum tension production averages 1,500 g/cm² of cross-sectional area in striated muscle, although values of 4,000 g/cm² have been recorded (5).

Kamiya (22) has measured tension production in living strands of *Physarum* plasmodia stretched so thin that the primary motion is linear contraction as in a muscle fiber. The force produced by these strands may be compared with that produced by vertebrate muscle. Kamiya has found that tension produced during isometric contraction in these thin plasmodial strands varies from 18 to 35 g/cm² of cross-sectional area, or about 100 times less than the force produced in isometric contraction of striated muscle. If contraction in the two systems is produced by the interaction of the S-1 subfragment of the myosin with the actin filaments, one would expect the force production per cross-sectional area to vary in proportion to the myosin concentration. As seen in Table VI, the myosin in rabbit skeletal muscle fibers is 120 times more concentrated than in *Physarum* plasmodia grown on rolled oats. It is evident that the ratio of force production per cm² of cross-sectional area during isometric contraction in striated muscle compared with thin plasmodial strands is similar to the ratio of their myosin concentrations. This supports the idea that the two systems possess a similar mechanism of force production. In comparing muscle contraction with plasmodial streaming, Hatano and Tazawa (16) believe, on the basis of less accurate values for *Physarum* actin and myosin concentrations, that the actomyosin (myosin B) rather than the myosin (myosin A) concentration varies in proportion to tension production. The results of the present investigation suggest that the myosin (myosin A) concentration is the critical factor in this comparison.

Myosin Evolution

The C′ fixation technique has been frequently used as a measure of the evolutionary relatedness of proteins (45, 46, 52). The variability in reaction of antiserum with homologous antigen and evolutionarily related antigens of similar conformation gives a good indication of change in amino acid sequence, but exact correlation is sometimes not possible (4, 32). For example, an amino acid substitution within a group of residues which includes the dominant antigenic determinant of the small protein cytochrome *c* often results in a greater change in immunologic reaction than an amino acid substitution elsewhere (41). This problem is diminished in large proteins containing numerous important antigenic determinants which all contribute importantly to the immunogenicity of the molecule. However, this effect may explain the lack of exact correlation in this study between taxonomic position of species within the family *Physaraceae*, e.g., *P. melleum* and *Fuligo septica*, and the cross-reaction measured by C′ fixation (Tables IV and V).

In this study, we have found that antibodies

produced in rabbits immunized with purified native myosin from *P. polycephalum* can cross-react only with plasmodial homogenates containing myosin from myxomycetes in the order *Physarales*, organisms closely related to *P. polycephalum*. We could detect no reaction of the antiserum within the limits of sensitivity of the C' fixation reaction against myosins from more distantly related myxomycetes, or against *Dictyostelium discoideum* myosin, chicken myosin, or rabbit myosin. This indicates that the amino acid sequences of the antigenic determinants against which the antibodies are directed are subject to considerable variation even within the class *Myxomycetes*. This finding contrasts with the ultrastructural and biochemical evidence suggesting that myosins from eucaryotic protists to vertebrates are very similar, with the exception of *Acanthamoeba* myosin (43). Thus, the myosins appear to be evolutionarily related proteins in which extensive amino acid substitutions have taken place with little change in overall conformation, similar to the globins (42), but unlike the actins which are much more conservative evolutionarily (9, 19, 50).

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