

Hybrids of *Physarum* Myosin Light Chains and Desensitized Scallop Myofibrils

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ABSTRACT The two light chains of *Physarum* myosin have been purified in a 1:1 ratio with a yield of 0.5–1 mg/100 g of plasmodium and a purity of 40–70%; the major contaminant is a 42,000-dalton protein. The 17,700 M_r *Physarum* myosin light chain (PhLC1) binds to scallop myofibrils, providing the regulatory light chains (ScRLC) have been removed. The 16,500 M_r light chain (PhLC2) does not bind to scallop myofibrils. The calcium control of scallop myosin ATPase is lost by the removal of one of the two ScRLC's and restored equally well by the binding of either PhLC1 or rabbit skeletal myosin light chains. When both ScRLC's are removed, replacement by two plasmodial light chains does not restore calcium control as platelet or scallop light chains do.

Purified plasmodial actomyosin does not bind calcium in 10^{-6} M free calcium, 1 mM $MgCl_2$. No tropomyosin was isolated from *Physarum* by standard methods. Because the *Physarum* myosin light chains can substitute only partially for light chains from myosin linked systems, because calcium does not bind to the actomyosin, and because tropomyosin is apparently absent, the regulation of plasmodial actomyosin by micromolar Ca^{++} may involve other mechanisms, possibly phosphorylation.

Plasmodia of the true slime molds exhibit a highly regulated form of cytoplasmic streaming in which the direction of endoplasmic flow reverses periodically at 30- to 100-s intervals. This rhythmicity is also apparent in the contraction and relaxation of stretched strands. These properties have been analyzed in works by Kamiya, Wohlfarth-Bottermann, and their colleagues (18–20, 43–47). It seems probable that the interaction of plasmodial actin and myosin is responsible for the contractility and the streaming. Both are present in the plasmodium and share several properties with muscle actin and myosin (1, 2, 12, 13, 32, 34). The microfilaments, identified as actin by their binding of heavy meromyosin (4), align in the direction of contractility in both suspended strands (16) and glycerinated threads, and reconstituted *Physarum* actomyosin threads contract with ATP (15). Increased numbers of fibrils correlate with the resumption of streaming in endoplasm or with increases in tension in stretched threads (8, 16).

It has been shown, by the microinjection of Ca-EGTA buffers, that calcium is required for the contraction of strands (44). Also, external calcium is necessary for the active fountain streaming of cytoplasmic droplets (11, 31, 36). The threshold level of free calcium necessary for strand contraction or fountain streaming is $\sim 1-2 \times 10^{-7}$ M (36, 44). Evidence that calcium

directly controls the rate of ATP splitting by affecting actin or myosin is, however, lacking. Data that seemed to show an actin-linked effect (21, 33, 35, 43) are unconvincing because of contamination by an ATP pyrophosphohydrolase that is activated by calcium (23), is present in actomyosin preparations, and (coupled with pyrophosphatases) leads to an apparent calcium-dependent ATPase (23). Purified myosin shows only some depression of actin-activated ATPase in 10^{-4} M calcium (32). Jacobson et al. (17) have discussed the lack of evidence for tropomyosin.

Given these uncertainties, it seemed valuable to examine the properties of the myosin in more detail. I previously reported that highly purified *Physarum* myosin had two light chains that remained associated with the heavy chain after gel filtration (32). The concept of these components as genuine light chains has been strongly questioned (17). However, antibodies to native myosin precipitate both the heavy and light chains from actomyosin in the presence of ATP (data of Eisenlohr and Kessler in 34), evidence that they are indeed part of the intact molecule. Nevertheless, the significance of these light chains can only be tested by a functional approach.

The desensitized myosin of scallop adductor muscles offers a unique test system for this purpose. The calcium control of

the myofibrils of these muscles is associated with the myosin (25). The intact myofibrils show a strong dependence on calcium for ATP hydrolysis and tension development under physiological conditions. The dependence on calcium for ATP hydrolysis and tension development is due to the presence of a myosin light chain which is easily washed away in 10 mM EDTA (26, 27, 42). Because of its effects, it is called a regulatory light chain (RLC) (26, 27, 40, 42). There are 2 mol of the RLC/mol of myosin. By varying the temperature of the EDTA treatment, one can remove different amounts of the RLC (5). When only 1 mol of RLC is removed by low temperature washes, the ATPase level of the myofibrils is no longer inhibited at 10^{-8} M calcium (26, 27). It has been shown that the removal of this first light chain is a negatively cooperative process (5). These myofibrils therefore contain a population of myosin molecules that largely possess a single regulatory light chain.

The inhibition of activity at low calcium can be restored if these desensitized myofibrils are incubated in 1 mM $MgCl_2$ with purified muscle myosin regulatory light chains, and restoration occurs in parallel with the rebinding of these heterologous light chains to the myofibrils (24). Every muscle myosin that has been tested has such a light chain (27, 38).

When 2 mol of RLC are removed from the scallop myofibrils, by EDTA treatment at 30–35°C (5), the activity of hybrids formed with heterologous light chains depends on the source of the light chain. Light chains from muscles with myosin-linked regulation restore calcium sensitivity whether they come from muscles with a calcium-sensitive myosin, like mollusc muscle, or from muscles regulated by phosphorylation of the myosin light chains, like gizzard muscle (15, 27, 38, 39, 41). Light chains from actin-regulated muscles such as rabbit skeletal or cardiac muscle or lobster fast muscle do not restore inhibition even though 2 mol of light chain are bound per mole of myosin (27, 38).

Because these properties have been established, the scallop myofibrils offer a unique way to test the function of light chains from nonmuscle myosins as well as from muscles. Here, I report the results of hybridizing *Physarum* myosin light chains with scallop myofibrils.

MATERIALS AND METHODS

Materials

Plasmodia of *Physarum polycephalum* were grown at 23°C in the dark on rolled oats; daily, 30–40 g were collected from the clean migrating sheets and frozen in sealed plastic in aliquots of 8–10 g. They were stored at –70°C for several weeks before use. The scallops *Aequipecten irradians* and *Placopecten magellanicus* were obtained from the Marine Biological Laboratory, Woods Hole, Mass. Human platelets were from the American Red Cross, Boston, Mass.

Procedures

The preparation and desensitization of washed scallop myofibrils were done as described by Kendrick-Jones et al. (26). Desensitization at 30°C (for *Placopecten*) and 35°C (for *Aequipecten*) were done as described by Chantler and Szent-Györgyi (5). ATPase activities were measured by following proton liberation at pH 7.5 in a pH stat (42). Scallop regulatory light chains were prepared as described by Chantler and Szent-Györgyi (5). Rabbit 5,5'-dithiobis-(2 nitrobenzoic acid) (DTNB) light chains and platelet light chains were prepared by the method of Kendrick-Jones et al. (26). Incubation with purified light chains was done in two ways. For all the early experiments, the light chains were mixed with an aliquot of desensitized myofibrils in 40 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 5 mM sodium phosphate, pH 7.0. The myofibrils were then gently rotated for 18 h at 5°C. The myofibrils were then washed in the same solution three times to remove unbound material. An aliquot was removed for gel electrophoresis and protein determination, and the pelleted myofibrils were dissolved

directly in 50 μ l of 4 M NaCl with added ATP and actin for the pH stat. In the second, later method, 0.1–0.2 ml of concentrated myofibrils containing 0.6 mg of *Placopecten* or 1 mg of *Aequipecten* myofibrils was mixed with the light chain preparation and brought to 0.5 ml in 0.4 M NaCl and 2.5 mM ATP, pH 7.0, together with actin, to yield 30% of the weight of the myofibril. The sample was then added to the pH stat containing 9.5 ml of 20 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EGTA. In this case, samples for gel electrophoresis were taken from the pH stat and were found to lack tropomyosin, which is solubilized under these conditions (Fig. 4).

Preparation of *Physarum* Myosin Light Chains

First, crude myosin and actomyosin were purified from frozen plasmodia by a procedure of Focant and Huriaux (9) slightly modified by J. Sellers and P. D. Chantler of Brandeis University (personal communications), and further adapted for plasmodia as follows.

(a) Frozen plasmodia (200–400 g) were fragmented with a wooden mallet, thawed in 6 vol of a wash solution containing: 40 mM NaCl, 20 mM $MgCl_2$, 10 mM sodium phosphate, pH 7.0, with 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) freshly diluted from stock stored at –20°C in isopropanol, and 5 mg of soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, N. J.) and 0.5 ml of aprotinin (Sigma Chemical Co., St. Louis, Mo.) per liter.

(b) Thawed mold was homogenized in the wash solution in a Sorvall omnimixer (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) with three 5-s bursts at low speed to minimize frothing. The pH was adjusted to 7.0 with 1 M Tris or 1 M NaOH.

(c) The homogenate was centrifuged at 16,000 g for 20 min and the supernate discarded.

(d) The precipitate was resuspended in fresh wash solution and steps 2 and 3 were repeated. A heavy layer of lipid was removed after each centrifugation.

(e) The precipitate was further homogenized with a motor-driven Teflon pestle in the extraction solution, used at 0.5–1 vol of the original mold: 0.4 M NaCl, 5 mM ATP, 0.1 mM PMSF, and aprotinin. Then EGTA was added to 10 mM and the pH was kept at 7.2 during EGTA addition, with 0.5 M disodium phosphate and 0.1 M NaOH as necessary. The homogenate was stirred for 10 min in ice.

(f) The extract was centrifuged at 40,000 g for 30 min. The precipitate was discarded and the clear supernate was carefully decanted and filtered through gauze to remove remaining lipid.

(g) To achieve a major separation of myosin from actomyosin, the supernate was made 5 mM in ATP and 20 mM in $MgSO_4$. Using saturated, neutralized ammonium sulfate containing 0.1 mM EGTA, proteins precipitating at 40% saturation were centrifuged out, comprising a crude actin-rich sample. Then the ammonium sulfate was increased to 65% saturation to yield a myosin-rich sample, in the precipitate.

(h) The precipitates were collected (20,000 g, 10 min), dissolved in 0.1 the original weight of mold of 0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 5 mM sodium phosphate, pH 7.0, and dialyzed overnight at 5°C against 200 vol of 20 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 5 mM phosphate, pH 7.0.

(i) The resulting white precipitates were collected at 40,000 g for 20 min, washed once or twice in the same volume of fresh dialysate, and used immediately for light chain preparation.

(j) The procedure for light chains was basically the method of Kendrick-Jones et al. (26) with minor but essential changes. The samples of crude myosin and actomyosin were dissolved at 5–10 mg/ml in 6 M guanidine-HCl, 5 mM dithiothreitol, 4 mM EDTA and 10 mM tris HCl and stirred at pH 7.9 for 18 h at 5°C.

(k) The solution was then diluted by adding an equal volume of cold water. Then 5 vol (per original solution) of ice-cold ethanol were added slowly to precipitate heavy chains and much of the actin. I obtained the most complete precipitation of actin by adding 1 mM of magnesium after the ethanol and stirring gently for 2–3 h.

(l) The precipitate was collected by centrifugation at 20,000 g for 15 min and the supernate carefully decanted.

(m) The ethanol was removed from the clear solution in a rotary evaporator at 30°C.

(n) The guanidine was removed by repeated dialysis against 10 mM tris HCl, pH 7.6, and 0.5 mM dithiothreitol, and further precipitates consisting of 42,000-dalton material were removed by centrifugation.

(o) The solution was then lyophilized, redissolved, and dialysed against 10 mM tris, pH 7.5, and finally lyophilized for final storage.

Gel Electrophoresis

To follow the purification procedure and for qualitative estimates of the rebinding of light chains, I used the microslab method of Matsudaira and Burgess (30), with 15% gels instead of gradient gels, and the buffer system of Laemmli (29). For stoichiometry, I used the alkaline urea system of Perrie and Perry (37)

as modified by Kendrick-Jones et al. (26). Because the scallop essential and regulatory light chains have been shown to be present in a 1:1 ratio, with 2 mol of each type of light chain per mole of myosin and equal dye binding (25), it is possible to estimate the stoichiometry of a hybrid light chain by measuring its density with respect to the essential light chain, which is not removed by the EDTA treatments used (only 2% of the essential light chain is lost; T. Walliman, Brandeis University, personal communication). Quantitative densitometry was done on Fast Green-stained gels after destaining in methanol-acetic acid, using a Gilford spectrophotometer with a gel scan attachment. Protein concentrations were estimated by the Folin-Lowry or biuret procedure.

Calcium Binding

Calcium binding was performed by the method of Chantler and Szent-Györgyi (5), but using a 10-min centrifugation of plasmodial myosin-enriched actomyosin (MEAM) (prepared as in reference 34), and was compared to calcium binding by scallop myofibrils. MEAM used for each test contained 7 mg/ml compared to 6.7 mg/ml myofibrils. Freshly prepared MEAM was provided by Dr. D. Kessler (Haverford College).

Tropomyosin

Evidence for the presence of tropomyosin was sought in crude actomyosin after one wash, using precipitation at pH 4.1 as a major criterion, and also with ethanol powders using the method of Cohen and Cohen (6) for the isolation of tropomyosin from platelets. Gels were examined for the presence of a band in the 30,000–38,000-dalton range.

Percent Calcium Sensitivity

Percent calcium sensitivity was calculated as the rate of ATP splitting in 10^{-4} M calcium minus the rate in EGTA, divided by the rate in calcium, times 100.

RESULTS

Purification

The separation of myosin from actin by ammonium sulfate fractionation under dissociating conditions was not so clean for plasmodial actomyosin as for muscle actomyosins. Nevertheless, considerable purification was achieved by this step. This can be seen by comparing the purity of the final light chains made from the myosin fraction, the 40–65% cut (Fig. 1, channel 2), with that of the 0–40% cut (Fig. 1, channel 3). The myosin fraction gives ~60–70% pure light chains, whereas the actin fraction yields light chains only ~40% pure. The yield was 0.5–1 mg of total light chains per 100 g of plasmodium. A persistent problem was degradation of the upper light chain, PhLC1, which occurred especially if the pH fell <6.8 during the preparation, but could be seen even (see splitting of PhLC1 on Fig. 1, channel 3) when the pH was kept >6.8. If the final light chains were stored at -20°C , as is usual for purified light chains, PhLC1 appeared as a smear on the gels or disappeared entirely after a week. PhLC2 was unaffected. The light chains were stable when stored lyophilized. Even during the overnight incubation of the redissolved light chains with myofibrils, however, some degradation occurred. The contaminating protease was not inhibited by a combination of PMSF, aprotinin, soybean trypsin inhibitor, and pepstatin. Therefore, incubation of light chains with myofibrils for short times in high salt at 22°C was tried; this resulted in better uptake of light chains without much degradation.

Hybrids with 10°C Desensitized Scallop Myofibrils

Fig. 1 shows actomyosin and two preparations of light chains. The plasmodial light chains (p1, p2) have mobilities close to that of the scallop light chains (which run as a single

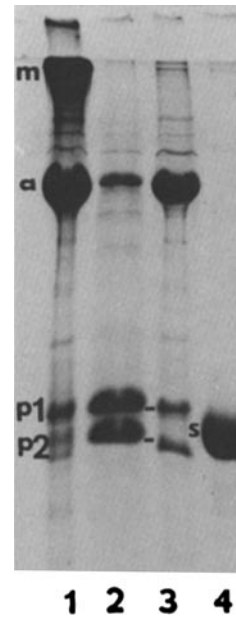


FIGURE 1 SDS microslab electrophoresis using the buffer system of Laemmli (29) and 15% acrylamide. Channels: 1, 0–65% ammonium sulfate cut from plasmodial actomyosin; 2, light chains prepared from the 40–65% cut; 3, light chains prepared from the 0–40% cut; and 4, regulatory light chain from scallop myosin. a: actin; p1: *Physarum* light chain 1; p2: *Physarum* light chain 2; s: scallop regulatory light chain; and m: myosin heavy chain.

band on SDS gels). However, they could be separated for qualitative purposes on 15% microslab gels, and Fig. 2 shows the rebinding of both *Physarum* light chain 1 and rabbit skeletal myosin DTNB light chains to desensitized scallop myofibrils. Note that the *Physarum* light chain (lanes 5 and 6) runs between scallop light chains (M_r , 17,000) and the DTNB light chain (M_r , 18,500). The fresh *Physarum* light chain preparation was as effective as the rabbit DTNB light chain in restoring inhibition of the scallop myofibril ATPase by EGTA, giving 81% calcium sensitivity as compared to 7% for the desensitized preparation (Table I). Similar results were obtained in two other experiments. Even the stored light chains had some effect (Table I), although on the SDS gels only a faint band corresponding to PhLC1 could be detected (channels 11 and 12, Fig. 2). Note that PhLC2 does not bind.

Alkaline urea gel electrophoresis gave an excellent separation of *Physarum* light chains from the myofibrillar components (Fig. 3) and made it possible to quantify the light chain binding to myofibrils by densitometry of stained gels (Table I). *Physarum* light chain was identified on these gels because it alone bound to the myofibrils (Fig. 3). The stoichiometry of the two light chains was 1:1. There appeared to be some degradation of the *Physarum* light chain during the binding experiment as indicated by a shoulder on the scan of the gel, appearing just below the main peak. This is only faintly visible on the gels. The lability of the preparations, the overnight incubation at 5°C and the incubation at 37°C of the samples before the urea gel run may all contribute to this.

Hybrids with 35°C Desensitized Scallop Myofibrils

I was not able to bind 2 mol of plasmodial light chains to the fully desensitized myofibrils at 5°C , even with two incu-

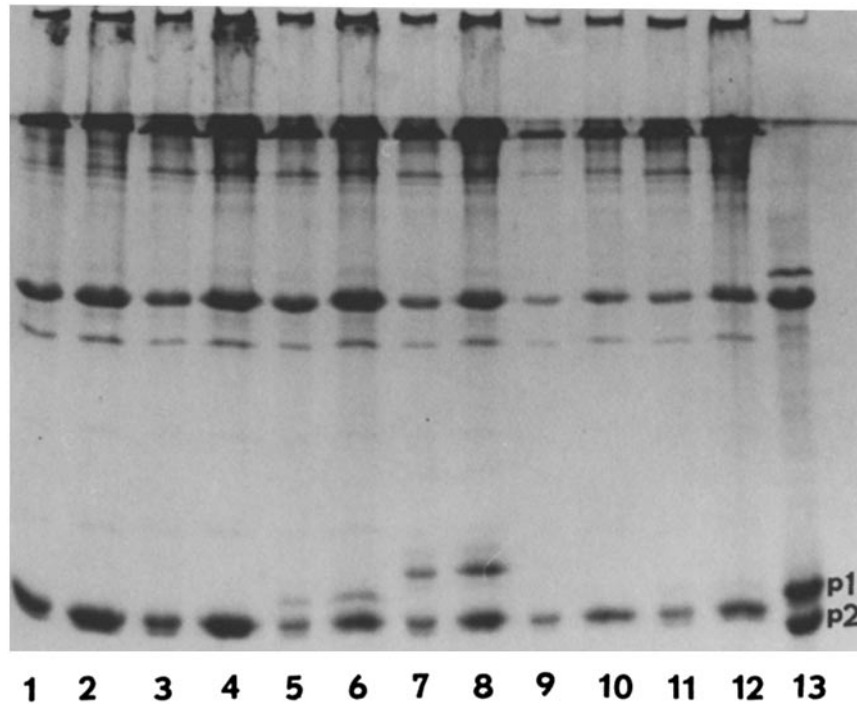


FIGURE 2 SDS microslab gel to show the binding of light chains to 10°C desensitized *Aequipecten* myofibrils. Channels: 1 and 2, intact scallop myofibrils; 3 and 4, desensitized myofibrils (note that the essential and regulatory light chains are not separated in *Aequipecten*); 5 and 6, desensitized myofibrils incubated with the plasmoidal light chain preparation demonstrated in channel 13; 7 and 8, desensitized myofibrils incubated with rabbit skeletal myosin DTNB light chains; 9 and 10, desensitized myofibrils incubated with purified scallop regulatory light chains; and 11 and 12, incubation with a sample of plasmoidal light chains which had been stored frozen at -20°C for 2 wk. A faint band can be detected at the same position as the strong band in 5 and 6; 13, preparation of plasmoidal light chains estimated as 60% pure.

TABLE I
Resensitization of 10°C Desensitized *Aequipecten* Myofibrils
by Light Chain Preparations

| Sample | ATP split/min/ mg | | Sensi- tivity % | RLC/myosin |
|--|----------------------|----------------------------|-----------------------|----------------|
| | 0.1 mM EGTA | 0.1 mM Ca ⁺⁺ | | |
| | μmol | | | |
| Control MF | 0.027 | 0.314 | 91.4 | 1.9 |
| Desens MF | 0.235 | 0.252 | 6.7 | 0.89 |
| Desens MF + fresh PhLC | 0.043 | 0.224 | 80.8 | 1.2* |
| Desens MF + stored PhLC | 0.173 | 0.271 | 36.1 | Diffuse band |
| Desens MF + rabbit DINBLC | 0.067 | 0.308 | 78.2 | ~2 (microslab) |
| Desens MF + <i>Ae-</i> <i>quipectin</i> RLC | 0.027 | 0.379 | 92.9 | 1.8 |

* The ratio of RLC to myosin in the hybrids is the sum of the remaining scallop light chain (0.89) plus the increment due to the amount of hybrid light chain bound, assuming equal dye binding capacity. The total in the case of *Physarum* does not include an additional fragment migrating below the main peak and not separable from the tropomyosin peak in this experiment.

bations and excess light chain; but, in several experiments more than 1 mol/mol myosin bound (Table II). The effect of plasmoidal light chains was not the same as that of either scallop or rabbit DTNB light chains. The scallop light chains, at a ratio to myosin of 1.3:1 mol, produced striking inhibition of the rate in EGTA and activation in 10^{-4} M calcium as compared to the desensitized myofibrils. Rabbit light chains in-

hibited the activity in EGTA and in calcium. Plasmoidal light chains had an activating effect in the presence of EGTA or calcium. A small degree of apparent sensitivity (27%) resulted because the activation in this experiment was greater in calcium, but there was no inhibition in EGTA.

To decide more certainly whether plasmoidal light chains were incapable of restoring inhibition in EGTA, it was necessary to obtain more complete stoichiometry. Incubations at 5°C for 12–18 h produced stoichiometries of only ~1–1.2, and some proteolysis of the plasmoidal light chains was generally seen. Therefore a brief incubation at 22°C in high salt in the presence of ATP was tried, the conditions used for the pH stat preparations. As shown in Table III (see also Fig. 4), this resulted in 2 mol of light chain bound, if the shoulder on the main peak is included, and even if the shoulder is not included, 1.48 mol of PhLC1/mol scallop myosin bound, fully enough to show inhibitory effects. Two different preparations were used and the results compared with those of scallop and human platelet light chains run in the same experiment. It is clear that plasmoidal light chains have a different effect than either scallop or platelet light chains. Both of these caused inhibition of the desensitized rate in the presence of EGTA, whereas plasmoidal light chains caused activation in both EGTA and calcium.

Because this apparent activation could be due to residual ATPase activity, I prepared a new stock of light chains and measured the ATPase activity under the conditions used for the pH stat. Despite the 6 M guanidine and the 70% ethanol, the final preparation contained enough residual ATPase activity to account for the apparent activation of Table III. (Note that in Table III the entire LC preparation is assayed together

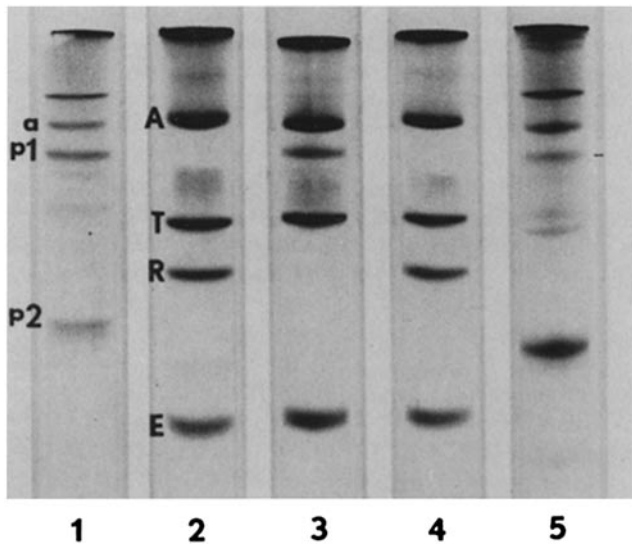


FIGURE 3 Alkaline urea gels used for stoichiometry. Channels: 1, plasmodial light chain preparation containing four major bands: these include a band migrating with actin, and a band below it marked p1, which is the regulatory light chain (see below); 2, scallop myofibrils containing A: actin; T: tropomyosin; R: regulatory light chain; E: essential or SH light chain; 3, desensitized scallop myofibrils incubated with a large excess of the preparation shown in channel 1, enough to give a threefold excess of PhLC1, and then washed to remove unbound components; 4, the same myofibrils incubated with a twofold excess of scallop regulatory light chains; 5, the supernate of the plasmodial light chain preparation after incubation with desensitized myofibrils. Note that there is specific reduction of the third, marked band which is bound to the myofibrils in channel 3. Myosin does not enter these gels, nor does paramyosin.

TABLE II

Effect of Added Heterologous Light Chains on 30 °C Desensitized *Placopecten* Myofibrils Using Overnight Incubation 5 °C in Low Salt

| Sample | ATP split/min/mg | | Sensitivity | RLC/myosin |
|---------------------------|------------------|-------------------------|-------------|----------------|
| | μmol | | | |
| | 0.1 mM EGTA | 0.1 mM Ca ⁺⁺ | | |
| Desens MF | 0.184 | 0.144 | (-27.7) | 0.012 |
| Desens MF + SC RLC | 0.114 | 0.540 | 78.9 | 1.26 |
| Desens MF + PhLC | 0.226 | 0.366 | 38.2 | 1.16 |
| Desens MF + rabbit DTNBLC | 0.116 | 0.113 | (-2.6) | ~2 (microslab) |

with the myofibrils.) The rate in the presence of EGTA was 3.35 μmol Pi/min/mg of light chain preparation that in 10⁻⁴ M calcium was 3.5. As ~250 μg total light chain preparation (~100 μg regulatory light chain) was used per 0.6 mg of *Placopecten* myofibrils, the rates seen in Table III can be ascribed to activity in the light chain preparation. Note that this residual ATPase did not show calcium sensitivity. The source of this activity is not clear.

Calcium Binding and Tropomyosin Extraction

Calcium binding of myosin-enriched actomyosin by the double-labeling method was found to be zero (slightly negative)

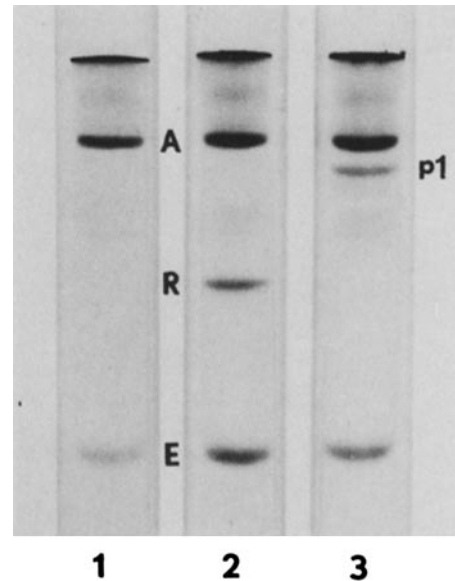


FIGURE 4 Alkaline urea gels of the hybridization shown in Table III. Here the myofibrils were recovered from the pH stat after the readings were made, so that tropomyosin has been solubilized (See Chantler and Szent-Györgyi [5]). Channels: 1, desensitized myofibrils; 2, after incubation with purified scallop regulatory light chains; 3, after incubation with plasmodial light chains. A, actin; E, essential light chain; R, regulatory light chain; P1, *Physarum* light chain one.

TABLE III

Effect of Light Chains on Fully Desensitized *Placopecten* Myofibrils Using a 5-min Incubation at 22 °C in 0.6 M NaCl, 5 mM ATP

| Sample | ATP split/min/mg | | Sensitivity | RLC/myosin |
|-------------------------|------------------|-------------------------|--------------|---|
| | μmol | | | |
| | 0.1 mM EGTA | 0.1 mM Ca ⁺⁺ | | |
| Desens MF | 0.321 | 0.281 | neg. (-12.5) | 0.048 |
| Desens MF + ScLC | 0.111 | 0.991 | 88.8 | 1.28 |
| Desens MF + PhLC (1st)* | 0.976 | 0.928 | neg. (-5.2) | 1.98 (+ shoulder) 1.48 (no shoulder) |
| Desens MF + PhLC (2nd)‡ | 0.688 | 0.703 | 2.1 | 1.84 (+ shoulder) 1.12 (no shoulder) |
| Desens MF + platelet LC | 0.044 | 0.378 | 88.4 | ~2 (microslab) |
| Desens MF | 0.258 | 0.273 | 5 | |
| Desens MF + ScLC | 0.046 | 1.564 | 97.1 | |

* PhLC (1st) is the better preparation with 75% in the main peak; 25% in the closely running shoulder after recovery from the pH stat.

‡ PhLC (2nd) contains 61% peak and 39% in shoulder.

in three tests where the conditions were 10⁻⁶ M free calcium and 1 mM magnesium chloride. Thus, no evidence for specific binding of calcium by the actomyosin was obtained. Tropomyosin could not be isolated from crude actomyosin, nor from the ethanol powders using the described method (6). Similar

results have been obtained by R. Fine (Boston University; personal communication).

DISCUSSION

My results show that two classes of light chains of different molecular weights can be purified from plasmodial actomyosin using essentially the same methods as for muscle actomyosins. When these light chains are electrophoresed on alkaline urea gels, the separation between the two classes is excellent and the stoichiometry (assuming equal fast green dye binding) is 1:1. On SDS gels, the molecular weights agree well with those reported previously when an antibody to native plasmodial myosin precipitated two light components estimated as 17,700 and 16,100 daltons along with the heavy chain (Eisenlohr and Kessler, in 34). Previous results reporting only one light chain (10) may be due to proteolysis of PhLC1, which has been observed to degrade very readily in the preparations studied here.

The conclusion that the bands on the gels indicate real light chain components has been challenged (17). However, plasmodial light chains, like other myosin light chains, are evidently stable in 6 M guanidine, soluble in 80% ethanol, and renature well after these treatments and after lyophilization, as shown by the fact that PhLC1 is as competent as rabbit DTNB light chain to resensitize 10°C desensitized scallop myofibrils by inhibiting the ATPase activity at low ($<10^{-8}$ M) calcium.

The susceptibility to degradation during the procedure used for urea gels (see Materials and Methods) probably resulted in some loss of light chain on the gel. Therefore in Table I, the stoichiometry must be regarded as a minimum estimate. Although I have not specifically shown that the plasmodial light chain binds to the myosin in the myofibril, another interpretation is most unlikely because PhLC1 does not bind to intact sensitive scallop myofibrils. Binding occurred whether tropomyosin was present in the myofibril or absent due to solubilization during the pH stat measurement. Binding occurred both at 5°C overnight or rapidly (5–10 min) at room temperature, but in either case it never exceeded 2 mol of light chain per mole of myosin, even if an excess of light chain was added or if the incubation was repeated with fresh light chain. *Physarum* light chain 1 can therefore be definitively classed as a member of the group of regulatory light chains. No clear-cut physiological role for PhLC2 is observed here, but Kessler et al. have recently reported (28) that PhLC2 shifts migration rate as does calmodulin in SDS in calcium vs. EGTA, suggesting calcium binding at mM calcium in the absence of $MgCl_2$.

Regulatory light chains from muscles can be further classified into two groups depending on whether they will restore calcium binding and sensitivity to scallop myofibrils from which both regulatory light chains per mole of myosin have been removed. As discussed in the Introduction, the generalization that has emerged is that light chains that can completely substitute for scallop light chains derive from systems which are themselves myosin linked (27, 38). *Physarum* light chains do not appear to do this, despite the presence of the residual ATPase from unknown source. Because this residual ATPase activity is not calcium sensitive, an inhibitory effect should be seen, and the difference between EGTA and calcium values should be at least 0.3 in Table III. The results show that the *Physarum* system does not operate like myosin-linked molluscan systems in this hybrid assay. On the other hand, the resensitization by platelet light chains is in agreement with data

showing regulation by phosphorylation of platelet myosin (3, 7).

Previous experiments showed that highly purified *Physarum* myosin or impure cytoplasmic supernates containing myosin could be activated by pure actin in the presence of EGTA (32, 35). These data were interpreted at that time to mean that a form of actin-linked control existed in *Physarum* that is, that the myosin itself was not inhibited from interacting with actin by removal of calcium and that inhibition must therefore be on the actin or related to the actin. Nevertheless, no tropomyosin component was detected in the plasmodial actomyosin, despite previous claims (22, 43). Results with smooth muscle myosin and platelet myosin show that a calcium control step can be exerted at the level of a kinase, which, by phosphorylating myosin, increases its activation by actin which is very low in the unphosphorylated state (3, 7, 15, 39, 41). A control of this type that works on plasmodial myosin could account for our previous data, because such activated myosins interact with actin in the absence of calcium. Perhaps the plasmodial light chains show some minor differences with respect to these systems, or perhaps the heavy chains of the myosin are involved. Three tests for specific binding of calcium by the myosin-enriched fraction were negative at a free-calcium level above that necessary for streaming. This, too, suggests that a mechanism involving phosphorylation is more likely to be involved than one requiring direct calcium binding.

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