A Calcium-Sensitive Preparation from *Physarum polycephalum*†

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ABSTRACT: Differential ultracentrifugation of an extract of the plasmodium of *Physarum polycephalum* yields a high-speed fraction which exhibits calcium-sensitive adenosine triphosphate activity at low ionic strength. The rate of inorganic phosphate production increased from 2- to 25-fold in different preparations when the calcium concentration was increased from about 10^{-8} to 10^{-5} M. Complement fixation using specific antibody to *Physarum* myosin showed the fraction to contain 3% myosin. By electron microscopy, actin-like microfilaments 50–150 nm long were present. Addition of pure rabbit F-actin or myosin to this fraction activated the ATPase measured in EGTA and so partially reversed the calcium sensitivity. If muscle myosin was added to the supernatant from which the fraction was centrifuged, a "hybrid complex" was obtained which included actin and additional protein from the plasmodium, and this hybrid was also calcium sensitive. Over 85% of the calcium-sensitive, magnesium-activated ATPase could be precipitated by sequential "hybrid" formation. The calcium sensitivity of the hybrid was maximal when formed at the lowest ratios of added myosin to *Physarum* proteins. It is concluded that the results do not allow a simple interpretation along the lines of either actin-linked or myosin-linked sensitivity. Evidence consistent with both a form of actin-linked and myosin-linked sensitivity is present in our results.

Calcium control of the interaction of actin and myosin has now been shown to be essentially universal in the muscles of animal organisms at all levels of the phylogenetic tree (Lehman, 1976). Recently, striking evidence for calcium effects on nonmuscle motility has been presented, especially in preparations of "native cytoplasm". That calcium has profound effects on cell motility has been realized for a long time, but the new demonstrations show that the thresholds are similar to those in muscle. Both Hatano (1970) and Taylor et al. (1973) showed, using respectively preparations of caffeine-treated *Physarum* cytoplasmic fragments and demembranated amoebae (*Chaos carolinensis*), that active cytoplasmic streaming required 5×10^{-7} to 10^{-6} M concentrations of calcium in the external medium. Both species have been shown to contain actin and myosin by biochemical criteria or by the specific morphological identification of characteristic thick filaments and thin filaments that will react with heavy meromyosin from rabbit muscle (Hatano and Oosawa, 1966; Hatano and Tazawa, 1968; Adelman and Taylor, 1969a,b; Wohlfarth-Bottermann, 1965; Komnick et al., 1973; Nachmias, 1964, 1974; Nachmias et al., 1970; Comly, 1973). Thus both cells contain typical forms of myosin as well as actin.

If the actomyosins present in these cells are causing cytoplasmic streaming, then the existence of this calcium requirement in these preparations implies that a calcium control of the actomyosins should be detectable. The work of Szent-Gyorgyi and colleagues (Lehman et al., 1973; Lehman, 1976) have shown that calcium can control muscle contractility by interacting with sites on either actin, myosin, or both. If these mechanisms are present in cells more generally one might expect that calcium control in nonmuscle (cytoplasmic) actomyosins would utilize a mechanism associated with the myosin component. This is an attractive idea because the myosin–actin ratio in motile cells is very low when compared with that in muscle, so that there is a large molar excess of actin (Kessler et al., 1976). However, Kato and Tonomura (1975) and Nachmias and Asch (1974) independently found some evidence that calcium sensitivity in *Physarum* appeared to be actin linked. Cohen et al. (1973) had previously found that the actomyosin isolated from human platelets was actin linked since inhibition of activity at 10^{-8} M calcium was fully reversed by added pure actin, while evidence from gel electrophoresis supported the idea that troponin-like proteins were found in platelets. Nevertheless, in both these cases, it is possible that a labile myosin-linked calcium sensitivity has been lost. The isolation of calcium-sensitive actomyosins generally requires rather lengthy procedures and dilution steps. In the dually regulated *Lethocerus* and locust muscles, the myosin sensitivity is lost by a single precipitation step (Lehman et al., 1974).

We had previously found that actomyosins made from *Physarum* are desensitized. We have turned to cruder preparations to look for calcium sensitivity and report here the properties of such a preparation of "cytoplasm" that is highly calcium sensitive. Inhibition of ATPase in low calcium is partially reversed by addition of high concentrations of pure actin, but other results cannot be explained by the simple assumption of an actin-linked system.

Experimental Procedure

Organism. Physarum polycephalum was grown as previously described (Nachmias and Asch, 1974).

High-Speed Fraction. Ten to fifty grams of fresh, migrating plasmodium was harvested, weighed, and immediately homogenized in an all-glass homogenizer in 1 volume of a low salt buffer containing 0.05 M KCl, 0.01 M imidazole, 5 mM dithiothreitol, pH 7.0, in ice. The homogenate was immediately centrifuged at 100 000*g* (average) for 60 min. Lipid was removed and the supernatant was then recentrifuged for an additional 120 min at 100 000*g* to yield a clear yellow pellet. It was used for experiments on the day of preparation.

Hybrids. Muscle myosin was added dropwise to ice-cold, rapidly stirred supernatants from the first (1 h) centrifugation, the reaction mixture was stirred 10 min, and the resultant

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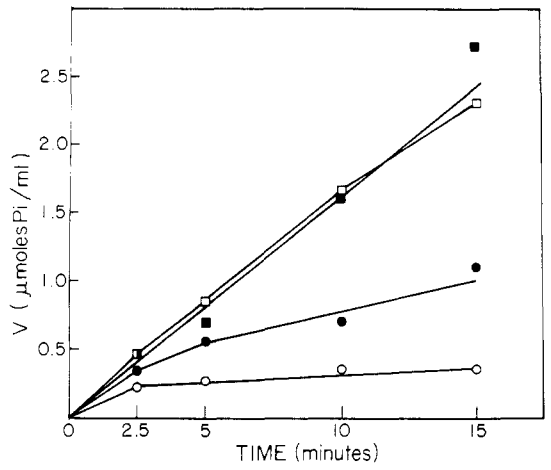

FIGURE 1: Rate of inorganic phosphate production by the pellet in EGTA (circles) or 10^{-5} M calcium (squares). The open symbols are the results when the fraction is tested alone; the closed symbols are the results when muscle myosin is added to the fraction and endogenous activity due to myosin alone is subtracted from the total. Final protein concentrations: myosin, 0.18 mg/ml; pellet, 0.376 mg/ml.

suspensions were collected at 15 000*g* for 30 min or were layered over 20% sucrose in buffer and centrifuged at 40 000*g* for 30 min to collect hybrid complexes. For controls, myosin was added to buffer and reprecipitated at pH 6.5.

Protein Preparations.[1] Vertebrate striated muscle myosin was prepared from rabbit leg muscle (Huxley, 1963) and stored in 0.25 M KCl, 0.05 M imidazole, 0.1 mM dithiothreitol, and 50% glycerol at −20 °C. Actin for reversal study was prepared from acetone powders of muscle and was shown, after two treatments with 0.6 M KCl, to be less than 2% contaminated with tropomyosin or troponin bands on heavily overloaded gels stained with Coomassie blue. For gel standards, actin was prepared by a single polymerization step. Actin was prepared from *Physarum* by the method of Hatano and Oosawa (1966).

Enzymatic Assays. Low ionic strength ATPase of either the high-speed fraction or the hybrids was determined in 0.07 M KCl, 0.01 M imidazole, 0.1 mM dithiothreitol, containing either 1 mM EGTA[2] or a Ca–EGTA buffer yielding a free calcium level of 10^{-5} M, or appropriate buffer mixtures to yield pCa values from 8 to 5. To calculate the free calcium concentration, we used the value of 10^6 M^{-1} for the association constant of the calcium–EGTA complex. The magnesium concentration in these reactions was 10 mM; the ATP concentration 2.5 mM. The reactions were run in a shaking water bath at 23 °C (100 strokes/min). The reaction was stopped with 8% (final) trichloroacetic acid. Inorganic phosphate was estimated in duplicate by the method of Taussky and Schorr (1952). For competition studies the actin was predialyzed against 0.04 M imidazole, 0.1 mM dithiothreitol, pH 7.0. Special care was taken to keep the ionic strength identical in control and experimental flasks. Results in which more than 60% of the substrate was used during the reaction were discarded.

Complement Fixation. The method of microcomplement fixation has been described (Levine, 1973; Kessler et al., 1976). The fixation of complement by the pellet in the presence of specific antibody is compared with the fixation due to *Physarum* myosin after purification to better than 90% by gel criterion (Nachmias, 1974). The proportion of myosin in the protein of the high-speed fraction is calculated by assuming that there is no interference of the complement fixation reaction by other proteins (Levine, 1973).

Sodium Dodecyl Sulfate Gel Electrophoresis. The sodium dodecyl sulfate buffer system was that of Laemmli (1970) using 10% acrylamide.

Protein determinations were by the Lowry method (Lowry et al., 1951) using bovine serum albumin (Sigma, fraction V) as standard. The anthrone reaction was by the method of Roe (1955) using glucose as standard. All chemicals were reagent grade. The phospholipase C was purchased from Sigma Chemical Company.

TABLE I: Hydrolysis of ATP by the High Speed Pellet Fraction in 10 mM Calcium, 10 mM Magnesium, or 10 mM EDTA.[a]

Expt	μmol of P_i min^{-1} (mg of pellet)$^{-1}$	μmol of P_i min^{-1} (mg of myosin)$^{-1}$ [b]
1. Calcium	0.035	1.16[c]
2. Calcium	0.024	0.80
3. Magnesium	0.130	4.29
4. EDTA	0.015	0.50

[a] All samples in 0.55 M KCl, 0.1 M Tris (pH 8.0), 22 °C. Means of duplicate determinations. [b] If pellet is 3% myosin. [c] Note: 0.98 μmol of P_i min^{-1} (mg of myosin)$^{-1}$ (mean of two calcium experiments) is equivalent to 7.84 mol of ATP s^{-1} (mol of myosin)$^{-1}$ if one assumes that the molecular weight of the *Physarum* myosin is 480 000.

Results

Properties of the Pellet Fraction. The protein content was 0.4 mg (±0.1 mg) of protein per original g of plasmodium, and approximately an equal amount of glucose equivalents of polysaccharide as determined by the anthrone test. The calcium sensitivity as defined by the ratio of ATP split in 10^{-4} M calcium as compared with that in 10^{-8} M calcium varied from 2 to 25. The rate of phosphate production was linear with time in calcium but in EGTA leveled off after an early rise (Figure 1, note open symbols only).

Increasing the known KCl concentration from 40 to 120 mM inhibited the ATPase activity in 10^{-4} M calcium salts by 50%. The fraction did not hydrolyze β-glycerophosphate, and endogenous phosphate production measured (20 min) was less than 1% of the phosphate produced from added ATP.

At low ionic strength, the hydrolysis of ATP at pCa 4 or 8 was unaffected by preincubation for 30 min at room temperature with phospholipase C. Phospholipase did not release inorganic phosphate when incubated alone with the pellet.

In 0.5 M KCl, the pellet fraction hydrolyzed ATP in the presence of 10 mM calcium, 10 mM magnesium, or 10 mM EDTA all at pH 8 at rates of 30, 130, and 15 nmol of P_i min^{-1} (mg of protein)$^{-1}$ (Table I). In 0.5 M KCl and in the presence of calcium, the pH optimum of the ATPase was 5.85 (two experiments) with a minimum at pH 7.5 and then a further increase to pH 9.

Evidence for Myosin and Actin in the Pellet Fraction. We examined the pellet for *myosin* by microcomplement fixation using previously prepared antibody specific for *Physarum* myosin (Kessler et al., 1976). By comparison of the peak position with the position of the maximum obtained with pure myosin, the pellet contains 3% myosin. Additional complement

[1] A brief report of early experiments with this preparation has appeared (Nachmias and Asch, 1974) and was presented at the Biophysical Society Meetings, February 1975, and at the Cold Spring Harbor Symposium on Motility, September 1975.

[2] Abbreviations used: ATP, adenosine triphosphate; EGTA, ethylene glycol bis(aminoethyl)tetraacetic acid; P_i, inorganic phosphate.

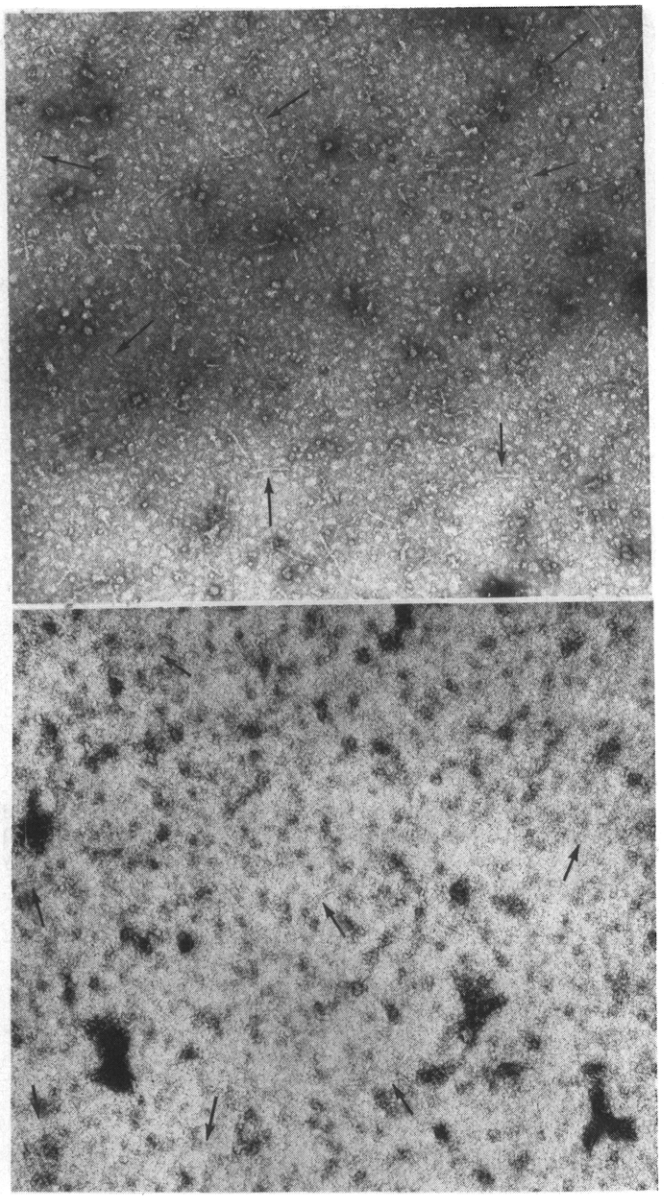

FIGURE 2: Negatively stained sample of the *supernatant* used to form the high-speed fraction (above) and the high-speed fraction itself (below) as examined by electron microscopy. The method has been described (Nachmias et al., 1970). The specimen was examined in a Siemens Elmiskop I at an accelerating voltage of 80 kV and initial magnification of 20 000×. Arrows point to short actin filaments, averaging 0.1 μm long (× 50 000).

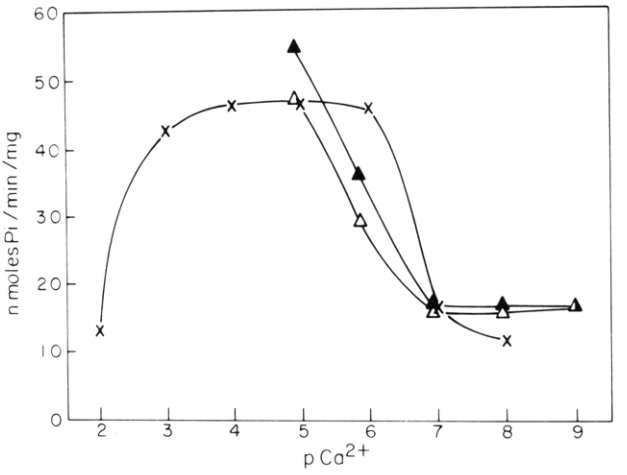

FIGURE 3: ATP hydrolysis as a function of calcium concentration. The calcium level was controlled with a calcium–EGTA buffer. For one experiment, the open triangles represent the 7.5-min values and the closed triangles, the 15-min values. For the second experiment, using a different preparation, the values shown (X) are the mean of two 10-min readings. Pellet concentration: 1 mg/ml. Conditions as described in text.

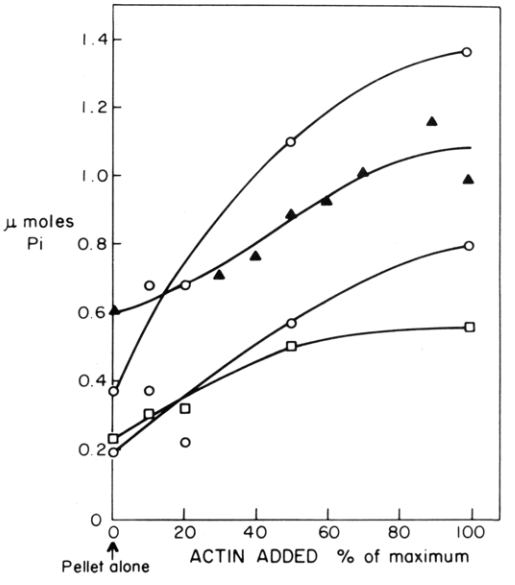

FIGURE 4: The ATPase activity of the high-speed fraction in EGTA as a function of added desensitized rabbit actin. Any small contribution to the activity from actin blanks has been subtracted. (Squares) Experiment 1, 7.5 min; (circles) experiment 2, 7.5 and 15 min; (triangles) experiment 3, 10-min values. The ratio of actin to pellet (mg/mg) at the highest added actin was 3.9 to 4.5 in the three experiments.

fixing activity was found in the supernatant fraction after an additional 3 h of centrifugation at 100 000*g*. A faint band was seen in the position of myosin when the pellet fraction was examined on sodium dodecyl sulfate gels.

The presence of *actin* in our preparation is shown by: (1) a main band on sodium dodecyl sulfate gels of the pellet in the position of muscle actin; (2) a band with the same mobility was precipitated out from solubilized pellet by the addition of muscle myosin at low ionic strength (Nachmias and Asch, 1974); and (3) electron micrographs of the high-speed fraction or of the supernatant from which it was prepared showed numbers of 50–150-nm long microfilaments (Figure 2).

Calcium Sensitivity and the Effect of Added Rabbit Actin. The ATPase activity of the pellet rose sharply between pCa 7 and 5 to a maximum. In two experiments, the slope of the rise differs but in both it occurs over 2 pCa units, with half activation between 0.5×10^{-6} and 1.0×10^{-6} M calcium salts. Figure 3 shows these effects.

If pure rabbit actin was mixed with the high-speed fraction before the assay, the ATPase activity in 1 mM EGTA was increased as a function of the added actin, leveling off at high actin (Figure 4). At these levels, however, the ATPase activity had not reached that of pCa 5. To see if complete reversal of the inhibitory effect would occur at infinite actin, double-reciprocal plotting (Eisenberg and Moos, 1968) seemed justified since in previous work (Nachmias, 1974) we had found that purified *Physarum* myosin behaved as if soluble in reacting with actin under comparable reaction conditions, and in the present preparation we appeared to be dealing with a soluble form of myosin. Figure 5 shows that linear results are obtained over a range of actin concentrations in the presence

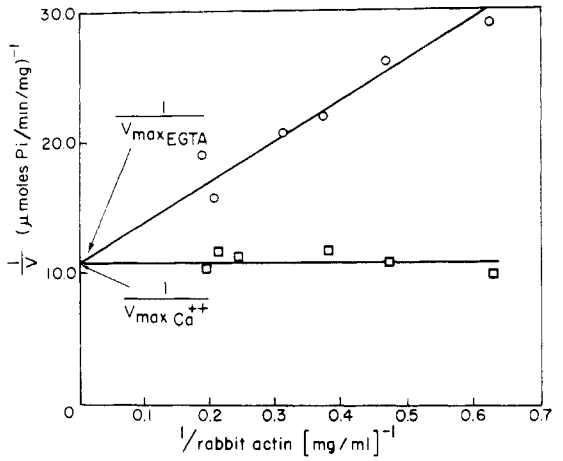

FIGURE 5: Double-reciprocal plot of the rate of inorganic phosphate production as a function of added actin. (Circles) In EGTA; (squares) in calcium. Pellet protein concentration 0.68 mg/ml. The extrapolation to V_{max} shown here is obtained by linear regression. For comparison with a weighted linear regression method according to Wilkinson, see text.

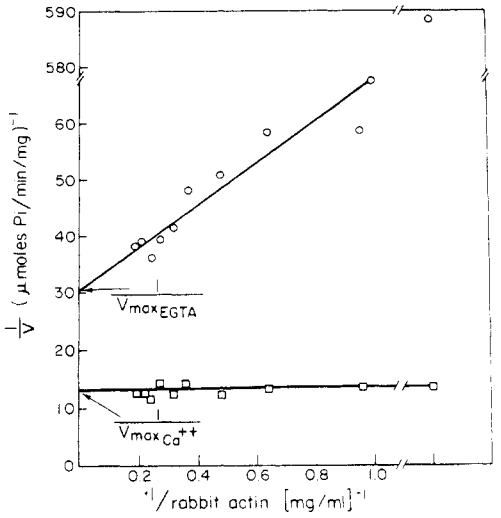

FIGURE 6: Double-reciprocal plot as in Figure 5, except that the values were obtained in the presence of 0.5 mM strophanthin K. (Circles) In EGTA; (squares) in calcium. Pellet final concentration is 1.3 mg/ml. Highest actin concentration is 5.2 mg/ml in Figures 5 and 6.

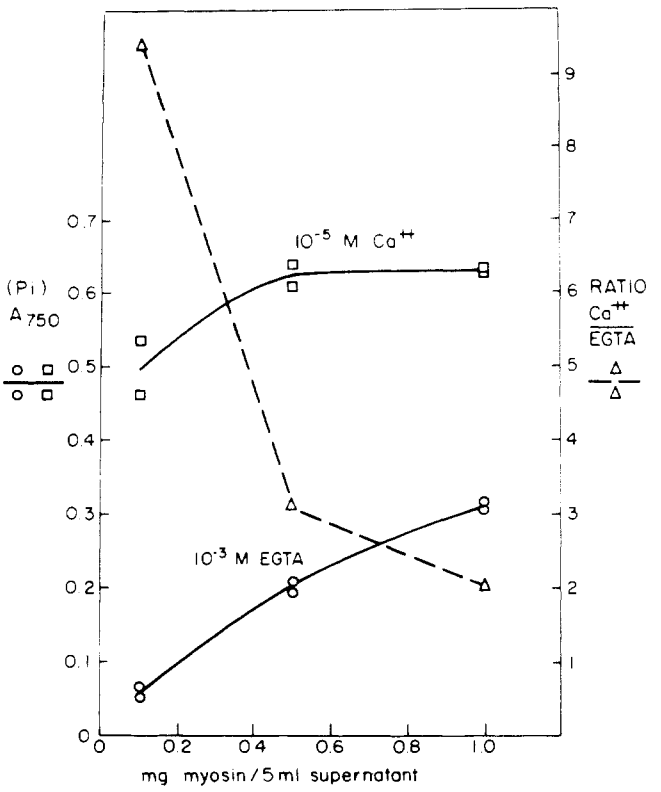

FIGURE 7: Calcium sensitivity of hybrid complexes made as described in the text at different ratios of myosin to *Physarum* supernatant. (Circles) In EGTA as shown; (squares) in calcium; (triangles) ratio of activity (10-min values).

TABLE II: Effect of Strophanthin K (0.5 mM) on Low Ionic Strength ATPase of High-Speed Fraction in EGTA (10^{-8} M Ca^{2+}) and Ca-EGTA Buffer (10^{-5} M Ca^{2+}).

	EGTA	Ca-EGTA
A Control	0.048	0.256
	0.056	0.244
B with strophanthin K	0.028	0.240
	0.032	0.236
% inhibition (mean)	44	5

[a] Numbers indicate μmol of P_i released in 10 min by 1 mg of high-speed fraction. Conditions as in text. [b] Percent inhibition (mean) is given by: (A − B)/A.

of EGTA, while little or no (as here) effect of added actin is observed in the presence of 10^{-4} M calcium. In several cases we found that complete reversal of the EGTA inhibition was indicated by the V_{max}.

If the reversal experiments were performed in the presence of strophanthin K, the original sensitivity was very high (due to depression of the EGTA activity) (Table II) and the reversal occurred, but was incomplete even at infinite actin (Figure 6). For both sets of experiments both straight linear regression and also a weighted version (Wilkinson, 1961) were used. The results by the latter method (not plotted) gave V_{max} of 11.44 (EGTA value, no strophanthin K) and 30.32 (EGTA, with strophanthin K). The results are therefore essentially identical with the V_{max} of the curves shown.

If bovine serum albumin was substituted for actin at a high (20:1) ratio to the protein present in the high-speed fraction, we found no activation but rather inhibition of the ATPase activity in both EGTA (56%) and in calcium (51%).

Results with Hybrids of Muscle Myosin. When rabbit striated muscle myosin was added sequentially in aliquots of 2 mg/10 ml supernatant from the first 1 h of centrifugation, the calcium-sensitive, magnesium-dependent ATPase was removed from the supernatant according to an exponential type of curve. After addition of 6 mg per 10 ml, more than 85% of the sequential ATPase was precipitated.

The calcium sensitivity of such hybrids made by a single addition of myosin was found to depend on the amount of myosin added to a fixed amount of supernatant, that is, on the ratio of the muscle myosin to the *Physarum* proteins in the final hybrid. As is shown in Figure 7, the highest sensitivity was obtained with the lowest ratio of added muscle myosin.

Figure 8 shows sodium dodecyl sulfate gels from one hybrid experiment; the band at 35 000 daltons is present in several experiments while in some a band of 55 000 daltons is also present (not shown here).

If muscle myosin is added directly to the pellet, it also causes a partial reversal of the EGTA inhibition. In Figure 1, com-

parison of open and closed symbols shows this. The activity of muscle myosin (which is low) is subtracted from the activity obtained with myosin plus pellet, and it is seen that the addition of the muscle myosin has a greater effect on the activity of the pellet in EGTA than in the calcium condition.

Discussion

Evidence for Actin and Myosin in the High-Speed Pellet Fraction. The results of the gels and the electron microscope examination of the pellet provide two independent proofs that actin is present in the pellet fraction. We also observed that actin was present in the supernatant from which the high-speed fraction was made when we made hybrids by coprecipitation with muscle myosin (Figure 8).

Conclusive evidence for the presence of myosin in the fraction comes from the complement fixation test and is supported indirectly by the enzymatic results. The complement fixation test gives more information than merely the presence of myosin. It indicates approximately 3% myosin in the fraction. This is of interest since this amount of *Physarum* myosin can account for all the high salt calcium activated ATPase. The specific activity of 35 nmol of P_i min^{-1} (mg of pellet)$^{-1}$ results in 1 μmol of P_i min^{-1} (mg of pure myosin)$^{-1}$ (cf. Table I). Purified plasmodial myosin yields specific activities of about 1 (Adelman and Taylor, 1969a,b; Nachmias, 1974). The complex pH–activity curve at high ionic strength is also similar to that of plasmodial myosin with an optimum at pH 5.9, a minimum at pH 7.5, and increasing activity in the alkaline range (Adelman and Taylor, 1969b); but magnesium-activated ATPase in 0.5 M KCl cannot be accounted for by myosin since purified plasmodial myosin is inhibited by magnesium (Adelman and Taylor, 1969b).

In summary, the evidence that the pellet contains myosin is conclusive, but other ATPases activated by magnesium are present.

Evidence in Favor of Some Form of Actin-Linked Regulation. The pellet had the characteristics of a calcium-sensitive actomyosin in several respects. Its activity was inhibited by increased KCl concentration; its half activation level was 0.5 to 1 $\times$ 10^{-6} M calcium, the range which controls the streaming effects described in the introduction; and the inhibition of ATPase at pCa 8 was partially reversed by pure actin. The hybrid complex made with muscle myosin at very low myosin/pellet ratios showed bands on gels in the region of conventional tropomyosins and troponins. However, a 55 000-dalton band also often coprecipitates (Kato and Tonomura, 1975; Nachmias, 1975) and suggests that the actin-linked effects may be unconventional. The subunit weight of 55 000 corresponds to a phosphohydrolase isolated by Kawamura and Nagano (1975) from *Physarum*.

The Reversal Gap and Myosin-Linked Sensitivity. Early in the actin reversal experiments we found that, when we had preparations of high (four or more) sensitivity, the reversal was incomplete even when the ratio of actin to total pellet protein was higher than that needed for complete reversal of pellets with the lower sensitivity levels (Nachmias and Asch, 1974). We had to exclude the possibility that muscle actin might be less effective than plasmodial actin in activating plasmodial myosin. Results at infinite actin should eliminate such differences. However, there were two problems with this approach: one, we could not convincingly demonstrate that plasmodial actin was more efficient than muscle actin at any given concentration. Second, the complete reversals at infinite actin were not obtained if we ran them in the presence of an inhibitor, strophanthin K, which inhibited the ATPase more strongly in EGTA than in calcium (Table II).

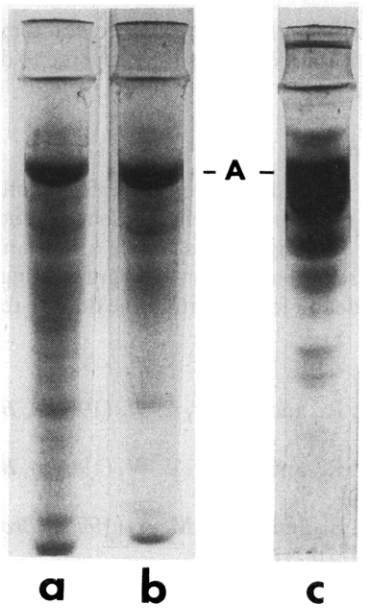

FIGURE 8: Sodium dodecyl sulfate gel electrophoresis of the hybrid complex formed by the addition of myosin at very low levels (0.1 mg of myosin/5 ml of supernatant). A marks the position of the actin band. (a) Directly precipitated calcium-sensitive hybrid; (b) calcium-sensitive hybrid after centrifuging through a step gradient of 20% sucrose; (c) actin from rabbit muscle prepared by a single ultracentrifugation.

Such a reversal gap, shown in Figure 6, could be due to another calcium-sensitive system unaffected by actin. For this reason we looked at the effects of phospholipase C, which Martonosi et al. (1968) found inhibited ATPase activity of muscle reticulum. This had no effect either on subsequent ATPase activity or on endogenous phosphate production. Part of the gap could be due to phosphohydrolase activity, which shows some calcium activation (Kawamura and Nagano, 1975).

The presence of the reversal gap caused us to look for some other evidence pointing to myosin-linked sensitivity. The data of Figure 7 are consistent with this, because the highest sensitivity of the hybrids is obtained with the lowest amounts of added myosin. This could occur if *Physarum* myosin, coprecipitating with the hybrid, were to represent a significant proportion of the myosin at the lower amounts, while at higher added muscle myosin the amount of sensitivity represents only the contribution from actin-linked factors. Additional data are given in Figure 1 (closed symbols) where added muscle myosin causes partial reversal of the calcium sensitivity of the pellet. Therefore, some of the actin is available for myosin activation.

In summary, competition studies of the pellet fraction yield evidence for both actin- and myosin-linked types of calcium sensitivity but the actin effects are atypical requiring very high levels of actin.

Mechanisms of regulation of cytoplasmic actomyosins have been proposed that do not seem to operate through calcium control, especially regulation by control of actin activation, either by a cofactor (Pollard and Korn, 1973) or via the phosphorylation of myosin light chains (Adelstein and Conti, 1975). These may in some cases be linked; a recent paper by Hartshorne and colleagues (Aksoy et al., 1976) shows that calcium can regulate the phosphorylation of gizzard actomyosin and that the actomyosin is desensitized when the phosphorylating system is removed. Now that we have evidence for

calcium sensitivity in the myxomycete actomyosin system, purification of components is the next step to determine how it is accomplished.

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