

# A 72,000-mol-wt Protein from Tomato Inhibits Rabbit Acto-S-1 ATPase Activity

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**ABSTRACT** Tomato activation inhibiting protein (AIP) is a molecule of an apparent molecular weight of 72,000 that co-purifies with tomato actin. In an assay system containing rabbit skeletal muscle F-actin and rabbit skeletal muscle myosin subfragment-1 (myosin S-1), tomato AIP dissociated the acto-S-1 complex in the absence of  $Mg^{+2}$ ATP and inhibited the ability of F-actin to activate the low ionic strength  $Mg^{+2}$ ATPase activity of myosin S-1. At a molar ratio of 5 actin to 1 AIP, a 50% inhibition of the actin-activated  $Mg^{+2}$ ATPase activity of myosin S-1 was observed. The inhibition can be reversed by raising the calcium ion concentration to  $1 \times 10^{-5}$  M. The AIP had no effect on the basal low ionic strength  $Mg^{+2}$ ATPase activity of myosin S-1 in the absence of actin. The protein did not bind directly to actin nor did it cause depolymerization or aggregation of F-actin but appeared, instead, to interact with the actin binding site on myosin S-1. Since AIP is a potent, reversible inhibitor of the rabbit acto-S-1 ATPase activity, it is postulated that it may be responsible for the low levels of actin activation exhibited by tomato F-actin fractions containing the AIP.

A group of proteins known as actin-associated proteins modulate the structural and biochemical role of actin in many cells (for a review see reference 7). Several of these proteins regulate the physical state of actin by controlling the polymerization, crosslinking, and overall length of actin in vitro.

In addition to modulating the assembly properties of actin, some actin-associated proteins appear to control the ability of F-actin to activate the low ionic strength  $Mg^{+2}$ -ATPase activity of myosin. Chicken gizzard smooth muscle filamin (4), *Dicystostelium discoideum* 120,000-dalton factor (3), and *Physarum* 36,000-dalton factor (10) are actin-binding proteins that inhibit the ability of F-actin to activate the low ionic strength  $Mg^{+2}$ -ATPase activity of myosin in vitro. This suggests that, in the presence of some actin-associated proteins, actin is removed from a biochemical role and is reserved for a structural function.

Tomato actin can activate the low ionic strength  $Mg^{+2}$ -ATPase activity of myosin subfragment-1 (myosin S-1) and of tomato myosin up to 20-fold (15). However, in some of the highly purified tomato actin fractions, a protein of 72,000 mol wt appears on overloaded SDS polyacrylamide gels of the tomato actin (14, 15). This protein is called tomato activation inhibiting protein (AIP) since the tomato actin that contained

traces of AIP gave disappointingly low levels of actin activation of the muscle myosin S-1 and of the tomato myosin (15).

In this paper, it is shown that the tomato AIP is a calcium-sensitive modulator of the in vitro actin activated low ionic strength  $Mg^{+2}$ -ATPase of rabbit myosin S-1 that appears to dissociate the acto-S-1 complex. The conclusions drawn here have strong implications for a similar role for AIP in the tomato contractile system.

## MATERIALS AND METHODS

**Purification of Tomato AIP:** 2 lb of tomato fruit was subjected to three, 5-s pulses at high speed in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT) containing 500 ml of extraction buffer consisting of 0.1 mM  $CaCl_2$ , 0.5 mM ATP, 0.75 mM 2-mercaptoethanol, and 3 mM imidazole, pH 8.0. The resulting slurry was diluted with an equal volume of cold extraction buffer and stirred for 1 h at 4°C. Insoluble debris was centrifuged into a pellet at 20,000 g for 30 min. The supernatant was filtered through cheesecloth and adjusted to pH 8.0. 100 ml of this supernatant was brought slowly and with stirring to 60% ammonium sulfate, using a saturated solution of ammonium sulfate (Mann-Schwarz Ultrapure, Spring Valley, NY) containing 10 mM EDTA. The ammonium sulfate fraction was centrifuged at 100,000 g for 60 min at 4°C. The pellet was resuspended in 15 ml of extraction buffer and dialysed against this buffer to remove the ammonium sulfate.

The dialysate was clarified by centrifugation at 10,000 g for 10 min and 6 ml of it was applied to a 10-ml column of DEAE-Sepharose-Cl 6B (kind gift of

Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) equilibrated in extraction buffer. Protein was eluted from the column by a 70-ml gradient from 0.0 to 0.5 M KCl followed by a step gradient of 2 M KCl.

The DEAE-Sepharose-Cl 6B fractions containing AIP, as detected by SDS PAGE, were pooled and concentrated 10-fold using an Amicon PM-10 membrane ultrafiltration unit (Amicon Corp., Scientific Systems Div., Danvers, MA). The concentrated pool was dialysed against a buffer containing 0.5 KCl, 0.75 mM 2-mercaptoethanol, and 3 mM imidazole, pH 8.0.

Approximately 1 ml of the dialysate was clarified by centrifugation at 20,000 g for 20 min. The resulting clear supernatant was applied to a 1 × 90-cm column of Sephacryl S-200 equilibrated in and eluted with 0.5 M KCl, 0.75 mM 2-mercaptoethanol, and 3 mM imidazole, pH 8.0. Fractions containing AIP, as determined by SDS PAGE, were concentrated 20-fold in an Amicon ultrafiltration unit (Amicon Corp.) and were dialysed against Buffer F containing 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT), and 10 mM Tris-HCl, pH 7.5.

**Preparation of Rabbit Skeletal Muscle Proteins:** Skeletal muscle myosin was isolated from the back and hind leg muscles of New Zealand white male rabbits according to the procedure of Kielley and Harrington (6). Myosin S-1 was prepared from the pure myosin using papain digestion as described by Margossian and Lowey (9). Rabbit skeletal muscle actin was prepared from an acetone powder according to the procedure of Spudich and Watt (13).

**Protein Analysis:** Protein concentration was determined by the method of Lowry et al. (8) Bovine serum albumin was used as standard. Absorbance at 290 nm was used to detect protein in the column fractions when ATP was a component of the buffer, since this wavelength minimizes absorbance by ATP (5).

**ATPase Assay:** Inorganic phosphate liberation was measured according to the method of Pollard and Korn (11). High ionic strength myosin ATPase activities used in analysis of the binding studies were assayed for in 1.5-ml aliquots with final concentrations of 0.5 M KCl, 2 mM ATP, 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA. Low ionic strength actin-activated myosin Mg<sup>2+</sup>-ATPase activities (referred to as actin activations) were measured in the presence or the absence of 0.20 mg/ml rabbit F-actin. Myosin S-1 was present at 0.020 mg/ml. Final ionic conditions were 33 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 1 mM ATP, and 10 mM imidazole, pH 7.0. Assays used to study the effect of increasing AIP concentration on the actin activation of myosin S-1 were carried out under these conditions except that the concentration of AIP was varied from 0 to 0.2 mg/ml (0–3 μM). Assays used to monitor the effect of calcium ion concentration on the inhibition of the myosin S-1 actin activation by tomato AIP were carried out as described above, but AIP was present at 0.6 mg/ml (50% inhibition level), and the free calcium ion concentration was varied using CaCl<sub>2</sub>-EGTA buffers. The pCa was calculated using a computer program developed by E. Freund and Dr. G. Fleck (Smith College, Northampton, MA) that accounted for pH, ionic strength, temperature, and using the binding constants of Potter and Gergely (12) for EGTA, ATP, Mg<sup>2+</sup>, and Ca<sup>2+</sup>.

Control assays substantiate that: (a) the AIP contained no inherent ATPase activity and (b) the activation of the myosin S-1 Mg<sup>2+</sup>-ATPase activity by F-actin in the absence of AIP was between 10- and 20-fold. The percent activity was calculated by dividing the specific activity of an individual assay point by the highest specific activity and multiplying the result by 100.

**Binding Studies Using High Ionic Strength K<sup>+</sup>-EDTA ATPase Assay:** Myosin S-1 (0.020 mg/ml) and rabbit F-actin (0.20 mg/ml) were mixed in the presence and absence of 10 mM Mg<sup>2+</sup>-ATP in 33 mM KCl, and 10 mM imidazole, pH 7.0. The pCa of the binding experiment mix was adjusted by using a Ca<sup>2+</sup>-EGTA buffer to 7.6 or 4.7. Identical tubes were prepared containing 0.6 mg/ml AIP. The tubes were incubated for 30 min at room temperature and centrifuged at 100,000 g for 30 min. The supernatants were assayed for ATPase activity in high ionic strength in the presence of K<sup>+</sup>-EDTA to detect myosin S-1.

**SDS PAGE:** Slab gel electrophoresis was performed according to the method of Blattler et al. (2), using a 5% stacking gel and a 15% separating gel. Gels were stained in Coomassie Brilliant Blue R-250 and scanned on a Beckman spectrophotometer (model II, Beckman Instruments, Fullerton, CA) at A<sub>590</sub>.

## RESULTS AND DISCUSSION

### Purification of Tomato AIP

A new protein has been isolated from the parenchymal cells of the fruit of the common tomato that appears to modulate the acto-S-1 interaction. The critical step in purifying tomato AIP was separating it from tomato actin, which is accomplished by fractionating the clarified ammonium sulfate dialysate on DEAE-Sepharose-Cl 6B. As shown in Fig. 1, the column

yielded two protein peaks. The larger peak, eluting between 0.1 and 0.5 M KCl, contained a protein fraction enriched for the 72,000-dalton AIP. Overnight dialysis and clarification of this fraction removed a great deal of insoluble material. Subsequent gel filtration of this peak on Sephacryl S-200 (Fig. 2) in high ionic strength yielded the pure AIP fraction. SDS PAGE (Fig. 3) shows that the ammonium sulfate fraction (a) contained 72,000- and 42,000-mol-wt bands on the gel. The DEAE column peak (b) was enriched for the 42,000-mol-wt tomato actin, whereas the second peak (c) was enriched for the AIP. The AIP fraction eluting from the gel filtration column (e) routinely exhibited a band of protein at an apparent molecular weight of 72,000.

Tomato AIP comprises >0.8% of the total tomato protein extractable, using this method of purification (Table I). Since tomato actin and myosin make up 6 and 0.2%, respectively, of the tomato protein fraction (15), AIP as a major component of the tomato cytoplasm, is likely to be an important factor in controlling the actomyosin interaction in the tomato. This is consistent with the original observation that column-purified tomato actin fractions, containing traces of the 72,000-mol-wt

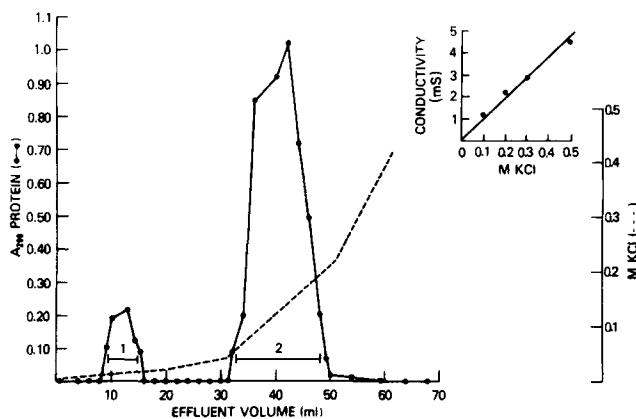


FIGURE 1 Elution profile of a DEAE-Sepharose-Cl 6B column. A 6-ml aliquot of the clarified ammonium sulfate fraction was applied to the column that was equilibrated in extraction buffer. The column was eluted with a linear salt gradient from 0.0 to 0.5 M KCl followed by a step gradient of 2 M KCl. 2-ml fractions were collected every 5 min. Fractions were assayed for protein at A<sub>290</sub> (●) and for conductivity (---). Pool 2 contains the 72,000-dalton AIP. Pool 1 contains tomato actin.

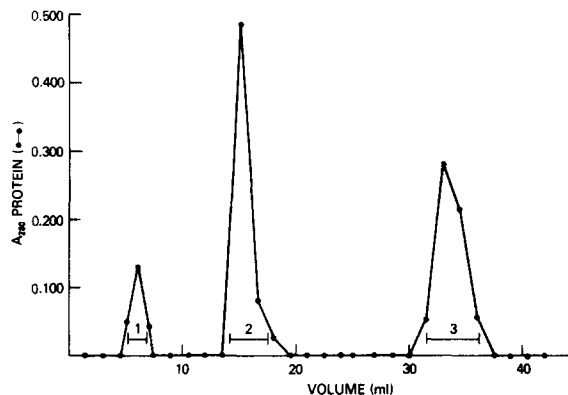


FIGURE 2 Gel filtration of the clarified DEAE 2 pool on Sephacryl S-200 in 0.5 M KCl, 0.75 mM 2-mercaptoethanol, and 3 mM imidazole, pH 8.0. Peak 2 contained the AIP fraction whereas peaks 1 and 3 contained high and low molecular weight proteins, respectively.

protein, exhibit low levels of actin activation of rabbit skeletal muscle myosin S-1 (15).

### Inhibition of Acto-S-1 ATPase Activity by AIP

As shown in Fig. 4, tomato AIP reduced the ability of rabbit F-actin to interact with myosin S-1 and enhance the S-1  $Mg^{+2}$ -ATPase activity. This inhibition can be increased by raising the concentration of AIP, whereas the concentration of actin and myosin S-1 were kept constant. At a molar ratio of one AIP to five F-actin, a 50% level of inhibition of the actin activation is observed.

To establish whether or not the AIP could be interacting with the myosin S-1 hydrolytic site to bring about inhibition, the basal low ionic strength  $Mg^{+2}$ -ATPase of myosin S-1, in the absence of F-actin, was examined in the presence of increasing levels of AIP. The results of the titration of myosin S-1 with AIP are shown in Fig. 5. The myosin S-1 maintained a basal  $Mg^{+2}$ -ATPase activity in low ionic strength regardless of the presence of tomato AIP. This suggests that the myosin S-1 hydrolytic site was not affected by AIP.

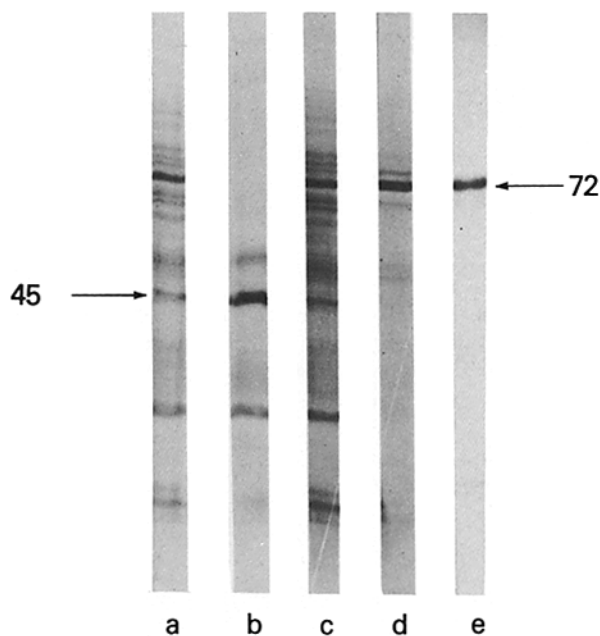


FIGURE 3 SDS PAGE of the purification of tomato AIP. 20  $\mu$ l of a 1 mg/ml sample were applied to a 5% stacking gel and a 15% separating gel. (a) Ammonium sulfate step; (b) the DEAE pool enriched for tomato actin; (c) the DEAE pool enriched for AIP; (d) the clarified DEAE 2 pool applied to S-200 column; (e) the purified tomato AIP. Molecular weights,  $\times 10^3$ .

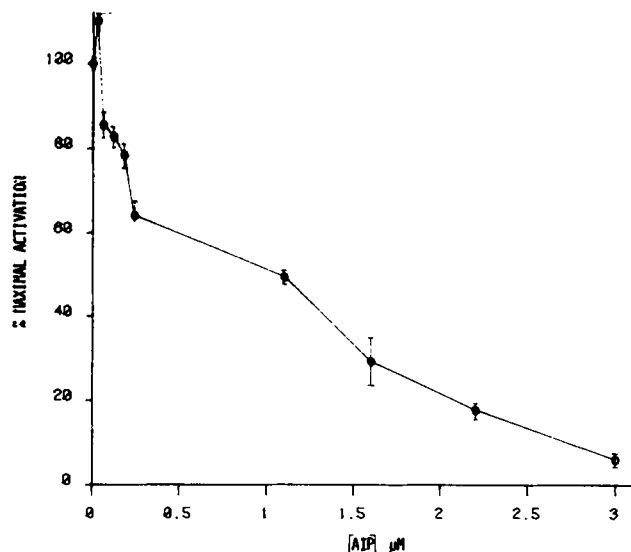


FIGURE 4 Titration of the actin activation of the myosin S-1  $Mg^{+2}$ -ATPase activity with AIP. The ordinate indicates the percent of maximal actin activation whereas the abscissa shows the final concentration of AIP in the tube. Conditions: 0.2 mg/ml F-actin, 0.02 mg/ml S-1, and AIP from 0 to 0.2 mg/ml (0–3  $\mu$ M). 50% inhibition occurs at molar ratio of one AIP to five actin. Ionic conditions are given in the text. Average 100% activity = 0.130  $\mu$ mol  $P_i$ /milligram/minute;  $n = 6 \pm SE$ .

### Regulation of the AIP-acto-S-1 Interaction

If the AIP constitutes an efficient regulatory mechanism, it seems reasonable to postulate that its inhibitory effects are reversible. Since calcium is a common ionic modulator of contractile events (1), the effect of increasing calcium ion concentration on the ability of AIP to inhibit acto-S-1  $Mg^{+2}$ -ATPase activity was studied.

An increase in the free calcium ion concentration to  $>1 \times 10^{-5}$  M resulted in restoration of 100% actin activation regardless of the presence of the 50% inhibition level of AIP (Fig. 6). The apparent calcium sensitivity of the inhibition of AIP accounts for a means of ready reversibility.

### AIP Dissociates the Rabbit Acto-S-1 Complex

To investigate the possibility that AIP might inhibit the rabbit acto-S-1  $Mg^{+2}$ -ATPase activity by dissociating the actin S-1 complex, a series of experiments studying the binding of actin and myosin S-1 in the presence and absence of AIP were carried out (Table II). As expected, the myosin S-1 bound to actin in the absence of ATP, but not in the presence of ATP. In the presence of ATP, myosin S-1 dissociated from the pelleted actin and remained in the supernatant.

TABLE I  
Purification of Tomato AIP

Step	Volume ml	Protein mg/ml	Total protein mg	Percent
Total homogenate	1,216 $\pm$ 28	4.8 $\pm$ 0.4	5,846 $\pm$ 578	
Extract supernatant	100 $\pm$ 0	3.5 $\pm$ 0.6	350 $\pm$ 61	100
Clarified 60% ammonium sulfate	17 $\pm$ 4	1.8 $\pm$ 0.6	30 $\pm$ 1.4	8.8
DEAE 1	12 $\pm$ 2	0.34 $\pm$ 0.1	4 $\pm$ 0.68	1.2
DEAE 2	17 $\pm$ 2	0.97 $\pm$ 0.4	17 $\pm$ 8.8	5.0
Sephacryl S-200	5.3 $\pm$ 0.60	0.46 $\pm$ 0.25	2 $\pm$ 0.17	0.8

The values given are the mean  $\pm$  standard error ( $n = 4$ ).

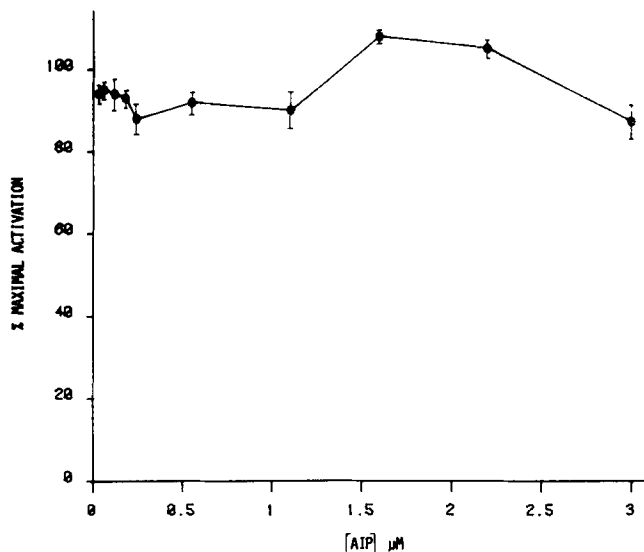


FIGURE 5 Titration of the basal low ionic strength  $Mg^{+2}$ -ATPase of myosin S-1 with AIP in the absence of actin. AIP does not inhibit the basal low ionic strength  $Mg^{+2}$ -ATPase activity of the myosin S-1 in the absence of F-actin. The concentration of myosin S-1 (0.02 mg/ml) is the same as that used in the assay done in the presence of F-actin. Ionic conditions are given in the text. Average 100% activity = 0.0140  $\mu\text{mol } P_i/\text{milligram}/\text{minute}$ ;  $n = 5 \pm \text{SE}$ .

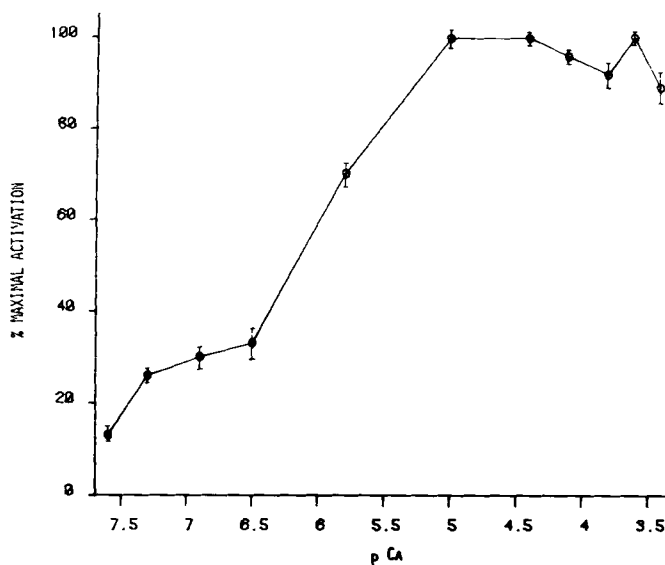


FIGURE 6 Reversal of the inhibitory effects of AIP by calcium. The ordinate indicates the percent of actin activation of the myosin S-1 and the abscissa indicates the increasing free calcium ion concentration from pCa 7.6 to 3.4. Conditions: 0.2 mg/ml F-actin, 0.02 mg/ml S-1, and 0.06 mg/ml AIP (50% inhibition level). Ionic conditions are given in the text. Average 100% activity = 0.136  $\mu\text{mol } P_i/\text{milligram}/\text{minute}$ ;  $n = 5 \pm \text{SE}$ .

When AIP was added to the acto-S-1 mixture at the molar ratio of one AIP to five actin at a pCa of 7.5, both the AIP and the myosin S-1 remained in the supernatant. Most important, the myosin remained dissociated from the pelleted actin, even in the absence of ATP. The appearance of myosin S-1 in the supernatants of high speed centrifugation experiments in the absence of  $Mg^{+2}$ -ATP and the presence of AIP strongly suggests that AIP keeps the acto-S-1 complex in the dissociated state.

TABLE II  
Binding Experiments

Sample	K-EDTA ATPase of myosin S-1 in supernatant $\mu\text{mol } P_i/\text{mg}/\text{min}$	Actin in pellet %
Mix pCa 7.6		
Acto-S-1 + AIP + ATP	0.245 $\pm$ 0.016	96
Acto-S-1 + AIP - ATP	0.231 $\pm$ 0.021	94.5
Acto-S-1 + ATP	0.258 $\pm$ 0.010	96.9
Acto-S-1 - ATP	0.021 $\pm$ 0.002	97.0
Mix pCa 4.7		
Acto-S-1 + AIP + ATP	0.138 $\pm$ 0.012	96.0
Acto-S-1 + AIP - ATP	0.015 $\pm$ 0.001	95.0
Acto-S-1 + ATP	0.133 $\pm$ 0.011	95.7
Acto-S-1 - ATP	0.014 $\pm$ 0.002	97.0

Conditions: 0.020 mg/ml S-1, 0.200 mg/ml F-actin + 0.060 mg/ml AIP, 33 mM KCl, 10 mM imidazole, pH 7.0  $\pm$  10 mM MgATP, and free calcium ion concentrations as indicated. Assay for myosin done in high ionic strength as indicated in Materials and Methods. The percent of actin in the pellet was determined from gel scans. Control tubes with actin alone show 96% actin in the pellet under these conditions. Values given are the mean  $\pm$  standard error ( $n = 3$ ), or the mean of two determinations for gel scans.

Furthermore, when this same experiment was carried out at pCa of 4.7 in conditions where AIP did not inhibit acto-S-1 ATPase activity, the myosin S-1 bound to actin in the absence of ATP and was dissociated from actin in the presence of ATP, regardless of the presence of AIP. Thus, the dissociation of the acto-S-1 complex by AIP exhibited the same calcium sensitivity as did the inhibition of acto-S-1 ATPase activity by AIP.

#### AIP Does Not Bind to Actin

Densitometric scans of gels of the supernatants and pellets of ultracentrifuge experiments (Table II) indicated that 96–98% of the F-actin was pelleted, regardless of the presence of AIP or of the free calcium ion concentration. AIP never appeared in the pellet, and it did not depolymerize or aggregate the actin as the amount of actin in the supernatant and the pellet remained constant, despite the presence of AIP. This suggests that AIP does not bind directly to actin or change the physical structure of actin.

#### Mechanism of ATPase Inhibition by AIP

Since AIP did not affect the hydrolytic site on myosin S-1 (Fig. 5) and did not appear to bind to F-actin (Table II), the possibility remains that AIP may alter the actin binding site on myosin S-1. As shown in Fig. 7A, when the AIP and F-actin concentrations were held constant, the inhibition by AIP could be reduced by increasing the myosin S-1 concentration. However, when the concentrations of AIP and myosin S-1 were held constant and the concentration of F-actin was increased, the inhibition could not be overcome (Fig. 7B).

Adding myosin S-1 may increase the population of myosin S-1 molecules that are free of AIP and able to interact with actin. On the other hand, increasing the F-actin concentration cannot change the number of myosin S-1 molecules blocked by AIP, so that the acto-S-1 ATPase activity remained at an inhibited rate over the entire range of F-actin concentrations. These results suggest that AIP interacts with the myosin S-1 molecule and changes the ability of myosin S-1 to interact with actin. Alternatively, the mechanism of the acto-S-1 AIP ATPase reaction may be more complicated since AIP does not completely inhibit ATP hydrolysis (Fig. 7B), nor does adding

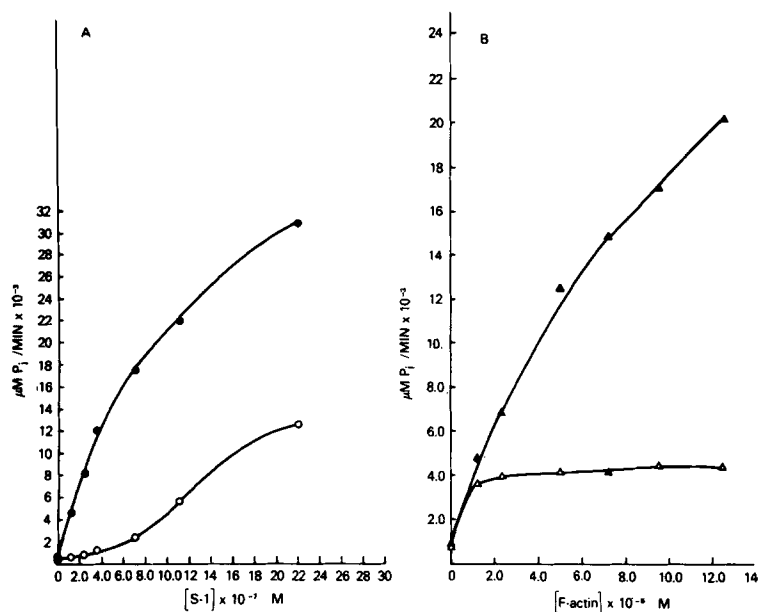


FIGURE 7 (A) Effect of AIP on the rate of acto-S-1 Mg<sup>2+</sup> ATPase activity over a range of myosin S-1 concentrations. Conditions: 33 mM KCl, 1 mM Mg<sup>++</sup> ATP, 0.2 mg/ml F-actin, 0.06 mg/ml AIP, pCa 7.4, and 10 mM imidazole, p.H 7.0. ●, rabbit acto-S-1 ATPase activity in absence of AIP; ○, rabbit acto-S-1 ATPase activity in presence of AIP. (B) Effect of AIP on the rate of acto-S-1 Mg<sup>2+</sup> ATPase activity over a range of actin concentrations. Conditions: 0.020 mg/ml S-1, and 0.06 mg/ml AIP, pCa 7.4. Ionic conditions were identical to those in 7A. ▲, rabbit acto-S-1 ATPase activity in absence of AIP; △, rabbit acto-S-1 ATPase activity in presence of AIP.

myosin S-1 cause complete recovery to 100% activity (Fig. 7A) in the ranges of protein concentrations studied.

### Conclusion

AIP resembles filamin (4), *Physarum* 36,000-dalton factor (10), and *Dictyostelium* 120,000-dalton factor (5) in inhibiting the actin-activated ATPase activity of myosin. Similar to the 36,000-dalton factor, the inhibition by AIP is calcium sensitive. Nevertheless, AIP is distinct from all of these proteins in that the physical structure of actin does not appear to be altered by AIP. Filamin and *Dictyostelium* 120,000-dalton factor crosslink actin into gels, and the 36,000-dalton factor curls and bundles actin filaments. It is postulated that altering the physical structure of actin by filamin, 120,000- and 36,000-dalton factor occur by direct binding of these proteins to the actin, resulting in the inability of actin to interact with myosin.

Tomato AIP is a unique calcium-sensitive modulator of the rabbit acto-S-1 Mg<sup>2+</sup>-ATPase activity. Tomato AIP causes the dissociation of acto-S-1 in the absence of ATP. AIP does not bind to actin nor does it change the physical structure of actin. The molecule has no effect on the hydrolytic site of the myosin S-1 but appears to alter the ability of the actin binding site on myosin S-1 to interact with actin. Whereas the inhibiting effects of AIP on the rabbit acto-S-1 interaction suggest a similar role for AIP in the tomato actin fractions with impaired ability to activate myosin S-1, it will be interesting to determine by direct means the precise step in the acto-S-1 ATPase kinetic scheme that is altered by AIP.

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