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 Mg2+ATP and inhibited the ability of F-actin to activate the low ionic strength Mg2+ATPase activity of myosin S-1. At a molar ratio of 5 actin to 1 AIP, a 50% inhibition of the actin-activated Mg2+ATPase activity of myosin S-1 was observed. The inhibition can be reversed by raising the calcium ion concentration to 1 x 10^{-5} M. The AIP had no effect on the basal low ionic strength Mg2+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 Mg2+ATPase activity of myosin. Chicken gizzard smooth muscle filamin (4), Dictyostelium 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 Mg2+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 Mg2+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 Mg2+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 CaCl2, 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 20,000 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 M KCl, 0.75 mM 2-mercaptoethanol, and 3 mM imidazole, pH 8.0.

Approximately 1 ml of the dialysate was centrifuged at 20,000 g for 20 min. The resulting clear supernatant was applied to a 1 x 90-cm column of Sephacyr 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 detected 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 MgCl₂, 0.5 mM diethytheritol (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 Kiely 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 Spandich 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 Mg²⁺-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 MgCl₂, 0.1 mM CaCl₂, 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 CaCl₂-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, Mg²⁺, and Ca²⁺.

Control assays substantiate that: (a) the AIP contained no inherent ATPase activity and (b) the activation of the myosin S-1 Mg²⁺-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⁺-EGTA 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 Mg²⁺-ATP in 33 mM KCl, and 10 mM imidazole, pH 7.0. The pCa of the binding experiment mix was adjusted by using a Ca²⁺-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⁺-EGTA to detect myosin S-1.

**SDS PAGE:** Slab gel electrophoresis was performed according to the method of Blatter 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 A595.

**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 Sephacyrl 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...
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 μl 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, x 10^3.](image)

![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 μM). 50% inhibition occurs at molar ratio of one AIP to five actin. Ionic conditions are given in the text. Average 100% activity = 0.130 μmol P_2/milligram/minute; n = 6 ± SE.](image)

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 × 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.

AlP 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 I). As expected, the myosin S-1 bound to actin in the absence of ATP, but not in the presence of AIP. In the presence of ATP, myosin S-1 dissociated from the pelleted actin and remained in the supernatant.

| Table 1 Purification of Tomato AIP |
|-----------------|---------|----------|----------|
| Step            | Volume  | Protein  | Total protein | Percent |
| Total homogenate| 1,216 ± 28 | 4.8 ± 0.4 | 5,846 ± 578 | 100     |
| Extract supernatant| 100 ± 0  | 3.5 ± 0.6 | 350 ± 61   | 8.8     |
| Clarified 60% ammonium sulfate | 17 ± 4 | 1.8 ± 0.6 | 30 ± 1.4 | 1.2     |
| DEAE 1          | 17 ± 2  | 0.34 ± 0.1 | 4 ± 0.68 | 5.0     |
| DEAE 2          | 5.3 ± 0.60 | 0.46 ± 0.25 | 2 ± 0.17 | 0.8     |

The values given are the mean ± standard error (n = 4).
suggests that AIP keeps the acto-S-1 complex in the dissociated state.

In ultracentrifuge experiments (Table II) indicated that 96-98% of the acto-S-1 complex by AIP exhibited the same calcium sensitivity as the inhibition of acto-S-1 ATPase activity by 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.

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 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...
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$^{2+}$-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 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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