# CONTROL OF INSECT MYOSIN ADENOSINETRIPHOSPHATASE BY THE BINDING OF THE SUBSTRATE TO AN ALLOSTERIC SITE*

R.A. Chaplain

Department of Zoology, University of Oxford, Oxford, England.

Received October 31, 1966

Evidence has recently been presented (Chaplain, 1966a) that insect myosin and actomyosin ATPase can exist in two states which differ in their affinity for the substrate, the one with higher affinity being catalytically inactive. It has been shown that actin changes the equilibrium between these two states of the myosin ATPase towards the active state and that in presence of low levels of $Ca^{++}$ the inactive state of the myosin ATPase becomes less readily accessible. A third enzymically inactive state which exhibits decreased affinity for the substrate is stabilized by ADP (Chaplain, 1966b); conditions which favour this state lead to superprecipitation of actomyosin gels and tension development of muscle fibres.

The present study provides additional support for the concept of the allosteric nature of substrate inhibition of insect actomyosin ATPase (Chaplain, 1966a) by demonstrating a correlation between the decreased enzyme activity and the binding of ATP to a site spatially distinct from the active site.

## Materials and Methods

Myosin and "natural" F-actin were prepared from freshly excised fibrillar flight muscles of the water bug Lethocerus cordofanus as described previously (Chaplain, 1966a, b). The reaction medium for the determination of ATPase activity and nucleotide binding contained 50-80 mM KCl, 20 mM Tris-maleate buffer, 2 mM EGTA and as an ATP-reconstituting system 1 mM [$^{32}$P] phosphoenolpyruvate and 0.025 mg pyruvate kinase, pH 7.0. To this 4.0 mg/ml myosin and 1.2 mg/ml. F-actin were added. A stabilized [$Ca^{++}$] was obtained by inclusion of Ca-EGTA (Portzehl et al. 1964). Equimolar amounts of $MgCl_2$ and [8-$^{14}$C, $\gamma$-$^{32}$P] ATP were added to the reac-

* This work was supported by a grant from the Agricultural Research Council.

tion medium. The ionic strength was adjusted to 0.1 with KCl to prevent any changes by increasing substrate levels. The final volume was 3 ml and the temperature 25°. The incubation time varied between 1-5 min. The reaction was terminated by filtration through Millipore filters (pore size 0.45μ), mounted on test tubes with side arms connected to a vacuum line. The filtrate was washed with a solution containing 75 mM KCl, 2 mM EGTA and 20 mM Tris-maleate buffer, pH 7.0. The filtration was complete within 2-3 seconds. No detectable protein appeared in the filtrate. The ATPase activity was determined from the amount of inorganic phosphate in the filtrate which was measured either by a semi-microadaptation of the Marsh method (Marsh, 1959), or after separation of the $^{32}P_i$ as described by Glynn and Chappell (1964). The protein trapped on the filter was extracted with 10% trichloracetic acid; the extract was neutralized by repeated ether extractions and concentrated in a flash evaporator. Samples were counted at three different dilutions. Internal standards of double-labelled ATP were used. The radioactivity was measured in an I. D. L. two-channel Tritomat 6020. The gate settings and values of $HV_1$ and $HV_2$ were such that at high $HV_1$ and $HV_2$ mostly $^{14}C$ and some $^{32}P$ was counted while at low $HV_1$ and $HV_2$ the counts gave a nearly true measure of $^{32}P$. The scintillation mixture was that of Bray (1960). ATP binding was estimated from the unchanged activity ratios of the two labels and by directly measuring the nucleotide concentration in the extract using the firefly luciferin-luciferase assay (Worthington). As the F-actin contains ADP as its bound nucleotide (Szent-Györgyi, 1951; Mommaerts, 1952) all the ATP bound to the actomyosin, as well as myosin, must reflect a binding to the myosin component.

The labelled [8-$^{14}C$] ATP (Sp. Act. 10.7 mC/mmole) obtained from Schwartz Bioresearch was converted to [$^{14}C$] ADP in presence of glucose and hexokinase (Sigma, crystalline). The ATP was then resynthesized by adding [$^{32}P$] inorganic phosphate (Radiochemical Centre, Amersham, Sp. Act. 1.3 mC/mmole) using the method of Glynn and Chappell (1964); [$^{32}P$] phosphoenolpyruvate of the same specific activity as the labelled ATP was synthesized by equilibration with pyruvate and pyruvate kinase (Sigma, crystalline).

## Results and Discussion

The results shown in Fig. 1 indicate that up to $2 \times 10^{-9}$ M ATP becomes bound/mg myosin; the decrease in myosin ATPase activity being paralleled by an increase in the binding of the substrate to the protein. The increase in ATP hydrolysis which occurs on interaction of actin with myosin is paralleled by a decrease in the amount of nucleotide bound. At $Ca^{++}$ concentrations of $10^{-7}$ M which markedly relieve substrate

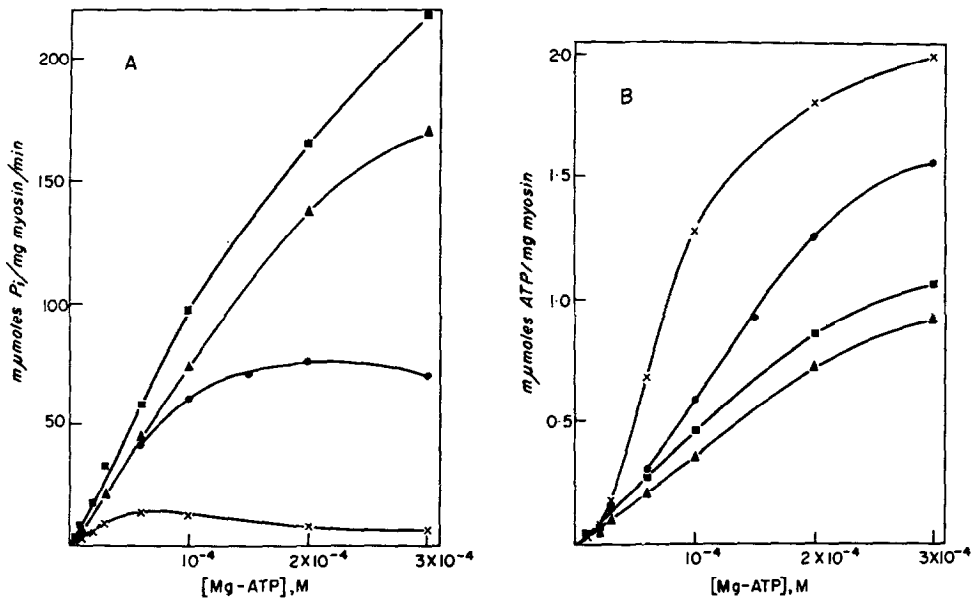


Fig. 1. ATPase activity (A) and ATP binding (B) of insect myosin and actomyosin ATPase. The experimental conditions are described in the text. Although ATP binding was determined both by measurements of radioactivity and by the fire-fly method, the differences in the results were not significant and only one symbol is given in part B of the Figure. The assay system and the $Ca^{++}$ concentrations of the reaction medium are as follows: x, myosin, pCa 8.5; ●, actomyosin, pCa 8.5; ■, actomyosin, pCa 7.0; ▲, actomyosin, pCa 7.0, in presence of 0.75 mM ADP.

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inhibition, the affinity of the regulatory site for ATP is further reduced. The decrease in ATP binding is particularly marked at Mg-ATP levels above $1.5 \times 10^{-4}$M where the actomyosin ATPase becomes strongly activated by $Ca^{++}$. The observed changes in ATP binding cannot reflect changes in affinity of the active site for the substrate, as the time interval of 2-3 sec. of the filtration step is long enough for the breakdown of the enzyme-substrate complex of both myosin and actomyosin to reach completion. The time constant for the breakdown of the enzyme-substrate complex for both myosin and actomyosin under fully activating conditions is about 30 msec; it never becomes slower than 90 msec (Chaplain, 1967).

Addition of 0.75 mM ADP resulted in inhibition of $Ca^{++}$-activated actomyosin ATPase in agreement with earlier findings (Chaplain, 1966b). However, under these conditions the amount of bound ATP was reduced. As ADP is known to stabilize a second inactive state (Chaplain, 1966b) it is most probable that it binds to the same effector site as ATP, thus resulting in decreases in enzyme activity and ATP binding.

The existence of a distinct ligand binding site for ADP was previously suggested from the observation that an ATP analogue, 6-morpholino-9 (2', 3'-0-isopropylidene)-β-D-ribofuranosylpurine 5'-tri-phosphate, antagonized the inhibition of the actomyosin ATPase by ADP (Chaplain, 1966b).

The results presented in this communication are in agreement with the conclusion that there are two ATP binding sites per myosin monomer of molecular weight of about $5 \times 10^5$ g, the active site and a stereospecifically distinct effector site. This conclusion is likely to apply equally well to myosin from vertebrate skeletal muscle, as the amino acid composition, the physicochemical parameters and enzymic activity of insect myosin are very similar, if not identical, to those observed for rabbit myosin (Maruyama, 1965). It is of interest that the possibility of two ATP binding sites on rabbit myosin was originally suggested by the binding of two molecules of pyrophosphate per $4.6 \times 10^5$ g myosin (Tonomura & Morita, 1959). On the basis of the present investigation it is possible to explain the observation of Nanninga and Mommaerts (1960) that only one ATP was bound per $4.2 \times 10^5$ g at $6 \times 10^{-6}$ M ATP by assuming that a major fraction of the myosin was in the active configuration, the effector site of most myosin molecules being unoccupied. Studies on the chemical structure of the myosin sites binding trinitrobenzenesulfonate have shown that one site is on each of the two polypeptide chains which constitute a myosin unit of $4.2 \times 10^5$ g and that the sites are not structurally equivalent (Kubo et al. 1965); this suggests that the active site of the myosin ATPase and the regulatory site may be localized on different subunits.

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