THE ALLOSTERIC NATURE OF SUBSTRATE INHIBITION OF INSECT ACTOMYOSIN ATPASE IN PRESENCE OF MAGNESIUM

R.A. Chaplain

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

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It has long been known for actomyosin preparations from vertebrate muscle that the Mg^{++}-activated ATPase activity is inhibited by overoptimal concentrations of ATP. This inhibition by excess substrate levels has been found remarkably affected by ionic strength, temperature and the concentration of divalent cations (Hasselbach, 1952). Similar results have also been obtained with myofibrils from rabbit skeletal muscle (Hasselbach and Weber, 1953; Perry and Grey, 1956). The substrate inhibition has been interpreted as being due to removal of free Mg^{++} or caused by an increase in the concentration of free ATP (Perry and Grey, 1956; Geske et al. 1957). The ATPase inhibition is reversed by Ca^{++} in myofibrillar systems (Perry and Grey, 1956) and isolated actomyosin preparations (Watanabe and Yasui, 1965). Overoptimal substrate levels equally affect structural changes in the actomyosin complex; the contraction of glycerinated fibres (Hasselbach and Weber, 1963) and actomyosin gels (Watanabe and Yasui, 1965; Maruyama and Gergely, 1962) is inhibited. This effect is also antagonized by Ca^{++}. Low concentrations of mercuribenzoate have been found to activate the ATPase activity (Blum, 1960) and to reverse the inhibition of actomyosin superprecipitation by high MgATP levels (Watanabe and Yasui, 1965; Maruyama and Gergely, 1962). The ATPase of the isolated myosin component is equally sensitive to substrate inhibition (Hasselbach, 1952; Baev, 1958).

A detailed kinetic analysis of the substrate inhibition of "natural" actomyosin and reconstituted actomyosin from insect fibrillar flight muscle has been carried out using equimolar concentrations of Mg^{++} and ATP as the substrate. The effects of low levels of mercurials, and Ca^{++} on the enzyme-substrate interactions are described.

Materials and Methods

Preparation of actomyosin: Freshly excised indirect flight muscle of giant water bugs (Lethocerus cordofanus or L. indicus) were homogenised in 0.3 M sucrose-15 mM Tris-

Cl, pH 7.2, containing 1 mM EDTA and 0.5 mM EGTA.* Myofibrils were separated by differential centrifugation of the homogenate at 600 g, 20 mins. in a MSE centrifuge. The myofibrillar fraction was then extracted with 10 times its volume of 0.7 M KCl - 20 mM Tris-Cl buffer, pH 7.8 for 4 hours. After separation of the structural residue by centrifuging at 8000 g, 20 min. the actomyosin was precipitated by dialysis against 0.05 M KCl - 10 mM histidine-HCl buffer, pH 7.0. The actomyosin pellet obtained on centrifugation was washed twice with 0.05 M KCl - 20 mM histidine-HCl buffer, pH 7.0.

Preparation of myosin and F-actin: Isolated actomyosin was used as starting material. Myosin was prepared from this by the method of A. Weber (1956) and natural F-actin by the method of Hama et al. (1965) without preliminary myosin extraction; this involves trypsin digestion.

Assay of ATPase activity: The basal medium was 0.075 M KCl - 10 mM histidine-HCl buffer, pH 7.0, containing 1 mM EGTA. CaEGTA was added to obtain a stabilized [Ca^{++}] (Portzehl et al. 1964). Unless otherwise stated K_2ATP (Sigma) was added together with equimolar amounts of $MgCl_2$ to the assay system. To prevent any change in ionic strength by the increasing substrate levels, the KCl concentration was correspondingly reduced. Protein concentrations of 0.3 - 0.5 mg actomyosin, 0.08 - 0.5 mg myosin and 0.03 - 0.5 mg actin were used. The incubation time was varied between 45 - 300 sec. to allow not more than maximal splitting of 20% of the total substrate in each incubation mixture. ATPase activity was estimated by measuring the amount of inorganic phosphate released using a small volume adaptation of the sensitive Marsh method (1959), the accuracy of which is $\pm$ 1 mμmole. Protein was determined by the biuret method of Gornall et al. (1949).

Results

The results were plotted as reciprocal activity-substrate plots according to Lineweaver and Burk (1934); such plots show marked curvatures at high concentrations of substrate, if equimolar amounts of Mg^{++} and ATP are added (Fig. 1). If [Mg^{++}] was 2mM in excess of [ATP] the substrate inhibition became marked already at lower ATP concentrations. In the presence of 4×10^{-7} M Ca^{++} the enzyme reaction obeyed normal Michaelis-Menten kinetics (Fig. 1). The curvature of the double-reciprocal plots disappears as low levels of p-chloromercuriphenyl sulfonate (Sigma) are added. The "desensitization" of the protein by mercurial with respect to the substrate inhibition can

* ethylene glycol bis- (β amino ethyl ether) N_1N^1 - tetraacetic acid.

FIG. 1

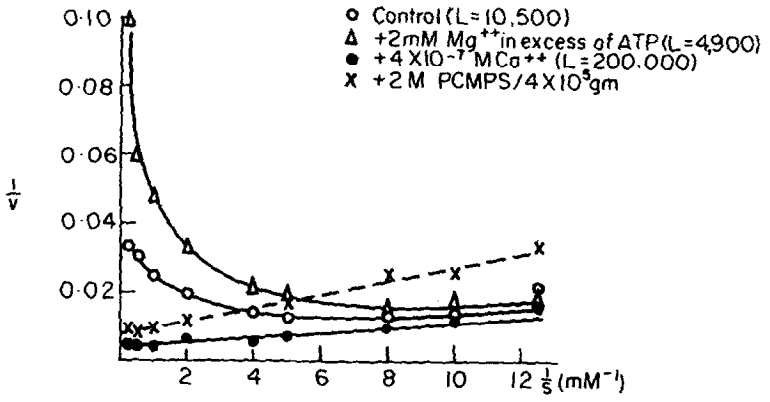

FIG. 2

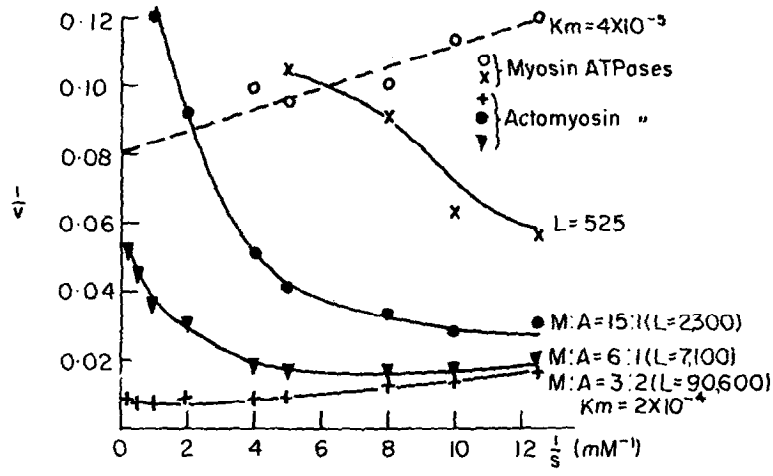

Fig. 1 & 2. Substrate inhibition of the ATPase activity of natural actomyosin and reconstituted actomyosin systems. The composition of the reaction mixture is described in the text. The final volume was 1.0 ml and the temperature 25°C. The reaction was stopped by addition of 10% trichloracetic acid. The reaction velocities are expressed in mμmoles/mg/min. The solid lines represent the theoretical curves for equation (1); the experimental points are superimposed. Results obtained with desensitized enzyme preparations are drawn as dotted lines.

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be simulated by heating the actomyosin to 50°C or ageing the actomyosin suspension for several days at 0°C.

If reconstituted actomyosin preparations were used in which the relative amounts of actin and myosin had been varied, reciprocal activity-substrate plots (Fig. 2) showed very marked substrate inhibitions for myosin/actin ratios of 15:1 or 6:1 (w/w). As the actin component of the actomyosin complex is increased, the inhibitory effect of over-

optimal substrate concentrations is gradually relieved. The kinetic results of actomyosin ATPase markedly differed from those of the isolated myosin component. First, Vmax increased 17-fold, from 210 mμmoles/g/sec to 3.6 mμmoles/g/sec, and second, the concentration at which the enzyme was half-saturated by ATP increased 5-fold (Fig. 2).

Results obtained with "pure" myosin preparations show considerable variations; while several preparations obeyed Michaelis-Menten kinetics, frequently strong substrate inhibition was observed (Fig. 2). It cannot at present be decided whether the isolation procedure for myosin might sometimes result in artificial "desensitization" of the protein or whether small impurities of actin are present in different preparations; such catalytical amounts of actin could be necessary to observe substrate inhibition of myosin ATPase.

Discussion

Since an equimolar ratio of Mg^{++} and ATP was maintained in the experiments described, removal of free Mg^{++} ions or inhibitory levels of free ATP, which is supposed to compete with the substrate MgATP for the active site on the enzyme (Perry and Grey, 1956), cannot account for the substrate inhibition of insect actomyosin ATPase. Further, the substrate inhibition observed for insect actomyosin ATPase does not obey classical kinetics.

The allosteric model of Monod, Wyman and Changeux (1965) predicts that in some instances, the phenomenon of substrate inhibition might be due to an allosteric mechanism. The inhibitory effect of overoptimal substrate concentrations can be explained if there are two states of the enzyme which differ in their affinity for ATP, the one with higher affinity being catalytically inactive; ATP is thus both substrate and feedback effector for actomyosin ATPase. On this model, the kinetic data obtained for the substrate inhibition should be fitted by an equation of the form

$$(1) \qquad \frac{V}{V_m} = \frac{LS/K_A(1+S/K_A)^{n-1}}{L(1+S/K_A)^n + (1+S/K_I)^n} ,$$

where K_A and K_I are the respective dissociations constants of ATP with the active and inactive states of the protein. The solid curves of Figs. 1 and 2 have been drawn out in this way, using values as follows: $n = 3$, $K_A = 3 \times 10^{-4}$, and $K_I = 6 \times 10^{-6}$. The Michaelis constant of myosin ATPase is a true dissociation constant (cf. Laidler, 1958). The chosen value of K_A was that obtained in presence of 7.5×10^{-7} M Ca^{++} which produces maximal ATPase activity ($V_m = 5.83$ μM/g/sec.) To convert as much as possible of the protein

into the inactive state experiments were carried out with myosin ATPase in presence of an excess of 2 mM Mg^{++} at pH 9. From the observed Km = 7 x 10^{-7} M the dissociation constant for the fully inactive state was estimated. The applicability of the allosteric model to the findings obtained with insect actomyosin ATPase is further emphasized by the fact that "desensitizing" treatments activate the enzyme activity in the presence of high levels of MgATP. The catalytically inactive state of the protein becomes less readily accessible when Ca^{++} is present. As the affinity of the allosteric site is reduced by Ca^{++} or following the destruction of the effector site by mercurials, heat or ageing, the enzyme reaction obeys normal Michaelis-Menten kinetics.

The results further indicate that actin binding to the myosin component displaces the equilibrium towards the active state of the enzyme. In terms of an allosteric model actin would act as an allosteric activator for myosin ATPase. Evidence in favour of such a model is provided by the observation that the centres of the myosin molecule which interact with ATP (active site) and actin are quite different and that the capacity of myosin to be activated by actin can be lost or inhibited without change in the actual actin binding (Kaldor et al. 1964; Barany et al. 1963; Perry and Cotterill, 1964). The possibility that the allosteric effector for certain proteins may be another cellular protein has previously been suggested (Lehninger, 1964).

An effective control of ATP splitting by high concentrations of MgATP offers an important advantage for the energetics of resting muscle. The level of magnesium in skeletal muscle, 6-7 mM (Long, 1961), is up to 2 mM in excess of that of ATP (Bendall and Davey, 1957). The fact that an excess of Mg^{++} potentiates the substrate inhibition would account for the much reduced activity of resting muscle and in this way explain that the heat production is as low as 1.2 mcal/g/min. (Hill, 1964).

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