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Nucleotide Regulatory Sites on Skeletal Myosin

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Myosin Ca^{2+}-ATPase activity decreased in the presence of ADP. Free ATP acted either as an activator or as an inhibitor depending on its concentration. The inhibition caused by ADP or ATP followed a competitive pattern with respect to the substrate. ATP, at activating concentrations, competed with dinitrophenol and with the anions SCN^-, CN^- and HCO_3^- for the same binding sites of myosin, whereas ADP did not compete with them. These results suggest that the nucleotide regulatory site or sites, different from the hydrolytic sites, seem to coincide with the anion binding sites.

Key words: Myosin Ca^{2+}-ATPase, Nucleotides, Regulatory sites of myosin Ca^{2+}-ATPase.

Myosin is a multimeric protein, which in its native state consists of two high molecular weight subunits, the myosin heavy chains (Mr = 200,000), and four low molecular weight subunits, the light chains (Mr = 15,000-27,000). Two structurally and functionally distinct regions of the protein may be identified. These are the NH_2-terminal portion, called subfragment 1, and the COOH-terminal half, usually referred to as the rod. The S1 fragment carries the site for ATP hydrolysis and interaction with actin (27). The binding and hydrolysis of ATP on one hand, and the interaction with actin on the other, induce significant localized changes in the structure of S1 (15, 35). BALINT *et al.* (1) and later MORNET *et al.* (25) showed that limited tryptic hydrolysis cleaves S1 into three major fragments of 27, 50 and 20 kDa which are aligned in this order within the heavy chain (22). The tryptic fragments of S1 have become a convenient framework in which to assign specific groups and functionalities. Several studies suggest that the actin binding sites are located at the 20 and 50 kDa peptides (25, 28, 29, 34, 41).

The ATPase site appears to be located in the 26 kDa peptide of S1 (30, 36). Therefore two types of ATP analogues are photochemically labeled mainly to the 50 kDa peptide in S1 (13, 23, 24). Although

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HIRATSUKA (13) has proposed that the myosin ATPase site is located between the 26 and 50 kDa peptides of S1, other authors have established the presence of regulatory sites on the myosin molecule capable of binding nucleotides (6, 11, 26, 37-39).

A variety of compounds of anionic nature affect several structural and functional properties of myosin. DNP is an activator of myosin Mg^{2+}-ATPase activity (7, 12). It has been suggested that DNP acts by competing with ATP for a non-hydrolytic site of the enzyme (18, 19) and it has also been shown (40) that DNP interacts with myosin at a site different from the binding site for actin. SCN^- affects the ATPase activity of myosin (4); besides, this anion has been found to bind to allosteric sites in S1, with specific inhibition of the acto-S1 ATPase (2). Several polyanions, such as RNA (5, 16, 31, 32) and heparin (3) have been reported to bind to the myosin heads and to act as strong inhibitors of the activation of the myosin ATPase by actin, leaving unaffected the Ca^{2+} or Mg^{2+} dependent ATPase of myosin.

The effect of polyanions could be due to electrostatic interactions with the clusters of cationic sites on the myosin head molecule. It is possible that electrostatic forces could be implicated in the transduction of chemical energy into mechanical energy in muscle contraction (17). The study reported here suggests the presence of regulatory sites of cationic nature on skeletal myosin capable of binding nucleotides as recently proposed (20).

Materials and Methods

Myosin was obtained from skeletal muscle of chicken (33). Protein determination was carried out following the reaction of Lowry *et al.* (21); crystalline bovine serum albumin was used as reference standard. Ca^{2+}-ATPase activity was assayed as described by RICHARD *et al.* (33) with slight modifications. Aliquots of the enzyme preparation were preincubated for 5 min at 30 °C in 0.7 ml of 0.1 M Tris-acetate (pH 6.5) and, when indicated, in the presence of the anions and/or nucleotides. The reaction was initiated by the addition of substrate (Ca^{2+}-ATP) at the appropriate concentrations. The incubation was continued for 3 min and stopped by the addition of 0.1 ml of 50 % trichloroacetic acid. Inorganic phosphorus was determined according to FISKE and SUBBAROW (10). Reagent blanks were determined in each experiment. Specific activity has been expressed as μmoles of Pi released/min per mg of protein.

The activation constants have been determined as follows: using a constant concentration of the substrate Ca^{2+}-ATP, plots of $1/v - v_0$ versus 1/(activator) (where v is the velocity in the presence of the activator and v_0, velocity in its absence) were constructed; slope/intercept has been defined as the k_a for the activador (9). The different k_i values were calculated according to DIXON (8).

Results

Effect of the nucleotides, ADP and ATP, on ATPase activity of myosin. — Figure 1 shows the effect of nucleoside diphosphate ADP on the hydrolytic activity of myosin at 2 and 0.2 mM substrate concentration. It may be seen that at either substrate concentration Ca^{2+}-ATPase responded with a decrease in activity, in the whole range of ADP concentrations used (0.1-1.5 mM).

Figure 2 shows the effect of varying concentrations of ATP on the reaction rate, while holding constant the concentration of the substrate Ca^{2+}-ATP (2 or 0.2 mM). Bell shaped curves were obtained, indicating that ATP behaved either as an activator or as an inhibitor depending on its concentration.

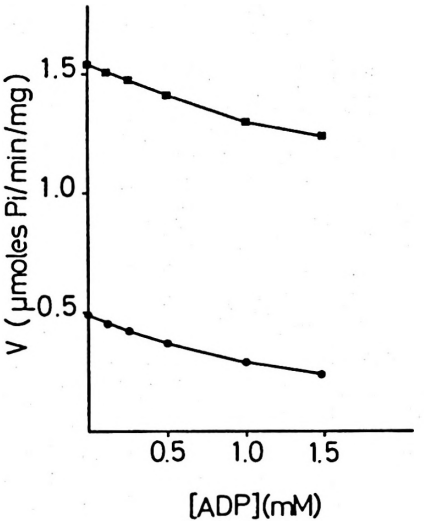

Fig. 1. *Effect of ADP on the Ca^{2+}-ATPase activity* • 0.2 mM Ca^{2+}-ATP; ■ 2 mM Ca^{2+}-ATP.

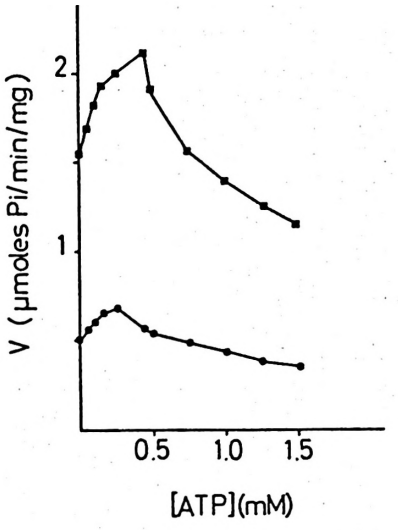

Fig. 2. *Effect of ATP on the Ca^{2+}-ATPase activity.* • 0.2 mM Ca^{2+}-ATP; ■ 2 mM Ca^{2+}-ATP.

Kinetic studies of the reaction catalyzed by myosin in the presence of nucleotides. — In order to determine whether inhibitors ADP and ATP compete for the catalytic site of enzyme, the kinetics of the hydrolytic reaction was studied in the presence of ADP or high ATP concentrations. The inhibition caused by ADP, at low substrate concentrations, follows a competitive pattern (fig. 3). A similar behavior was observed for inhibitory concentrations of ATP (at high and low substrate concentrations), and for ADP at high substrate concentrations (diagrams not shown).

K_i values obtained according to DIXON (8) were as follows: at high substrate concentrations, K_i ADP was 0.4 mM and K_i ATP 1.38 mM, and at low substrate concentrations, K_i ADP was 0.87 mM and K_i ATP 0.96 mM.

The constants of activation for ATP, both at low and high substrate concentrations, were calculated from the diagram described by EBEL and LARDY (9). The following values were obtained: at 2 mM

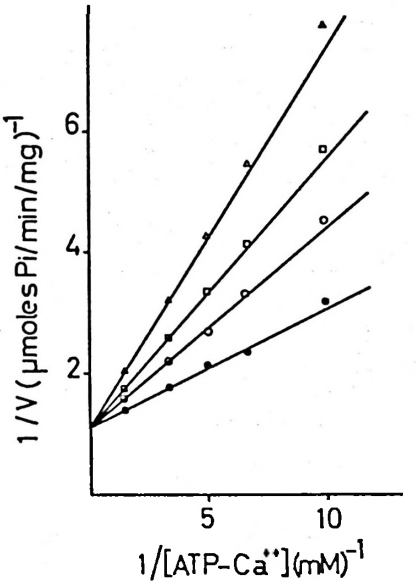

Fig. 3. *Inhibition of Ca^{2+}-ATPase by ADP at low Ca^{2+}-ATP concentration.* • absense of ADP; ○ 0.5 mM ADP; □ 1 mM ADP; △ 1.5 mM ADP.

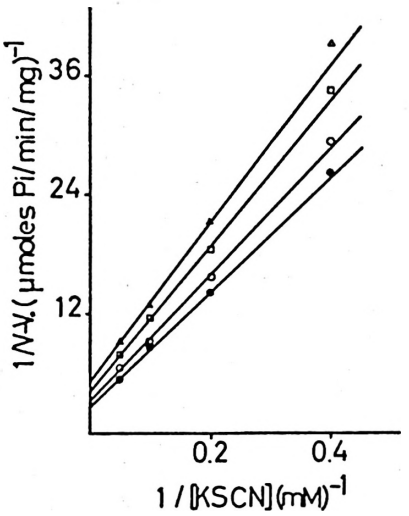

Fig. 4. *Inhibition by ADP of Ca^{2+}-ATPase activity at 0.2 mM Ca^{2+}-ATP and varying SCN^- concentration.*
• absence of ADP; ○ 0.25 mM ADP; □ 0.5 mM ADP; △ 1mM ADP.

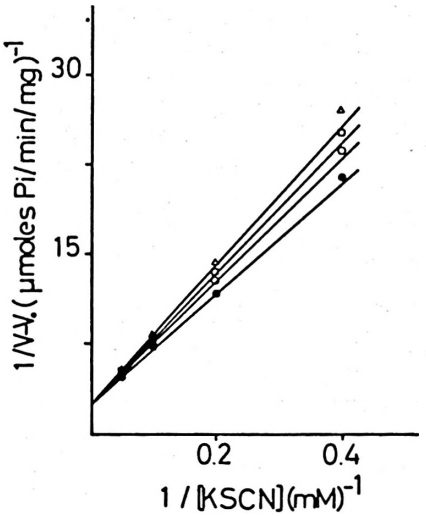

Fig. 5. *Activation by ATP of Ca^{2+}-ATPase activity at 0.2 mM Ca^{2+}-ATP and varying SCN^- concentration.*
△ absence of ATP; □ 10 µM ATP; ○ 25 µM ATP; • 50 µM ATP.

Ca^{2+}-ATP, K_a ATP was 2 mM, and at 0.2 mM Ca^{2+}-ATP, K_a ATP was 0.42 mM.

Effect of anions on the Ca^{2+}-ATPase activity of myosin in the presence of ADP or ATP. — To study the possible interrelation between nucleotide binding sites and anion binding sites, the hydrolytic activity of myosin was determined in the presence of varying concentrations of DNP, SCN^-, CN^- and HCO_3^-, both in the absence and in the presence of either ADP or low ATP concentrations (activating concentrations), at 0.2 mM Ca^{2+}-ATP. The results have been plotted as double reciprocal diagrams. In figure 4 the lines representing $1/v-v_0$ versus $1/(SCN^-)$, in the absence or in the presence of three fixed ADP concentrations, intersect with the y-axis at different points, thus indicating that ADP and SCN^- do not bind at the same sites. However, in the absence or in the presence of three ATP concentrations (fig. 5), the different straight lines intersect at the same point on the y-axis. This should be interpreted as an indication that ATP and SCN^- compete for the same sites.

DNP, CN^- and HCO_3^- behaved in a similar way to SCN^-, when either ADP or ATP were present.

Discussion

Previous results from different groups have shown that the steady state kinetics of myosin ATPase deviates significantly from Michaelis-Menten behavior (14, 15, 42). Among the several interpretations advanced to explain this fact, Yee *et al.* (42) suggested that it could be due to an allosteric activation caused by the binding of substrate at the regulatory sites. More recently evidence from our laboratory suggests the presence on myosin of regulatory sites of cationic nature capable of binding substrate molecules (Ca^{2+}-ATP), but with

lower affinity than the catalytic sites (20). Therefore, it could be assumed that, at low Ca^{2+}-ATP concentrations, the regulatory sites would be free and therefore capable of binding ligands other than the substrate; whereas at high Ca^{2+}-ATP concentrations, the regulatory sites would be occupied by substrate molecules, thus hindering the binding of other ligands.

In order to ascertain if nucleotides ADP and ATP bind to the enzyme inducing some modifications of its hydrolytic activity some experiments were carried out. As shown in Results, ADP caused a decrease in Ca^{2+}-ATPase activity of myosin; the inhibition constants were different at low and high substrate concentrations indicating that the binding of substrate at the regulatory sites affects the affinity for ADP. The competitive relationship between ADP and substrate, both at low and high substrate concentrations, suggests that ADP binds to the enzyme at the catalytic sites.

The ATPase activity of myosin was modified by the presence of free ATP, i.e. ATP in excess of Ca^{2+}-ATP (fig. 2). This nucleotide behaved as an activator or as an inhibitor of the hydrolytic reaction, depending on its concentration; at low concentrations, ATP had an activating effect, whereas increasing its concentrations, the activating effect decreased and an inhibitory effect appeared. These activating and inhibitory effects of free ATP could be interpreted as the result of an interaction of the nucleotide with two kinds of sites exhibiting different affinities. The interaction with the site (or sites) of higher affinity would lead to an activation, whereas the interaction with the site (or sites) of lower affinity would cause an inhibition of the enzyme.

The values of the activation constants for ATP were 0.42 mM and 2 mM at low and high substrate concentrations respectively. This fact could be explained, assuming that free ATP binds to myosin with a high affinity at the same regulatory sites at which the substrate binds with a low affinity; thus at low substrate concentrations ATP would bind more readily at the regulatory sites, since these sites should be free of substrate molecules.

The competitive nature of the inhibitory effect of ATP was interpreted as a consequence of its possible binding with a low affinity at the catalytic sites; the fact that the values of the inhibition constants are different at low and at high substrate concentrations confirms that the binding of the substrate at the regulatory sites affects the properties of the catalytic sites.

It has been suggested that DNP (18, 19) competes with ATP for a non hydrolytic site of myosin. BALISH and DREIZEN (2) in studies on steady state actin activated ATPase of myosin-S1 reported that ATP is a competitive inhibitor with respect to actin activation, acting at two independent allosteric sites linked to the acto-S1 interaction zone and away from the hydrolytic site; from the effects of varying ATP/Cl^- ratios on acto-S1 ATPase they proposed a model in which ATP and monovalent anions occupy the same inhibitory sites. On the other hand the kinetics studies carried out in our laboratory are consistent with a model in which substrate (Ca^{2+}-ATP) and anionic modifiers occupy the same non-hydrolytic regulatory sites (20). With the purpose of studying if ATP caused its activating effect by binding to the enzyme through the same type of regulatory sites of cationic nature, we have analyzed the relationship between ATP and several anions: DNP, SCN^-, CN^- and HCO_3^-. The results obtained indicate that ATP and anions compete for the same regulatory sites. Moreover we have confirmed that anions and ADP bind at independent sites, since they show a non-competitive relationship.

From the comparison of data related to the tryptic digestion of S1 in the presence of nucleotides or polyanions, it has been suggested that the myosin-head-nucleotide interactions bear some resemblance to

the polyanion-S1 interactions; but the interaction of ATP exhibits an additional factor of specificity (17).

The findings reported in this paper support the existence on myosin of binding nucleotide sites of cationic nature, different from the catalytic sites.

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Resumen

La actividad Ca^{2+}-ATPasa de la miosina disminuye en presencia de ADP. El ATP actúa como activador o como inhibidor en función de su concentración. La inhibición causada por ADP o por ATP es de naturaleza competitiva con respecto al sustrato. El ATP, a concentraciones en que actúa como activador, compite con el dinitrofenol y con los aniones SCN^-, CN^- y HCO_3^- por el mismo sitio de unión a la miosina, mientras que el ADP no compite con ellos. Los resultados sugieren que el centro o centros reguladores de unión de nucleótidos, diferentes de los centros activos responsables de la hidrólisis, coinciden con los sitios de enlace de aniones.

Palabras clave: Miosina:Ca^{2+}-ATPasa, Nucleótidos, Centros reguladores de la miosina-Ca^{2+}-ATPasa.

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