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THE EFFECT OF MECHANICAL STRETCHING OF THE MYOSIN ROD COMPONENT (FRAGMENT LMM + HMM S-2) ON THE ATPase ACTIVITY OF MYOSIN

B.F. POGLAZOV, G.P. SAMOKHIN, A.M. KLIBANOV, D.I. LEVITSKY, K. MARTINEK and I.V. BEREZIN

A.N. Belozersky Laboratory of Bioorganic Chemistry and Molecular Biology, Lomonosov State University, Moscow 117234 (U.S.S.R.)

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Summary

The binding of myosin to nylon fiber gives immobilized myosin with a considerable ATPase activity. Treatment of immobilized enzyme with papain results in the entire ATPase activity (known to be concentrated in myosin heads, (fragment HMM S-1)) being replaced from the fiber into the solution; this means that myosin is chemically bound to the fiber via its rod part (fragment LMM + HMM S-2). When nylon fiber is mechanically stretched, the ATPase activity of myosin attached to it sharply decreases; after relaxation of the fiber the enzymatic activity returns to the initial level. The detailed study of this phenomenon has shown that reversible inactivation of myosin upon fiber stretching is not the result of an altered microenvironment of the enzyme. The discovered regulatory effect is ascribed to deformation of myosin molecules induced by support stretching. Thus deformation of the myosin tail (not indispensable for ATPase since its cleaving-off does not alter the enzymatic activity) leads to decrease in the ATPase activity of the enzyme. The possible role of the above phenomenon in the mechanism of muscle contraction is discussed.

Introduction

There exist abundant data that the molecule of myosin may be divided into a number of fragments performing different functions in muscle contraction. Mihalyi and Szent-Györgyi were the first to carry out tryptic cleavage of a myosin molecule into two halves [1,2]. The fragments formed thereby were called L-meromyosin (LMM) and H-meromyosin (HMM) [3]. Later, using a higher concentration of trypsin, one succeeded in further cleaving HMM into two subfragments: HMM S-1 and HMM S-2 [4,5]. Kominz and Lowey with

coworkers [6—8] reported that HMM S-1 may be broken off from the whole myosin molecule by papain digestion. It has been found thereby that ATPase activity and the centre reacting with actin are localized in subfragment S-1 and remain almost unaltered when the latter is removed from the myosin molecule. This result was actually the reason why the possible effect of the rod of myosin molecule (LMM + HMM S-2) on the myosin ATPase activity has not been considered for a long time and only recently, in the work of Werber et al. [9], attention was drawn to this problem.

In spite of the fact that subfragment S-1 had been believed to be autonomous we are apt to think that fragment LMM + HMM S-2 does take part in the regulation of the ATPase activity of subfragment HMM S-1, since the phenomenon of muscle contraction implies functional relationship to exist between the catalytic state of subfragment HMM S-1 (ATP hydrolysis) and the conformation of subfragment HMM S-2 [10], and it is most likely therefore that these fragments are connected by a feedback mechanism. In order to solve all these questions we applied in this work the previously developed [11,12] mechanochemical method of studying the mechanism of enzyme action. The gist of this method is the following. Enzyme molecules are covalently bound to an elastic support (e.g. to a partially hydrolyzed nylon fiber with the help of glutaraldehyde). On stretching this support mechanically, the enzyme molecules attached to it (by multiple bonds) may be deformed. Thus, under permanent conditions one may change the structure of the enzyme to follow the changes in its function.

Experimental

Preparation of myosin and its attachment to nylon fiber

Myosin was prepared from rabbit skeletal muscle and immobilized on the surface of nylon fiber with the help of glutaraldehyde as described previously [13].

ATPase activity assay

(1) To determine the ATPase activity of free myosin, 0.7 ml of 0.3 M $NH_4Cl/5 \cdot 10^{-3}$ M EDTA/$2 \cdot 10^{-3}$ M ATP was added to 0.3 ml of solution containing 0.3 mg/ml myosin, 0.06 M borate buffer (pH 7.5). The solution was incubated for 5 min at 25°C. The specific activity was expressed in μmol P_i/mg of protein per min.

(2) Determination of the ATPase activity of fiber-bound myosin treated with papain was carried out as follows. Nylon fiber containing 0.05—0.1 mg of immobilized myosin was incubated for 30 min at 25°C in 0.3 ml of 0.06 M borate buffer (pH 7.5)/0.5 M KCl, and 0.7 ml of 0.3 M $NH_4Cl/5 \cdot 10^{-3}$ M EDTA/$2 \cdot 10^{-3}$ M ATP. Then the fiber was taken out and the remaining solution was tested for the presence of orthophosphate.

(3) The ATPase activity assay of immobilized myosin in the nylon fiber stretching experiments was the following.

60 cm of myosin-carrying (0.18 mg) fiber was wound around a stretching device (see below), placed in a thermostatted cuvette containing $2 \cdot 10^{-3}$ M ATP/$5 \cdot 10^{-3}$ M EDTA/0.3 M NH_4Cl and preincubated for 10 min at 25°C. The

total volume was 30 ml at pH 8.0. Then at certain intervals, 1-ml aliquots were taken and the concentration of inorganic phosphate was determined [14]. In all experiments, the degree of ATP conversion in the reaction did not exceed 5% and the time dependence of the inorganic phosphate liberation was linear. We have shown that 15% reduction in the volume of the reaction mixture (for the samples) may be neglected.

In a typical experiment, the activity of myosin bound to 60 cm nylon fiber corresponded to 0.05—0.1 μmol P_i/min.

Papain treatment

To cut off HMM S-1 from immobilized myosin, 25 cm nylon fiber carrying myosin (0.05 mg) was placed into 1 ml of 0.06 M borate buffer (pH 7.5) containing 20 μg of papain + 0.1 mg of cysteine and incubated at 25°C with constant stirring for 10 min.

The weight ratio between the active papain and the immobilized myosin was 1 : 300; this means that the specificity of action of papain was ensured and resulted in subfragment HMM S-1 being cleaved off [8].

Then the fiber was taken out of the solution, thoroughly washed in 0.5 M KCl and its ATPase activity was assayed. At the same time, the ATPase activity in the papain solution where the fiber had been incubated was assayed. The fiber was then thoroughly washed in 0.5 M KCl, placed in fresh papain solution and incubated at 25°C for 10 min. This treatment was performed several times, and the data on the ATPase activity on the fiber and in the solution were plotted against the time of incubation with papain.

Stretching of nylon fiber

Experiments elucidating the effect of the stretching of the fiber on the enzymatic properties of the myosin bound to it were carried out with the use of a special stretching device described previously [11,12].

Study of the kinetics of inactivation of myosin bound to nylon fiber by picryl sulfonic acid

Inactivation of the ATPase of the myosin bound to the nylon fiber by picryl sulfonic acid was carried out by the known method [15,16], somewhat modified by us. The myosin-carrying fiber, stretched or unstretched, was placed in a thermostatted cuvette at 20°C, containing 10^{-4} M picryl sulfonic acid (30 ml) in 10^{-2} M Tris · HCl buffer (pH 7.5), and incubated for a certain time. The fiber was removed, washed quickly with $5 \cdot 10^{-3}$ M EDTA/0.3 M NH_4Cl (pH 8.0) and the ATPase activity of the immobilized myosin was determined with the fiber unstretched.

Study of the kinetics of inactivation of nylon fiber-bound myosin by 5,5'-dithio-bis(2-nitrobenzoic acid)

Inactivation of the ATPase of myosin by 5,5'-dithio-bis(2-nitrobenzoic acid) was carried out according to the method of Seidel [17]. The fiber, stretched or unstretched, was incubated at 6°C for some time in 30 ml of $7 \cdot 10^{-5}$ M 5,5'-dithio-bis(2-nitrobenzoic acid)/$3 \cdot 10^{-2}$ M KCl/$5 \cdot 10^{-2}$ M $MgSO_4$/10^{-2} M Tris · HCl (pH 8.0). Then the fiber was taken out, quickly washed with 5 ·

10^{-3} M EDTA/0.3 M NH_4Cl (pH 8.0) and the ATPase activity of the myosin bound to it was determined, with the fiber being unstretched.

Results and Discussion

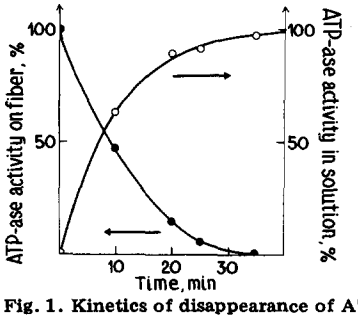
Effect of papain on immobilized myosin

Immobilization of myosin on the nylon fiber resulted in a preparation with a sufficiently high catalytic activity. There are good grounds for believing that the ATPase activity revealed is caused by the action of those myosin molecules which are bound to nylon with their rod part. This is in a way supported by the previously reported fact that immobilization of subfragment HMM S-1 leads to inactivation of ATPase [13]. To prove unambiguously that in immobilized total myosin heads with ATPase activity are not covalently bound to the support, we tried, by hydrolyzing the protein partially, to remove the ATPase activity and make it go to the solution. For this purpose, the immobilized enzyme was incubated with papain in a ratio ensuring specific cleavage of the subfragment HMM S-1.

Fig. 1 illustrates the results of the ATPase activity assay on the fiber and in the bathing solution. As can be noted from the figure, 35 min incubation of the fiber with papain causes its complete inactivation with the ATPase activity being now in the solution. Both these processes obey the first-order kinetics and are characterized by the same rate constant. Hence, they are interrelated and are, in fact, the consequences of the single phenomenon.

Thus the experimental data obtained evidence that the fiber ATPase activity is caused by those myosin molecules which are bound to nylon fiber with their rod parts (LMM + HMM S-2) and have free heads which readily go into the solution when treated with papain.

The rod part of the myosin molecule seems to be bound to the nylon surface via multiple covalent linkages, since the amount of lysine residues in LMM and HMM S-2 involved in the protein-support binding is very great [18]. Therefore, nylon fiber stretching is expected to cause deformation of the rod part of those myosin molecules which are bound on the fiber longitudinally. We wondered



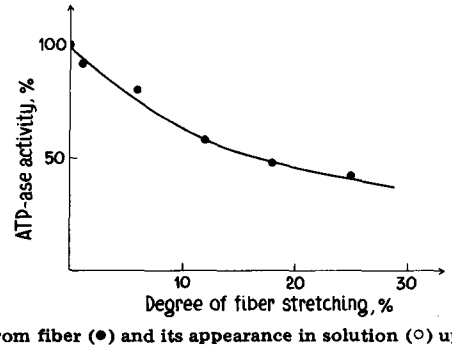


Fig. 1. Kinetics of disappearance of ATPase activity from fiber (•) and its appearance in solution (○) upon treatment of myosin bound to nylon fiber by papain. For conditions see Experimental.

Fig. 2. Dependence of ATPase activity of myosin bound to nylon fiber on the degree of fiber stretching. For conditions see Experimental.

what effect the stretching of the rod part of these molecules would have on their ATPase activity.

Effect of nylon fiber stretching on the ATPase activity of bound myosin

Upon nylon fiber stretching the ATPase activity of bound myosin significantly decreases (Fig. 2) and is only 40—50% of the initial value. After cessation of stretching and fiber relaxation, the ATPase activity reaches the initial level. The "stretch-relax" procedure may be repeated endlessly with the "decrease-increase" of ATPase activity being retained.

We studied the dependence of the rate of ATP enzymatic hydrolysis on the substrate concentration for the myosin bound to unstretched and stretched fiber. Analysis of the Lineweaver-Burk plots showed (Fig. 3) that, when fiber is stretched, the apparent Michaelis constant for ATP hydrolysis with immobilized myosin does not change, whereas the maximum rate of this enzymatic process decreases. The results obtained cannot be ascribed to the altered diffusion conditions of the enzyme system [11,19], since (as can be inferred from the fact that the Michaelis constant remains the same upon fiber stretching) the value of the mechanochemical effects in Fig. 2 is not substrate concentration-dependent.

Another explanation for the decrease in myosin activity when the fiber is stretched (Fig. 2) may be that the microenvironment of the immobilized enzyme alters when the support is deformed, i.e. the parameters of the micromedium in which the enzymatic reaction proceeds (e.g. local pH, dielectric constant etc.) change. The obvious way to verify this possibility is to bind myosin to a support whose surface possesses properties other than those of nylon fiber and to repeat the mechanochemical experiments. To this end, to the surface of the nylon fiber treated with glutaraldehyde not myosin but bovine serum albumin was bound. The amino groups of the immobilized albumin were treated with glutaraldehyde, then another layer of albumin was bound. This

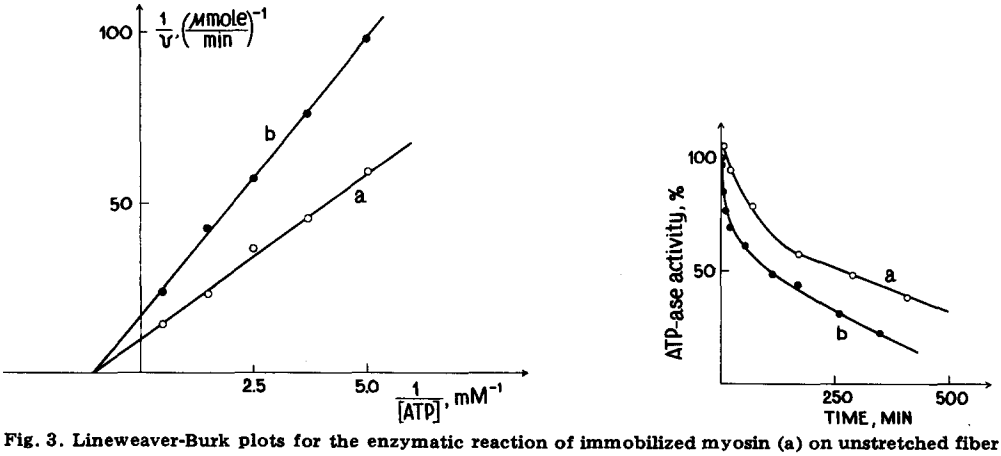


Fig. 3. Lineweaver-Burk plots for the enzymatic reaction of immobilized myosin (a) on unstretched fiber and (b) on fiber stretched by 12%. For conditions see Experimental.

Fig. 4. Kinetics of inactivation of myosin bound to nylon fiber by picryl sulfonic acid. Inactivation was performed with (a) unstretched and (b) stretched fiber. For conditions see Experimental.

procedure was repeated three times. As a result, we had nylon fiber coated with protein (albumin); this surface is different from that of nylon fiber. This fiber was again treated with glutaraldehyde and myosin was bound to it. When the fiber is stretched, the ATPase activity of the bound myosin decreases in the same way as when myosin is bound to a nonmodified nylon fiber (Fig. 2). Thus the reversible inactivation of myosin induced by deformation of the fiber does not depend on the nature of the surface to which the enzyme is bound.

Another mechanism of inactivation of the immobilized myosin on fiber stretching may be that the enzyme's active centres become less accessible for the substrate when the support is deformed (e.g. due to the enzyme being pressed against the surface of the fiber). To verify this possibility, we studied the effect of fiber stretching on the interaction rate between the bound myosin and picryl sulfonic acid which reacts specifically with ϵ-amino groups of lysine in the active centre of myosin and inactivates the enzyme [15,16]. The rate of interaction between immobilized myosin and picryl sulfonic acid on fiber stretching proved to sharply increase (Fig. 4). Thus the reversible inactivation of myosin occurring on fiber stretching is not to be accounted for by steric blocking of the active centre when the support is deformed. Apparently, according to this mechanism, when fiber is stretched, the rate of interaction between immobilized myosin and any specific reagent affecting the active centre must decrease.

So the most likely explanation for the mechanochemical effect obtained (Fig. 2) is that myosin loses its catalytic activity as a result of deformation of its native structure. It should be emphasized that it is the part of enzyme molecule which is remote from the active centre and is bound to the nylon fiber, namely, fragment LMM + HMM S-2, that is primarily subjected to deformation.

Molecular model of the mechanochemical effect

Considering that the rod part of the myosin molecule is capable of being bound to nylon fiber in many points, the following schematic representation of immobilized molecules retained their ATPase activity should be suggested (Fig. 5A). With this method of immobilization, the stretching of fiber will result in the points where the myosin molecule binds to the support beginning to diverge, which will entail deformation of the myosin rod (Fig. 5B). In terms of the suggested model, relaxation of the fiber will be accompanied by relaxation of the rod fragments of myosin molecules until the initial conformation is attained.

As can be noted from Fig. 2, when the nylon fiber is stretched, the ATPase activity of the bound myosin decreases by only 60%. This may be explained by the fact that not all the molecules of immobilized myosin can respond to fiber stretching (e.g. myosin molecules bound transversally on the fiber cannot). This conclusion is supported by our experiments with 5,5′-dithio-bis(2-nitrobenzoic acid). This reagent is known to modify specifically SH-groups of myosin active centre, which results in the ATPase activity of the enzyme being inhibited [17]. We have shown that, when the fiber is stretched, the rate of inactivation of the bound myosin by 5,5′-dithio-bis(2-nitrobenzoic acid) decreases (Fig. 6A). This result may mean that on fiber-stretching-induced

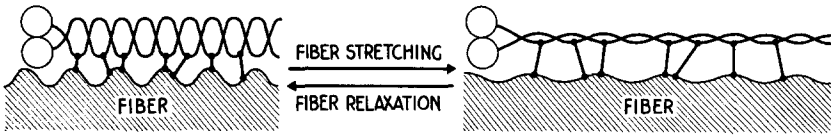


Fig. 5. Schematic representation of fiber-stretching-induced deformation of an immobilized myosin molecule.

deformation of myosin, the reactivity of catalytically essential groups of myosin active centre decreases. In terms of our molecular model, it would be logical to consider that the reactivity of 5,5′-dithio-bis(2-nitrobenzoic acid) becomes lower only with respect to the molecules of immobilized myosin which are capable of being deformed when fiber is stretched, and remains unaltered in the molecules unaffected by the support stretching.

From the above suggestion it may be inferred that if the fiber with myosin bound to it is stretched and then treated with 5,5′-dithio-bis(2-nitrobenzoic acid), it is the immobilized stretching-insensitive myosin molecules that will be primarily inactivated. On the other hand, the enzyme molecules which are sensitive to stretching (thereby, both their ATPase activity and the capacity for interaction with the inhibitor decrease) will hardly be subjected to inactivation. This will result in an increase in the value of the mechanochemical effect in the case when myosin bound to stretched fiber is treated with 5,5′-dithio-bis(2-nitrobenzoic acid).

The mentioned effect, as shown, does take place (Fig. 6B). As a result of incubation of myosin on stretched fiber with 5,5′-dithio-bis(2-nitrobenzoic acid), the mechanochemical effect increased from 2 (with the degree of stretching of 17%) to 8-fold. This result shows that our molecular model is adequate.

The results obtained allow one to conclude that the decrease in the ATPase activity observed on fiber stretching occurs due to deformation of the rod part of the myosin molecule.

Changes in the ATPase activity of myosin on stretching were reported to

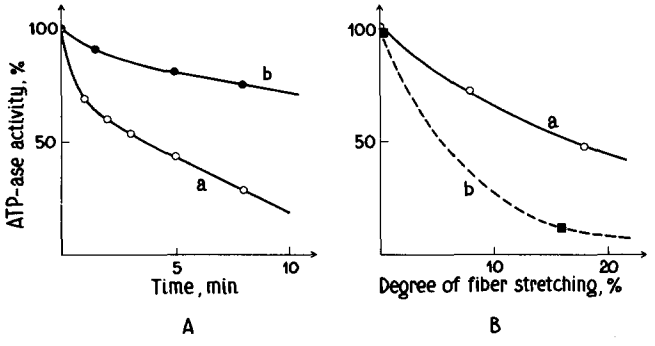


Fig. 6. A. Kinetics of inactivation of myosin bound to nylon fiber by 5,5′-dithio-bis(2-nitrobenzoic acid). Inactivation was performed with (a) unstretched and (b) stretched fiber. B. Dependence of the ATPase activity of myosin bound to nylon fiber on the degree of stretching of fiber (a) before treatment and (b) after 70 min treatment with 5,5′-dithio-bis(2-nitrobenzoic acid). For conditions see Experimental.

take place in insect flight muscles [20—23]. On stretching these muscles, their ATPase activity increased, and so did the tension in them and the work they perform.

On contraction, the above parameters in these muscles returned to the initial level. As in insect flight muscles myosin protofibrils are connected with Z-lines [24,25], the increase in the ATPase activity on stretching the muscles [20—23] should be the result of direct mechanical deformation of tail portions of myosin molecules that form the backbone of the protofibril. Chaplain [21] showed that activation of ATPase of insect flight muscles on their stretching is associated with enhanced binding of Ca^{2+} ions by myosin molecules. On stretching the muscle, growth of tension on it (associated with increase in ATPase activity) lags behind elongation (just as decrease in tension lags behind shortening) [26—28]; this means that the contractile apparatus of flight muscles is capable of performing its own periodical contractions (oscillations). Thus, the functional correlation between the ATPase activity of myosin heads and the mechanical state of its tail part play a very important role in insect flight muscles; this is actually the molecular basis of myogenic rhythm of their contraction.

In our experiments, changes in the ATPase activity of myosin heads on mechanical deformation of the tail part was also found to take place in myosin from rabbit skeletal muscle. In what direction the ATPase activity of myosin on deformation of the tail part in an intact muscle will change can be predicted neither from the results of our experiments (as we determined NH_4^+-EDTA-activity of myosin and not the Mg^{2+}-activity of actomyosin), nor from the data on stretching flight muscles of insects (because skeletal muscles, unlike flight ones, have no myosin-mediated Ca^{2+}-regulation of ATPase, and changes of the ATPase activity on stretching myosin may proceed via a basically different mechanism). In addition, in skeletal muscles of vertebrates, myosin protofibrils are not connected with Z-lines and mechanical tension is transmitted to the tail parts of myosin not directly but only through its heads. That is why it is very hard to name the processes in which the discovered regulatory property of skeletal myosin reveals itself. We can only offer speculations.

It should be noted, for example, that the drop in the ATPase activity on myosin stretching is in conformity with the theory of muscle contraction put forward by Davies [29]. This theory suggests that ATP cleavage occurs after shortening of myosin molecule. This means that if we stretch myosin (i.e. do not permit it to shorten), the rate of ATP hydrolysis in the system will drastically decrease.

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