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THE ADENOSINE TRIPHOSPHATASE ACTIVITY OF THE MEROMYOSINS

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SUMMARY

1. Mg^{2+} does not activate the ATPase (ATP phosphohydrolase, EC 3.6.1.3) of actin–heavy meromyosin complex (acto–HMM) at low ionic strength.

2. The interaction inhibitor heparin, which dissociates actomyosin and inhibits its ATPase activity, also inhibits the ATPase of acto–HMM.

3. Acto–HMM does not show Ca^{2+} sensitivity, *i.e.* its ATPase activity is not inhibited by the removal of Ca^{2+} with ethyleneglycol-bis(aminoethyl)-tetraacetic acid.

4. The enzymic characteristics of the ATPase-active component of light meromyosin (LMM) preparations are the same as those of heavy meromyosin (HMM).

5. The ATPase-active component of LMM preparations can be removed from the preparation by ultracentrifugation in the presence of actin in 0.5 M KCl.

INTRODUCTION

Several investigators have studied the ATPase activity of the different fragments resulting from the proteolysis of myosin[1–7]. Experiments of this kind can give some insight into the changes undergone in the catalytic center of myosin as a result of proteolysis on the one hand and in the mechanism of the ATPase activity of the more complex parent myosin on the other. The published observations, however are incomplete and partly contradictory. On the ATPase activity of acto–HMM *e.g.* LEADBEATER AND PERRY[4] report an activating, and other authors[3,5,6] an inhibiting, effect of Mg^{2+}. There is no clarity on the Ca^{2+} need of acto–HMM either, a question which has an outstanding importance regarding the essential role of Ca ions[8–12] in the enzymic and physicochemical behavior of actomyosin. The nature of the low ATPase activity found in LMM is a subject that has received only little consideration. The aim of this paper is to report additional evidence concerning the questions outlined above.

Abbreviations: HMM, heavy meromyosin; LMM, light meromyosin; EGTA ethyleneglycol-bis(aminoethyl)-tetra-acetic acid; acto–HMM, actin–heavy meromyosin complex.

Materials and methods

Myosin was prepared according to Portzehl, Schramm and Weber[13] and actin according to Bárány *et al.*[14] followed by the purification of Mommaerts[15].

Meromyosins were prepared with trypsin or chymotrypsin essentially as described by Szent-Györgyi[16], proteolysis being inhibited after digestion by addition of diisopropylfluorophosphate up to 0.01 M or 0.001 M final concentration[17].

The proteolytic enzymes were lyophilized preparations of Köbányai Gyógyszergyár, Budapest. The ATP used was a product of Reanal, Budapest.

ATPase activity was measured at 20° in 2-ml samples containing 0.01 M Tris–HCl buffer (pH 7.2), 0.01 M KCl, 1 mM ATP and, if not otherwise stated, 1 mM $MgCl_2$. Incubation was terminated by addition of 2 ml 10% trichloroacetic acid. The concentration of proteins and the time of incubation was chosen so as to obtain a splitting of the terminal phosphate of ATP of less than 25%.

Inorganic phosphorus was measured according to Fiske and SubbaRow[18], and protein according to Gornall, Bardawill and David[19].

For preparative ultracentrifugation a Spinco Model D apparatus was used.

Results

ATPase activity of HMM

It has been suggested by several authors that the functionally important enzymic activity of actomyosin is the one observed under conditions of Mg^{2+} activation[20,21]. Hence this activity in myosin fragments merits a special interest. Leadbeater and Perry[4], on the ground of activability by Mg^{2+} of acto–HMM, concluded that the mechanism of ATP splitting by the fragment is essentially identical with that of the parent myosin under these conditions. These authors first used HMM prepared by chymotrypsin, but the effect was observed to some extent with the fragment obtained by trypsin too. In contrast to this, Nagai *et al.*[3] and Yagi, Nakata and Sakakibara[5] report an inhibiting action of Mg^{2+} on acto–HMM or at least no activation. Mueller[6], using the actin complex of the subfragment obtained from HMM by further tryptic digestion, obtained inhibition by Mg^{2+}. In most of our eighteen experiments we obtained practically no effect of Mg^{2+} on the ATPase activity of acto–HMM prepared by either proteolytic enzyme and with an actin to HMM ratio varied over a wide range. With some of the preparations we obtained a marked inhibition (unpublished).

The addition of ATP diminishes the viscosity of acto–HMM solutions, which suggests that the complex dissociates. Paradoxically, actin enhances the ATPase activity of HMM under the same conditions[3]. The results obtained with "interaction inhibitors"[22,23], substances that dissociate actomyosin at low ionic strength in the presence of ATP, offer a simple means to probe further into this intriguing question. Heparin giving no inhibition on myosin alone, as shown in this laboratory[12], seemed well suited to this kind of experiment as its inhibiting effect on the ATPase activity of actin complexes might be supposed to result uniquely from its dissociating influence. As shown in Fig. 1, the ATPase activity of acto–HMM is depressed by heparin to the level of activity obtained with HMM alone.

Another aspect of the relation between inhibition and dissociation of the actin complex is represented by the role of Ca^{2+}. Many investigations have led to the con-

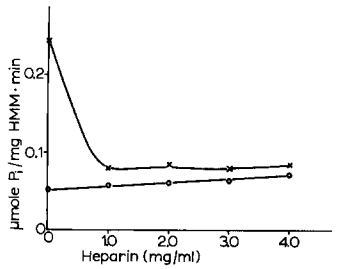

Fig. 1. Effect of heparin on the ATPase activity of HMM and acto–HMM. 0.44 mg HMM (prepared by trypsin), 0.2 mg actin, incubation 3 min. X, with actin; ○, without actin. For other details see METHODS.

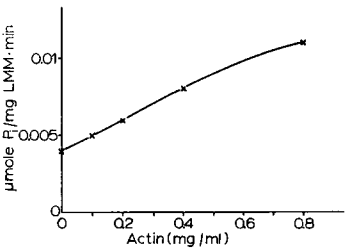

Fig. 2. Effect of actin on the ATPase activity of LMM. 0.6 mg/ml LMM (prepared by trypsin), incubation 60 min. For other details see METHODS.

clusion that the low activity of actomyosin systems observed on removal of trace amounts of Ca^{2+} is the result of dissociation[8–12]. Experiments with acto–HMM on the essentiallity of Ca^{2+} have not been reported as yet. We tried to clarify the question by ensuring a low concentration of Ca^{2+} by the use of the chelating agent EGTA. This agent has a negligible affinity for Mg^{2+}, at about pH 7, offering the possibility of reducing the concentration of Ca^{2+} (present as impurity) without influencing the Mg^{2+} concentration[24]. In Table I we summarize the results of an experiment in which the concentration of EGTA was varied at two different actin: HMM ratios. As may be seen, the removal of ionized Ca^{2+} from the milieu had no influence on the ATPase activity.

TABLE I

DEPENDENCE OF THE ATPASE ACTIVITY OF ACTO–HMM ON EGTA CONCENTRATION

HMM (prepared by trypsin), 0.3 mg/ml. Incubation 3 min. For other conditions see METHODS.

EGTA (M)	*HMM mg per actin mg*	*μmole P_i per mg HMM per min*
0	2.2	0.25
$1 \cdot 10^{-4}$	2.2	0.25
$5 \cdot 10^{-4}$	2.2	0.26
$1 \cdot 10^{-3}$	2.2	0.24
$5 \cdot 10^{-3}$	2.2	0.25
0	1.1	0.24
$1 \cdot 10^{-4}$	1.1	0.24
$5 \cdot 10^{-4}$	1.1	0.23
$1 \cdot 10^{-3}$	1.1	0.23
$2 \cdot 10^{-3}$	1.1	0.23

We carried out an analogous experiment at 0°, as this low temperature favors dissociation[25] and enhances the Ca^{2+} level needed to prevent dissociation[12] of actomyosin systems. No effect was obtained with acto–HMM at 0° (Table II).

As reported by WEBER AND WINICUR[29], with actomyosins formed from different actin preparations, the removal of Ca^{2+} caused inhibition only with certain of the actin

TABLE II

DEPENDENCE OF THE ATPase ACTIVITY OF ACTO–HMM ON EGTA CONCENTRATION AT 0°

HMM (prepared by trypsin) 0.45 mg/ml. Incubation 60 min, 0°. For other conditions see METHODS.

EGTA (M)	*HMM mg per actin mg*	*μmole P_i per mg HMM per min*
0	2.2	0.0109
$1 \cdot 10^{-4}$	2.2	0.0116
$5 \cdot 10^{-4}$	2.2	0.0119
$1 \cdot 10^{-3}$	2.2	0.0119
$2 \cdot 10^{-3}$	2.2	0.0112

preparations whereas others did not need Ca^{2+}. This was corroborated later by EBASHI and was traced to the effect of an impurity in actin tentatively identified by him as tropomyosin[26]. It seems that the experiments on the Ca^{2+} requirement of acto–HMM are conclusive only when the actin component used gives an actomyosin inhibited by the removal of Ca^{2+}. Table III shows the influence of EGTA on the ATPase activity of actomyosin formed with the actin preparations used for experiments of Tables I and II together with the analogous experiments for acto–HMM.

ATPase activity of LMM

It is generally accepted that the ATPase center of myosin goes with the HMM fragment in the course of fragmentation. Nevertheless a small but readily measurable ATPase activity is found in LMM preparations in spite of repeated precipitations. This activity was found to be inseparable even in the course of fractionation of LMM on ion exchanger cellulose[27]. A more precise characterization of this ATPase as well as the elucidation of the question whether it results from a contamination of LMM (by myosin or HMM) have not been found in the literature. Our experiments on these questions will now be reported.

As Fig. 2 shows, in the presence of Mg^{2+}, LMM is activated by actin in the manner of myosin and HMM. On the other hand, as shown by the experiment of Table IV, Mg^{2+} inhibits the ATPase of LMM in the presence of actin, *i.e.* LMM be-

TABLE III

EFFECT OF EGTA ON THE ATPase ACTIVITY OF MYOSIN, HMM AND LMM IN THE PRESENCE OF ACTIN

Concentration of myosin, HMM and LMM (both prepared by trypsin) 0.45 mg/ml, actin 0.2 mg/ml; EGTA $2.5 \cdot 10^{-4}$ M. Incubation: myosin 5 min; HMM 3 min; LMM 60 min. Activity: μmole P_i per min per mg myosin, HMM and LMM respectively. For other conditions see METHODS.

Conditions	*Activity*
Actomyosin	0.19
Actomyosin + EGTA	0.09
Acto–HMM	0.26
Acto–HMM + EGTA	0.24
Actin + LMM	0.0065
Actin + LMM + EGTA	0.0062

TABLE IV

EFFECT OF Mg^{2+} ON THE ATPASE ACTIVITY OF LMM IN PRESENCE OF ACTIN

LMM (prepared by trypsin) 0.46 mg/ml, actin 0.2 mg/ml. Incubation 60 min. For other conditions see METHODS.

Composition of the samples	*μmole P_i per mg LMM per min*
LMM + actin	0.011
LMM + actin + 0.001 M $MgCl_2$	0.008
LMM	0.003
LMM + 0.001 M $MgCl_2$	0.003

haves like HMM and differs from myosin. LMM ATPase activated by actin is not inhibited by EGTA (Table III) in this respect parallelling HMM and deviating from myosin.

In several recent investigations it was found that after further proteolytic degradation of HMM, the ATPase-active center and the actin-binding center of myosin are still intact and in the same subfragment[6,28]. Thus if the ATPase activity found in LMM is derived from the HMM part of myosin and is not a property of LMM proper, it can conceivably be separated with the help of actin as described by MUELLER AND PERRY for "subfragment 1" (ref. 28) provided that LMM does not bind actin in such circumstances. In fact when LMM is ultracentrifugated in 0.5 M KCl with the addition of actin practically all ATPase activity sediments out, in spite of the fact that the bulk of the protein of LMM remained in the supernatant (Table V). The ATPase activity of LMM is thus due to contamination by some protein in which the ATPase-active and actin-binding centers of myosin are coexistent.

TABLE V

SEPARATION OF LMM FROM THE ATPASE-ACTIVE COMPONENT BY MEANS OF ULTRACENTRIFUGATION IN THE PRESENCE OF ACTIN

LMM and myosin dissolved in 0.5 M KCl were clarified by ultracentrifugation at 205 000 × *g* for 2 h. Ultracentrifugation at 205 000 × *g* for 2 h was repeated after the addition of actin. Samples of actin, myosin and LMM were also ultracentrifuged in the same way in order to estimate the amount of non-sedimenting protein present. Before and after ultracentrifugation, protein and ATPase of the supernatant were measured on suitable aliquots. Ca^{2+}-activated ATPase was measured as this is uninfluenced by the presence of actin. Conditions of ATPase measurement: 1 mM $CaCl_2$, 1 mM ATP, 0.5 M KCl, 0.01 M Tris–HCl (pH 7.2), LMM or myosin concentration in the test 0.5 mg/ml, 30 min incubation at 20° (LMM was prepared by trypsin).

Composition of the samples	*Protein content (mg/ml)*		*Activity (μmole P_i per min per mg) myosin or LMM*	
	Before centri-fugation	*After centri-fugation*	*Before centri-fugation*	*After centri-fugation*
LMM	1.95	1.84	0.046	0.041
Myosin	1.98	1.79	0.320	0.315
Actin	0.98	0.12	—	—
LMM + actin	2.87	1.82	0.037	0.004
Myosin + actin	3.01	0.45	0.280	0.007

DISCUSSION

The experiments presented in this paper show to important differences between the enzymic behavior of the actin complex of myosin and HMM. (1) Acto–HMM is not activated by Mg^{2+}; in fact sometimes it is inhibited. (2) No requirement for trace amounts of Ca^{2+} was found for acto–HMM ATPase. These results show clearly that the enzymic site of myosin is not entirely unaffected by the proteolytic process. The effect of Ca^{2+} can well be explained for actomyosin as a supression of dissociation by this ion. Hence for acto–HMM it is reasonable to assume that its actin complex with enhanced activity can be formed without the intervention of Ca^{2+}. Perry[4] and Yagi, Nakata and Sakakibara[5] found that, while activation by actin shows clearly that HMM interacts with actin in the presence of ATP, the viscosity drop is indicative of dissociation of the complex. The two observations can be reconciled if it is assumed that actin is attached to HMM at two distinct sites. One "static" site has no direct role in the splitting of ATP and is dissociated by it. This dissociation gives the viscosity effect. The other "dynamic" site binds actin only concomitantly with the binding (and enzymic splitting) of the substrate[29]. This second binding has the character of an intermediary complex formed of actin, HMM and ATP. The splitting in the presence of Mg^{2+} at low ionic strength is faster *via* this complex than through the action of the ATPase site of HMM alone. The requirements for Ca^{2+} of this assumed "dynamic site" would be abolished by the changes in the protein molecule that occur during fragmentation.

According to some experiments in progress in this laboratory, the loss of activability of the actin complex of the myosin digest by Mg^{2+} precedes fragmentation to some extent. These experiments (to be published later) suggest that the change in the enzymic characters of the active center is a consequence of proteolysis independent of fragmentation, *i.e.* it would be a change in the HMM part of the myosin molecule. The ATPase-active component of LMM resembles HMM in enzymic character (inhibition by Mg^{2+} of the actin complex and no requirement for Ca^{2+}; see Table III). On the other hand, it is inseparable by solubility differences from LMM proper. LMM has solubility characteristics much resembling those of the intact myosin molecule. It could thus be tentatively assumed that the ATPase-active protein fraction of LMM is a form of the myosin molecule which is not yet fragmented but has undergone the change affecting the enzymic center of myosin during the course of proteolysis. Tentative work on the isolation of this fraction of LMM is in progress in our laboratory.

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