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ACTIVATION BY ACTIN OF ATPase ACTIVITY OF CHEMICALLY MODIFIED GIZZARD MYOSIN WITHOUT PHOSPHORYLATION

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SUMMARY: The ATPase activity of myosin from chicken gizzard measured in the presence of either Mg^{2+} or Ca^{2+} is increased in the absence of dithiothreitol or upon reaction with Cu^{2+}, o-iodosobenzoate, or N-ethylmaleimide. Iodosobenzoate or Cu^{2+} produce no change in K^{+}(EDTA)-ATPase while N-ethylmaleimide produces a decrease. These treatments also make the actin-activated ATPase insensitive to Ca^{2+} when assayed in the presence of tropomyosin and a partially purified myosin light chain kinase. Phosphorylation of N-ethylmaleimide modified myosin remains dependent on Ca^{2+} and therefore appears not to be required for activation by actin of the ATPase activity of modified myosin.

There is convincing evidence that the interaction of actin with myosin from smooth muscle can be regulated by Ca^{2+}-dependent phosphorylation of the 20,000-dalton light chain (1-4), however, there is also evidence that actin activation of some myosin preparations from smooth muscle does not depend on phosphorylation but does respond to Ca^{2+} in the presence of actin-bound regulatory proteins (5,6). Some of the enzymatic properties of gizzard myosin have been reported to change with time (6) raising the possibility that oxidation might influence the ATPase activity and the interaction with actin. In addition the activity of myosin alone is influenced by thiol modification and there is evidence that myosins from gizzard and from skeletal muscle may differ in their response to thiol modifiers (7-9). The present work shows that oxidation of gizzard myosin or its modification with N-ethylmaleimide increase the Ca^{2+}-ATPase activity and make the actin-activated ATPase activity insensitive to Ca^{2+} and to phosphorylation of myosin.

METHODS

Myosin was prepared from fresh chicken gizzards essentially as described by Sobieszek and Small (10) with 0.1 to 0.5 mM 1,4-dithiothreitol (DTT) being

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included in all steps of the preparation. Tropomyosin from chicken gizzards was obtained by one isoelectric precipitation (at pH 4.9) of the 37-55% ammonium sulfate fraction from "native" tropomyosin (11) described by Aksoy et al. (1). F-actin was prepared from rabbit skeletal muscle as described by Spudich and Watt (12). The partially purified fraction containing myosin light chain kinase used in this work was the 37-55% ammonium sulfate fraction of "native" tropomyosin referred to as P_{37-55} (1).

Ca^{2+}-activated ATPase activity was determined by incubation of gizzard myosin in 25 mM NaCl, 40 mM Tris, pH 7.5, 10 mM $CaCl_2$, and 5 mM ATP, K^+(EDTA)-ATPase in 0.6M KCl, 5 mM EDTA, 40 mM Tris, pH 7.5 and 5 mM ATP and Mg^{2+}-ATPase in 80 mM NaCl, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, and either 0.1 mM EGTA or 0.1 mM $CaCl_2$. The reaction was terminated by adding an equal volume of 10% trichloroacetic acid and released inorganic phosphate determined by the method of Fiske and Subbarow (13). Incorporation of ^{32}P from γ-[^{32}P] ATP into myosin was measured as described previously (14).

Treatment with N-ethylmaleimide was carried out for 10 min at 0° in a solution containing 0.5M KCl, 40 mM morpholinopropane sulfonic acid (MOPS), pH 7.5, 1 mM N-ethylmaleimide and 10 mg of myosin per ml, the reaction being terminated by adding 0.01M 1,4-dithiothreitol. The reaction with o-iodosobenzoate was initiated by adding iodosobenzoate to a final concentration of 0.01M in a solution containing 0.3M NaCl, 0.1M MOPS, pH 7.5, and 10 mg of myosin per ml. The reaction was carried out for 10 min at 25°, the samples placed in ice, and Mg^{2+}-, Ca^{2+}-, or K^+(EDTA)-ATPase activities were determined within one hour. For measurement of ATPase activity in the presence of actin and regulatory proteins excess reagent was removed by adding 14 volumes of 1 mM MOPS, pH 7.0, containing 0.1 mM EDTA. The myosin was allowed to settle, the supernatant removed and the myosin either washed with a solution containing 40 mM NaCl, 1 mM MOPS, pH 7.0, and 0.1 mM EDTA or resuspended in the same solution without the washing step. Treatment with Cu^{2+} was done by adding $Cu(NO_3)_2$ to a final concentration of 3 µM for activity of myosin alone and 10 µM for actin-activated ATPase in a solution containing 0.5M NaCl, 1 mM MOPS, pH 7.5. ATPase activities were determined without removal of Cu^{2+}. DTT was removed either by dialysis against 400 volumes of a solution containing 0.5M NaCl, 1 mM MOPS, pH 7.5, and 0.1 mM EDTA or by the dilution procedure described above.

RESULTS AND DISCUSSION

The Ca^{2+}-activated ATPase activities of gizzard myosin prepared with DTT present throughout the preparation are consistently low when measured at low ionic strength (25 mM NaCl) but removal of DTT by dialysis after completion of the preparation can produce as much as a 4-fold increase in activity (Table I). This increase is not observed in 0.6M KCl and it is not accompanied by any change in the K^+(EDTA)-activated ATPase activity. Because it was difficult to define standard conditions under which we could consistently observe this "spontaneous" increase, we attempted to produce the effect with sulfhydryl modifiers. Both Cu^{2+} (15) and iodosobenzoate (16) produce 2- to 3-fold increases in both Ca^{2+}-ATPase and Mg^{2+}-ATPase measured in 25 mM NaCl and little or no change in

TABLE I

ATPase Activities of Myosin Alone

Additions	ATPase Activity ΔP_i, μmoles/mg myosin/min			
	Ca^{2+}	Ca^{2+}+KCl	Mg^{2+}	K^+(EDTA)
DTT (in preparation)	0.037	0.144	0.0051	0.39
None (DTT removed)	0.176	0.154	-	0.45
$Cu(NO_3)_2$	0.097	0.114	0.0188	0.34
o-iodosobenzoate	0.270	0.182	0.0110	0.37
N-ethylmaleimide	0.140	0.182	0.0119	0.11

K^+(EDTA)-ATPase (Table I). With N-ethylmaleimide the increase in Ca^{2+}-ATPase is accompanied by a loss of K^+(EDTA)-ATPase as in the case of myosin from skeletal muscle (17). When the assay is done at 25° N-ethylmaleimide affects Ca^{2+}-ATPase only at low ionic strength (Table I) but at 37° N-ethylmaleimide increases Ca^{2+}-ATPase in 0.6M KCl (9), a result we also have obtained. Treatment of rabbit skeletal muscle myosin with iodosobenzoate under conditions used for gizzard myosin increased Ca^{2+}-ATPase in 0.6M KCl from 0.17 to 0.29 μmoles/mg/min without a change in the K^+(EDTA)-ATPase. Iodosobenzoate increased Ca^{2+}-ATPase of gizzard heavy meromyosin from 0.033 to 0.137 μmoles P_i/mg/min indicating that the increase is not related to formation of myosin filaments.

Initial measurements of ATPase activity in the presence of actin, tropomyosin and P_{37-55}, an ammonium sulfate fraction of "native" tropomyosin (1), showed a decrease in the Ca^{2+}-dependence upon removal of DTT used in the preparation of myosin (Table II), suggesting that oxidation of sulfhydryl groups might influence the activation by actin and that sulfhydryl modifiers might have similar effects. Indeed, treatment of gizzard myosin with Cu^{2+}, iodosobenzoate, or N-ethylmaleimide increases the actin-activated ATPase in the presence of EGTA and there is no further activation by micromolar concentrations of Ca^{2+} (Table II). It may be noted that either N-ethylmaleimide or Cu^{2+} produce some decrease

TABLE II

ATPase Activities in the Presence of Actin

Additions	ATPase Activity ΔP_i, nmoles/mg myosin/min		
	Myosin Alone + EGTA	Complete System + EGTA	Complete System + Ca^{2+}
	Experiment 1		
DTT (in preparation)	3.2	10.2	27.5
None (DTT removed)	2.9	18.5	29.3
o-iodosobenzoate	10.6	29.9	35.7
N-ethylmaleimide	10.5	19.0	19.6
	Experiment 2		
DTT	2.4	7.2	47.1
$Cu(NO_3)_2$	7.0	24.0	25.0

The assay system contained 1.3 mg of myosin per ml. The complete system also contained 0.2 mg of tropomyosin per ml, 0.37 mg of P_{37-55} per ml, and 1.0 mg of actin per ml with N-ethylmaleimide, 1.1 mg/ml with Cu^{2+}, and 1.5 mg/ml with and without DTT and with iodosobenzoate.

in the actin-activated ATPase in the presence of Ca^{2+}, tropomyosin and P_{37-55}, which also was observed in some experiments with iodosobenzoate. That three different sulfhydryl modifiers produce a loss of the dependence of activity on Ca^{2+} suggests that modification of sulfhydryl groups accounts for the effect and suggests that oxidation of protein sulfhydryls produces the decrease in Ca^{2+} sensitivity that is seen upon removal of DTT. This change occurs readily in the absence of reducing agents and has been observed on storage of gizzard myosin for several weeks after adding 0.1 mM DTT. It may be related to changes in the ATPase activity previously reported on storage of gizzard myosin (6). The effects of N-ethylmaleimide resemble those observed in the rabbit skeletal muscle system where reaction of myosin with N-ethylmaleimide can abolish the Ca^{2+} sensitivity of the actin-activated ATPase, which in this case is regulated by the tropomyosin-troponin system (18).

TABLE III

Effects of Tropomyosin and an Ammonium Sulfate Fraction Containing Myosin Light Chain Kinase on Actin-Activated ATPase of N-Ethylmaleimide Modified Myosin

	ATPase Activity ΔP_i, nmoles/mg myosin/min			
	NEM Myosin		Control Myosin	
Additions	+EGTA	$+Ca^{2+}$	+EGTA	$+Ca^{2+}$
-	10.5	-	3.2	-
Actin	11.8	11.1	-	-
Actin + P_{37-55}	17.7	20.4	-	-
Actin + tropomyosin	12.4	14.4	-	-
Actin + P_{37-55} + tropomyosin	19.0	19.6	10.2	27.5

Assay conditions as described in Table II.

Although modification of myosin with N-ethylmaleimide abolishes the Ca^{2+} requirement for activation by actin, actin alone does not activate unless fraction P_{37-55} is present. The active substance in P_{37-55} cannot be replaced by purified gizzard tropomyosin, which itself has no effect on actin activation in the presence or absence of P_{37-55}, indicating that a factor other than tropomyosin still is required for activation by actin (Table III).

To determine whether modification alters enzymatic phosphorylation of myosin we measured the incorporation of ^{32}P from γ-[^{32}P] ATP into native and N-ethylmaleimide modified myosin in the presence of P_{37-55}. Although with both native and modified myosins ^{32}P incorporation requires Ca^{2+} (Table IV), the actin-activated ATPase of native myosin depends on Ca^{2+} but that of modified myosin does not (Table II). These results studies strongly suggest that phosphorylation is not required for actin-activated ATPase with N-ethylmaleimide modified myosin. Activation without phosphorylation has been previously reported (5) but unlike the present modified myosins those preparations were dependent on Ca^{2+}. Whether modifications having slightly different effects than

TABLE IV

Effect of N-ethylmaleimide on Phosphorylation of Myosin

Myosin	^{32}P Incorporation moles per mole myosin +EGTA	+Ca^{2+}
Control	0.39	0.86
N-ethylmaleimide treated	0.23	0.76

The incubation medium contained 0.7 mg of myosin per ml, and 0.46 mg of P_{37-55} per ml, under conditions described for measuring Mg^{2+}-ATPase activity. No incorporation occurred in the absence of myosin.

does oxidation might contribute to previous results (5,6) and whether modifications other than phosphorylation might have physiological significance are questions which remain to be answered.

The effects of sulfhydryl reagents provide a third example of a chemical modification of myosin which permits activation of ATPase activity by actin, the other two being proteolytic digestion with papain or trypsin (14,19-21) and phosphorylation (1-4). That such diverse modifications can mimic the effect of phosphorylation suggests a molecular mechanism involving a structural change in the actin binding site but in which the phosphoryl group does not participate directly in actin binding.

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