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CHYMOTRYPTIC HEAVY MEROMYOSIN FROM GIZZARD MYOSIN: A PROTEOLYTIC FRAGMENT WITH THE REGULATORY PROPERTIES OF THE INTACT MYOSIN

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SUMMARY: Proteolytic cleavage of gizzard myosin using α-chymotrypsin under conditions which produce HMM when applied to skeletal muscle myosin yields an enzymatically active fragment which behaves like the original myosin in that its ATPase activity is activated by actin but only with the participation of Ca^{2+} and additional regulatory proteins. In contrast, papain-subfragment-1 from gizzard myosin has an actin-activated ATPase that is dependent neither on Ca^{2+} nor on regulatory proteins. The chymotryptic fragment can be phosphorylated by a partially purified preparation of light chain kinase, and contains intact 20,000 and 17,000-dalton light chains and a heavy chain with an apparent molecular weight of approximately 120,000, based on gel electrophoresis in sodium dodecyl sulfate.

Myosin of vertebrate smooth muscles differs from that of skeletal muscles in that its ATPase activity is not activated by actin without the participation of Ca^{2+} and regulatory proteins (1-4). The nature of these proteins remains controversial but most evidence implicates a Ca^{2+}-dependent myosin light chain kinase (4-10, cf. 11,12). In contrast, proteolytic fragments of myosin from smooth muscles do not exhibit these requirements for activation by actin (13-15). The actin-activated ATPase of S-1* prepared by digestion of gizzard myosin with papain requires neither regulatory proteins nor Ca^{2+} and is not influenced by the Ca^{2+} concentration (13,14), while S-1 from vascular smooth muscle myosin is either insensitive or partially sensitive to Ca^{2+}, depending on the method of preparation (15). We have digested gizzard myosin with α-chymotrypsin and found that the resulting fragment exhibits the same type of Ca^{2+}-sensitive actin-activated ATPase as the parent molecule.

*Abbreviations used: EGTA, 2,2'-ethylenedioxybis[ethyliminodi(acetic acid)]; HMM, heavy meromyosin; S-1, subfragment-1; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Myosin was prepared from chicken gizzards essentially as described by Sobieszek and Small (13) using a single precipitation by $MgCl_2$. Actin (16) and tropomyosin (17) were prepared from rabbit skeletal muscle by established methods. The light chain kinase preparation from chicken gizzard used in this work was the 37 to 55% ammonium sulfate fraction of "native tropomyosin" (1) described by Aksoy et al. (3).

Chymotryptic digestion of gizzard myosin was carried out under conditions approximating those used by Weeds and Pope (18) to prepare HMM from myosin of rabbit skeletal muscle. Digestion was carried out at 25° for 10 min in a solution containing 0.5M KCl, 0.01M $MgCl_2$, 40 mM morpholinopropane sulfonic acid, pH 7.5 and 0.1 mM 1,4- dithiothreitol with myosin at a concentration of 15 to 20 mg/ml and α-chymotrypsin at concentrations of 0.01 to 0.25 mg/ml. The reaction was terminated by adding sufficient 0.1M phenylmethylsulfonyl fluoride in ethanol to give a final concentration of 0.3 to 0.5 mM. The digest was dialyzed overnight against 100 to 200 volumes of a solution containing 0.04M NaCl, 0.1 mM EDTA, 0.2 mM 1,4-dithiothreitol and 5 mM morpholinoproprane sulfonic acid, pH 7.0, and the suspension centrifuged at 50,000 xg for 60 min to remove undigested myosin and insoluble fragments. Measurements of enzymatic activity and ^{32}P incorporation were carried out with the supernatant fraction from this step. Further purification was carried out by chromatography on a 1x30 cm column of DEAE cellulose (Whatman DE-52) using a gradient of 0.04 to 0.5M NaCl in 0.1 mM EDTA, 0.2 mM 1,4-dithiothreitol and 5 mM morpholinoethane sulfonic acid, pH 7.5. Papain S-1 was prepared from gizzard myosin as described by Sobieszek and Small (13) using 0.2 mg of papain per ml, a 10 min digestion and termination of the reaction by addition of N-α-p-tosyl-L-lysine chloromethyl ketone to a final concentration of 5 mM. Polyacrylamide gel electrophoresis in SDS was carried out according to Weber and Osborn (19).

ATPase activity was determined by incubating myosin or chymotryptic fragments at a concentration of 1.5 to 2.5 mg/ml in a medium containing 35 mM KCl, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$ for 15 min, terminating the reaction with an equal volume of 10% trichloroacetic acid, removing the precipitate by low-speed centrifugation and determining inorganic phosphate by the method of Fiske and Subbarow (20). ^{32}P incorporation was determined by incubation for 10 min under the conditions used for the ATPase assay modified to include 0.5 mM -labelled [^{32}P] ATP (1 μCi/μmole). The reaction was terminated by adding an equal volume of 2% SDS and dialyzing exhaustively against a solution containing 1% (w/v) SDS, 0.1M NaCl and 10 mM sodium phosphate, pH 7.0. Radioactivity was determined by Cerenkov counting (21).

RESULTS AND DISCUSSION

When gizzard myosin is digested with α-chymotrypsin, dialyzed to lower the salt concentration to 0.04M NaCl, and centrifuged to remove insoluble proteins, the ATPase activity of the soluble fraction is not activated by actin unless both Ca^{2+} and a partially purified preparation of gizzard light chain kinase, a preparation that also contains tropomyosin, are included in the assay system, the activity in the presence of the kinase and EGTA being essentially that of the

TABLE I

Enzymatic Activities of Myosin and Its Proteolytic Fragments

Additions to Assay	ATPase Activity ΔP_i, n moles/mg/min		
	Myosin	Chymotryptic HMM	Papain S-1
None	7	6	41
Actin	-	6	94
Actin + kinase + Ca^{2+}	24	33	93
Actin + kinase + EGTA	8	7	85

ATPase activities were measured in 35 mM KCl, 10 mM $MgCl_2$, 20 mM Tris, pH 7.5, 2 mM ATP, 0.1 mM DTT and when added, 2 mg rabbit skeletal muscle actin per ml, 0.05 to 0.2 mg chicken gizzard kinase per ml, and when added, 0.1 mM $CaCl_2$ or 0.1 mM EGTA. Chymotryptic digestion was carried out for 10 min with 0.05 mg of α-chymotrypsin per ml.

chymotryptic fragment alone (Table I). Thus the fragment has the same requirement for Ca^{2+} and regulatory proteins as intact gizzard myosin. In contrast, the ATPase of S-1 prepared by digestion of gizzard myosin with papain is activated by actin alone and is not significantly influenced by the addition of the kinase either in the presence or absence of Ca^{2+} (Table I). Digestion with papain also produces a 6-fold increase in the Mg^{2+}-ATPase activity measured in the absence of actin, a larger change than can be attributed to removal of the rod portion of myosin, suggesting that digestion with papain alters the active site of the molecule. No increase in Mg-ATPase is observed on digestion with chymotrypsin.

The ATPase activity of the chymotryptic fragments in the presence of kinase (Fig. 1) increases with actin concentration in the presence of Ca^{2+} while actin has no effect in the presence of the Ca^{2+} chelator, EGTA. Double reciprocal plots of activities measured in the presence of Ca^{2+} are linear (Fig. 1, inset);

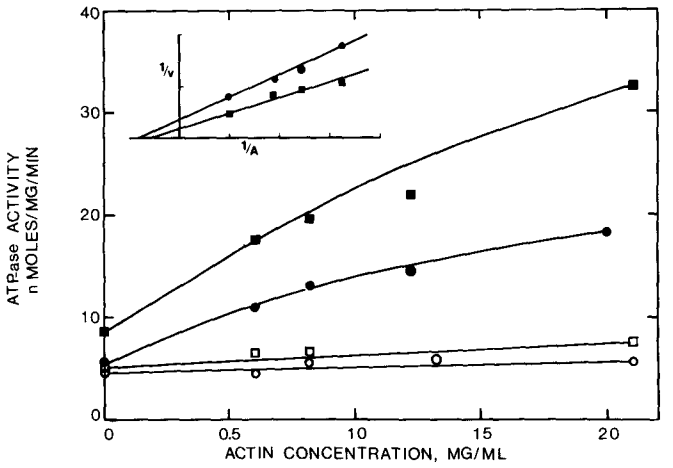

Fig. 1. ATPase activity of fragments prepared by digestion with varying concentrations of α-chymotrypsin, assayed in the presence of 0.1 mM $CaCl_2$, closed symbols, or 0.1 mM EGTA, open symbols. The concentrations of chymotrypsin were 0.05 mg/ml, □ , ■ ; and 0.25 mg/ml, ○ , ● . Inset represents data obtained in the presence of Ca^{2+}.

TABLE II

ATPase Activity and Phosphorylation of Myosin and Its Chymotryptic Fragments

Concentration of Chymotrypsin	ATPase Activity ΔP_i, nmoles/mg myosin/min		^{32}P Incorporation
mg/ml	Mg	Mg+Actin + Kinase	moles/10^5g
0	1.4	25	0.36
0.01	-	-	0.46
0.05	1.6	51	0.23
0.25	1.1	26	0.08

The ATPase activity and phosphorylation of the chymotryptic fragments were determined on the protein fraction soluble in 0.04M NaCl. The 37 to 55% ammonium sulfate fraction of native tropomyosin was used as a source of light chain kinase in the phosphorylation and actin-activated ATPase assays. The actin-activated ATPase of the chymotryptic fragments is the maximal velocity determined from a plot of 1/v vs. 1/actin concentration.

the maximal velocity, determined by extrapolating to infinite actin concentration, decreases when the concentration of chymotrypsin used in the digestion is increased to 0.25 mg/ml (Table II). This decrease is somewhat greater than that observed in the absence of actin, suggesting that the loss of actin activation is not correlated with the loss of the activity of myosin alone. The extent of enzymatic phosphorylation of the chymotryptic fragments by myosin light chain kinase also decreases with increasing concentrations of chymotrypsin (Table II). The greatest incorporation of ^{32}P -- 0.46 moles per 10^5g of protein -- corresponds to 1.5 moles per mole of protein using a molecular weight of 320,000, twice the sum of the molecular weights of the heavy and light polypeptide chains estimated by gel electrophoresis in SDS (Fig. 2).

Electrophoresis of the soluble fragments produced by chymotryptic digestion (Fig. 2) indicates that low concentrations of chymotrypsin produce very limited digestion; essentially a single band is observed in the region of the heavy chains with an apparent molecular weight of approximately 120,000, this value suggesting that the fragment corresponds to HMM produced by digestion of myosin from rabbit skeletal muscle. More extensive digestion leads to cleavage of additional peptide bonds and formation of fragments of approximately 70,000 and 65,000 daltons. Bands are also observed at positions corresponding to molecular weights of 20,000 and 17,000 which represent the L_{20} and L_{17} light chains, respectively. Little or no L_{20} light chain is observed in digests made with higher concentrations of chymotrypsin, which exhibit low levels of enzymatic phosphorylation and low actin-activated ATPase activities.

The increase in actin-activated ATPase with concomitant cleavage of L_{20}, which occurs on digestion of arterial myosin with papain, led Mrwa and Ruegg to suggest that L_{20} inhibits the actin-myosin interaction and the inhibition can be overcome reversibly and physiologically by phosphorylation or irreversibly by digestion with papain (15). On the other hand, Okamoto and Sekine (22) have observed that tryptic digestion of gizzard myosin leads first to inhibition of the Ca^{2+}-regulated, actin-activated ATPase, which is then followed by a reactiva-

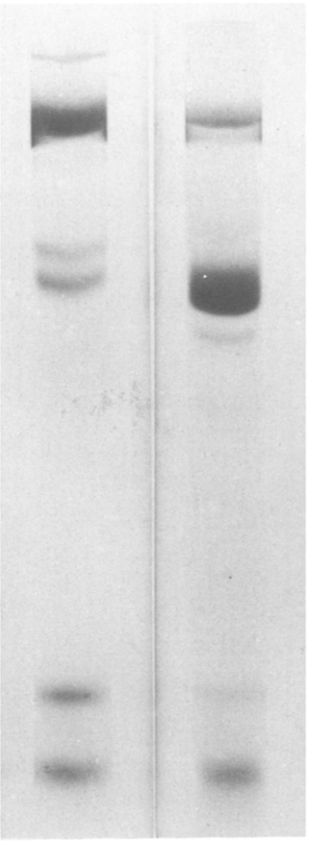

Fig. 2. Polyacrylamide gel electrophoresis in SDS. 7.5% polyacrylamide gels were used. Samples are material soluble in 0.04M NaCl and subsequently purified by chromatography on DEAE-cellulose. Sample A was prepared by digestion with 0.05 mg chymotrypsin/ml and B with 0.25 mg chymotrypsin/ml.

tion, and disappearance of the Ca^{2+} requirement for activity. Our results indicate that chymotryptic digestion of gizzard myosin initially produces a soluble fragment without major changes in the enzymatic properties, while more extensive digestion leads to a loss of the actin activation and cleavage of the L_{20} light chain. However, loss of activation by actin also is accompanied by proteolysis of the heavy chains, and therefore our present results do not provide a critical test of whether or not the L_{20} light chain acts as an inhibitor of

actin-activated ATPase activity. They do indicate that proteolytic cleavage of the light chain is not sufficient to produce Ca^{2+}-independent actin activation of the ATPase activity.

The soluble chymotryptic fragment of gizzard myosin described here, having actin-activated ATPase activity dependent on Ca^{2+} and regulatory proteins, should be a valuable tool in enzymatic and physico-chemical studies on the nature of the actin-myosin interaction and its regulation by Ca^{2+} in smooth muscle.

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