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AN EFFECT OF TRYPSIN ON THE ACTIN–MYOSIN INTERACTION*

DARREL E. GOLL, R. M. ROBSON**, JOANNE TEMPLE AND M. H. STROMER

Departments of Animal Science, Biochemistry and Biophysics, and Food Technology, Cooperating, Iowa State University, Ames, Iowa 50010 (U.S.A.)

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SUMMARY

Three different lines of evidence were obtained to show that trypsin modifies the actin–myosin interaction: (1) At trypsin to actomyosin or myosin ratios between 1 to 300 and 1 to 500, 30 min of trypsin treatment causes an 8-fold increase in the Ca^{2+}-modified ITPase activity of actomyosin but has no effect on the Ca^{2+}-modified ITPase activity of myosin alone. At these same trypsin to actomyosin ratios, the $Mg^{2+} + Ca^{2+}$-modified ATPase activity increases by 10–30% during the first 1–2 min of trypsin digestion, and then decreases rapidly to less than 20% of its original activity after 60 min of digestion. Trypsin has no effect on the $Mg^{2+} + Ca^{2+}$-modified ATPase activity of pure myosin. (2) The rate of turbidity response of reconstituted actomyosin suspensions is first increased and then decreased by trypsin treatment. At trypsin to actomyosin ratios of 1 to 3000, rate of turbidity response is maximal after 5 min of trypsin digestion and then decreases; after 60 min, the turbidity response is much slower than the response of the control actomyosin. (3) Supercontracted sarcomeres, shortened to less than 50% of their initial length, are lengthened to 70% of their initial length by 4 min of trypsin treatment. Myosin B from such lengthened sarcomeres has less than 35% of its myosin converted to light meromyosin and heavy meromyosin.

These results show that trypsin modifies the actin–myosin interaction in at least two ways: (1) a very rapid initial modification that increases the $Mg^{2+} + Ca^{2+}$-modified ATPase activity and the rate of turbidity increase, and (2) a slower modification that decreases the $Mg^{2+} + Ca^{2+}$-modified ATPase activity and rate of turbidity response, and that lengthens contracted sarcomeres. Tryptic modification is not due to cleavage of myosin to light and heavy meromyosin. Since tryptic modification occurs more rapidly than conversion of myosin to light and heavy meromyosin, all heavy meromyosin preparations will be modified.

Abbreviations: EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane; myosin B, natural actomyosin or an actomyosin made by direct extraction of myofibrils.

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** Present address: Muscle Biochemistry Group, 124 Animal Science Laboratory, University of Illinois, Urbana, Ill. 61801, (U.S.A.).

INTRODUCTION

The usefulness of proteolytic enzymes as probes of the molecular architecture of proteins has been well established[1–4]. Among the muscle proteins, proteolytic enzymes have been particularly beneficial in studies on the structure of myosin[5–10]. For the most part, these studies have been based on the early finding of GERGELY[11] and PERRY[12] that trypsin very rapidly lowered the viscosity of myosin solutions. It was subsequently shown that myosin possesses two proteolytically labile areas, one near the center of the molecule and another near the junction between the "head" or globular portion of the molecule and the "tail" or rod portion of the molecule[13–16]. Cleavage at the center of the molecule results in fission of myosin into two parts, called light meromyosin and heavy meromyosin[17]. The heavy meromyosin fragment contains the "head" part of the parent myosin molecule together with a short section of the tail portion, whereas the light meromyosin fragment is composed only of the remaining tail portion of myosin[18,19]. Moreover, the ATPase and actin-binding properties of myosin remain with the heavy meromyosin fragment, which now differs from myosin by being water soluble.

Tryptic cleavage at the second proteolytically labile site of myosin, near the junction between the head and tail sections of the molecule, ordinarily requires longer digestion times and higher trypsin-to-myosin ratios than cleavage into light and heavy meromyosin[19–21]. Recently, however, several different conditions have been discovered that make it possible to cleave intact myosin between its head and tail portions before the molecule is split into light and heavy meromyosin. These conditions include proteolytic digestion using an insoluble complex of papain with cellulose[15,22,23] or tryptic digestion at an ionic strength of 0.02 in the presence of 10 mM $CaCl_2$ (refs. 24–26). Evidently, under these conditions, cleavage at the light meromyosin–heavy meromyosin site is suppressed and the largest proportion of peptide bonds split are located near the head region of the myosin molecule.

Extensive studies on the proteolytic dissection of myosin have shown that each myosin molecule contains only one light meromyosin fragment and one heavy meromyosin fragment[26–28], and that the heavy meromyosin portion of the molecule forms the cross-bridges extending from the surface of the thick (myosin) filament toward the thin (actin) filament[18]. These findings have increased interest in the actin–heavy meromyosin interaction in the hope that this interaction would possess all the characteristics of the actin–myosin interaction but in a simpler system. There have, however, been a number of studies indicating that the actin–heavy meromyosin interaction may differ subtly from the actin–myosin interaction. YAGI *et al.*[29] found that the complex of F-actin with heavy meromyosin was more easily dissociated by ATP than the complex of F-actin with myosin. This finding was supported by EISENBERG AND MOOS[30] who have demonstrated that raising either the ATP or KCl concentration causes acto–heavy meromyosin to dissociate before any effect is noticed on actomyosin. EISENBERG AND MOOS[30] also confirmed an earlier report by LEADBEATER AND PERRY[31] that actin modified the ATPase activity of heavy meromyosin even when viscosity measurements indicated that actin and heavy meromyosin had not physically combined. On the other hand, actin activation of myosin ATPase is always accompanied by (or the result of) a physical interaction between actin and myosin. Additional evidence that the actin–heavy meromyosin interaction differs from the actin–myosin

interaction was provided by the report of PERRY AND COTTERILL[32] that under selected conditions, phenylmercuric acetate treatment of heavy meromyosin causes a complete loss of ATPase activity without affecting the ability of heavy meromyosin to interact with F-actin; the same conditions, however, cause simultaneous loss of ATPase and actin-binding activities in purified myosin. This response of heavy meromyosin to phenylmercuric acetate is probably related to the finding that maximal stimulation of the Ca^{2+}-modified ATPase activity of myosin requires 3 times more phenylmercuric acetate on a molar basis than is needed to cause maximal stimulation of the Ca^{2+}-modified ATPase activity of heavy meromyosin under identical conditions[33], even though myosin has a lower molar content of cysteine than heavy meromyosin.

Collectively, these results suggest that heavy meromyosin has undergone some conformational change during its proteolytic release and that the actin–heavy meromyosin interaction may differ subtly from the actin–myosin interaction. This hypothesis is supported by the finding of MARUYAMA *et al.*[34] who concluded on the basis of ATPase activities, that trypsin-treated myosin B (as distinguished from acto–heavy meromyosin) dissociated into actin and myosin at a lower KCl concentration than did untreated myosin B. Consequently, we have studied the effects of very brief tryptic digestion of different actomyosin-containing systems, using NTPase activities, rate of superprecipitation, and changes in contraction state as indices of the actin–myosin interaction.

MATERIALS AND METHODS

Protein preparations

All preparations described in this paper were obtained from the back and leg muscles of rabbits; essentially identical results have been obtained with preparations from bovine or porcine skeletal muscle. All solutions were prepared with double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers. Rabbits were handled as described by ARAKAWA *et al.*[35].

Myosin and α-actinin-free actin were prepared as described by SERAYDARIAN *et al.*[36], and reconstituted actomyosin (1 part of actin to 2 parts of myosin by weight) was made within 1 h after fresh actin and myosin became available[35]. Myosin B was made as described by ROBSON *et al.*[37] and myofibrils were made according to STROMER *et al.*[38] or by a modification of the method of PERRY AND GREY[39]. All protein preparations were used within 7 days. Protein concentration was estimated by using the biuret method[40] as modified by ROBSON *et al.*[41].

Trypsin treatment

Both trypsin and trypsin inhibitor were the purest, crystalline, salt-free preparations available from Sigma Chemical Co. Stock trypsin solutions were made by dissolving trypsin (2–5 mg/ml) in 0.001 M HCl. These solutions were stable for several months at 2°. Soybean trypsin inhibitor (4–10 mg/ml) was dissolved in water. The activity of all trypsin preparations was routinely monitored by using rate of change of absorbance at 247 nm with *p*-toluenesulfonyl-L-arginine methyl ester as a substrate. All trypsin preparations used in this study had 14000–21000 *p*-toluenesulfonyl-L-arginine methyl ester units of activity[42] per mg. Protein preparations (myofibrils, myosin B, reconstituted actomyosin, or myosin) were incubated with trypsin for

specified periods of time at 25° and pH 7.6, and the reaction stopped by addition of a 4-fold excess of soybean trypsin inhibitor. The ionic composition and amount of trypsin used during trypsin treatment will be given in the individual experiments.

NTPase and turbidity assays

ATPase and ITPase activities were assayed immediately after trypsin treatment. After trypsin had been inactivated by soybean trypsin inhibitor, the protein in the digestion mixture was diluted to a concentration appropriate for NTPase assay, the specified modifiers added, and the assay conducted at 25°. NTPase activity was measured according to the procedure of GOLL AND ROBSON[43]. Turbidity measurements were also made immediately after trypsin treatment and were done as described by ARAKAWA *et al.*[35]. The electrolyte medium will be specified in the individual experiments.

Analytical ultracentrifugation

Analytical ultracentrifuge studies were conducted on a Spinco Model E ultracentrifuge equipped with a phase plate and rotor temperature indicator control unit. All runs were done with Kel-F centerpieces, and the plates were measured with a Nikon 6C profile projector.

Microscopy

Phase microscopic observations were made with a Zeiss photomicroscope with a green interference filter and a 100X planapochromatic objective in the light path. After each treatment, an aliquot of the myofibril suspension was removed, pelleted at 2000 rev./min for 10 min and the supernatant discarded. The pellet was covered with 2.5% glutaraldehyde until it could be removed intact from the tube for subsequent cutting into 1-mm cubes. Glutaraldehyde–O_8O_4 fixation was used for all samples. Sections mounted on uncoated grids were stained with uranyl acetate and lead citrate and examined in a RCA EMU-4 electron microscope.

RESULTS

NTPase experiments

The conclusion that trypsin modifies the actin–myosin interaction will be supported in this paper by three lines of complementary evidence. The first of these is the effect of trypsin on the NTPase activities of actomyosin preparations (Figs. 1–3). When assayed at 10 mM KCl, the 1 mM Ca^{2+}-modified ATPase activity increases by 125–150% during the first 30 min of trypsin treatment (trypsin to actomyosin ratio of 1 to 300, by weight), whereas the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase activity decreases by approx. 30% during the same period of time (Fig. 1). When the assays are done at 50–100 mM KCl, however, the 1 mM Ca^{2+}-modified ATPase activity changes very little during 30 min of tryptic action (Fig. 1) and the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase activity exhibits a biphasic response (Fig. 2). The 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase of reconstituted actomyosin increases by 10–30% during the first 1–2 min of tryptic digestion and then decreases rapidly (Fig. 2). After 60 min of tryptic treament, the specific activity is only 20% as high as the initial activity before trypsin treatment.

The marked decrease that occurs in specific activity of the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase after 5–10 min of trypsin digestion is easily obtained under many different conditions of tryptic treatment and occurs in myofibril or myosin

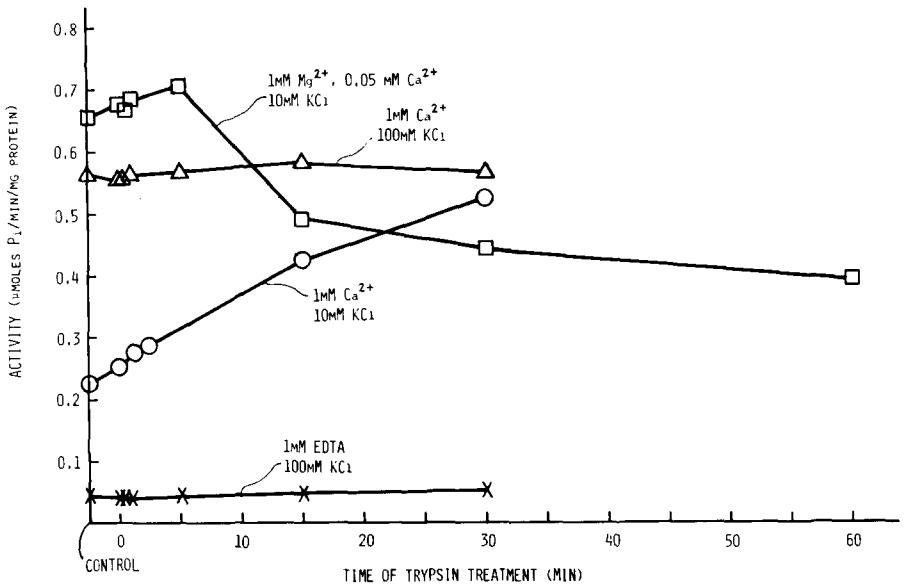

Fig. 1. Effect of trypsin treatment on the ATPase activity of reconstituted actomyosin suspensions. Reconstituted actomyosin was incubated with trypsin (1 part of trypsin to 300 parts of reconstituted actomyosin, by weight) for the times shown on the abscissa. Control samples had water added in place of trypsin and trypsin inhibitor; zero-time samples had trypsin that had first been mixed with four parts (w/w) of trypsin inhibitor added to them. Conditions for trypsin treatment: 2.5 mg reconstituted actomyosin per ml, 100 mM KCl, 50 mM Tris–HCl (pH 7.6) 25°. Reaction was stopped by a 4-fold (w/w) addition of soybean trypsin inhibitor, the suspension diluted, and aliquots removed immediately for ATPase assay. Conditions of ATPase assay: 0.2 mg actomyosin per ml, 20 mM Tris–HCl (pH 7.6.), 1 mM ATP, 25°, KCl and activators as shown specifically by labels in the figure.

B preparations as well as in reconstituted actomyosin (Fig. 2). However, the conditions of tryptic digestion must be more carefully controlled if the small increase in specific activity after 1–2 min of tryptic treatment is to be observed. When using reconstituted actomyosin, this increase is most easily detected if the tryptic digestion is done at ionic strengths below 0.15 and in the presence of 6–10 mM Ca^{2+}. The increase is larger (30–50% higher than control activities, Fig. 2) and more easily detected at higher ionic strengths when myofibrils are used in place of reconstituted actomyosin (Fig. 2). Even when myofibrils are used, however, the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase assay must be done immediately after tryptic digestion if the rapid initial increase in specific activity is to be observed. Assay immediately after trypsin treatment is necessary because the tryptic-induced increase in activity occurs very quickly, within the first 2–5 min of treatment at trypsin to actomyosin ratios as low as 1 to 3000 by weight. Furthermore, a 4-fold excess of soybean trypsin inhibitor does not completely stop tryptic proteolysis but instead lowers its rate approximately a 100-fold (Table I). Evidently, even this markedly lower rate of hydrolysis is sufficient to cause an increase in 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified

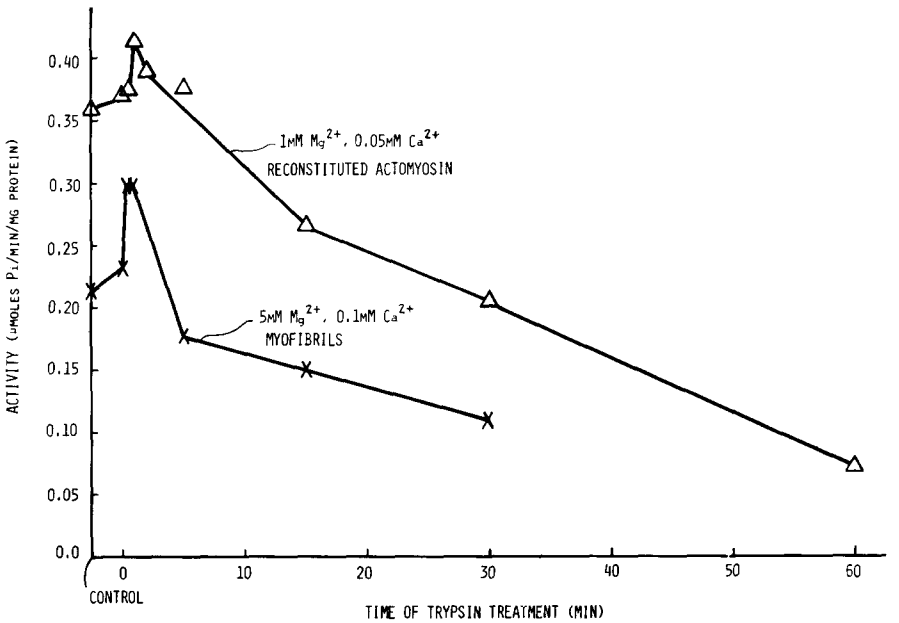

Fig. 2. Effect of trypsin on the Mg^{2+} + Ca^{2+}-modified ATPase activity of reconstituted actomyosin and myofibrils. Control and zero-time samples handled as described in legend to Fig. 1. Conditions for trypsin treatment of reconstituted actomyosin: actomyosin suspended in 20 mM $CaCl_2$, 50 mM Tris–HCl (pH 7.6), 350 mM KCl at 7.0 mg protein per ml and 0° for 14 h; then treated with 1 part of trypsin to 500 parts of actomyosin (w/w) in 100 mM KCl, 50 mM Tris–HCl (pH 7.6), and 5.7 mM $CaCl_2$ at 2.5 mg protein per ml and 25°. Reaction stopped by a 4-fold addition of soybean trypsin inhibitor, and samples diluted for ATPase assay. Conditions for ATPase assay of trypsin-treated actomyosin: 0.2 mg actomyosin per ml, 50 mM KCl, 20 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM ATP, 25°. Conditions for trypsin treatment of myofibrils: 2.8 mg myofibrillar protein per ml, 110 mM KCl, 110 mM Tris–HCl (pH 7.6), 1 part of trypsin to 100 parts of myofibrillar protein, 25°. Reaction stopped by 4-fold addition of trypsin inhibitor and samples diluted for ATPase assay. Conditions for ATPase assay of myofibrils: 0.8 mg myofibrillar protein per ml, 108 mM KCl, 46 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM ATP, 25°. △—△, trypsin-treated reconstituted actomyosin; ×—×, trypsin-treated myofibrils.

TABLE I

ACTIVITY OF TRYPSIN IN THE PRESENCE OF SOYBEAN TRYPSIN INHIBITOR AND AFTER DENATURATION

Conditions for assay of trypsin activity: 0.87 mM *p*-toluenesulfonyl-L-arginine methyl ester, 50 mM sodium phosphate (pH 8.0), 0.5 μg of trypsin, 25°; total volume, 3.0 ml. A unit of trypsin activity was defined as that amount which would cause an increase in $A_{247\,nm}$ of 0.001 per min at 25° (*cf.* ref. 56). In tube with soybean trypsin inhibitor, 2.0 μg of inhibitor were mixed with 0.5 μg of trypsin before addition to the cuvette. Trypsin was denatured by refluxing at 100° at pH 4.0 for 3 h, followed by freezing, and then refluxing at 100° for 6 h. This treatment did not cause any precipitation so the trypsin solution was diluted and 0.5 μg of the diluted solution added directly to the cuvette.

Treatment	*Units of tryptic activity*
Untreated trypsin	15400 (6)*
Trypsin + soybean trypsin inhibitor	130 (4)
Denatured trypsin	430 (1)

* Figures are averages of determinations done on several different trypsin preparations, the numbers in the parentheses represent the number of different preparations used in calculating the mean.

ATPase activity after 60–90 min, since activity of control samples (when trypsin and trypsin inhibitor were mixed prior to addition to the actomyosin) was 20–50% higher if assays were done several hours after addition of trypsin *plus* trypsin inhibitor than if assays were done within 15 min after such addition. An indication of this rapid increase in specific activity can be observed in Fig. 2 where the control or zero-time samples had slightly higher specific activities than the samples that received no trypsin or trypsin inhibitor addition.

Although the increase in specific activity of the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase is small, repeated experiments have left no doubt that it occurs in all three contractile preparations that we have tested (myofibrils, myosin B and reconstituted actomyosin). The inclusion of 0.1 mM Ca^{2+} in our assays of trypsin-treated myofibrils and myosin B (Fig. 2) eliminates the possibility that tryptic digestion of troponin contributed to the results obtained with these preparations. The 1 mM EDTA modified ATPase activity is also changed by tryptic treatment, increasing gradually by 15–25% during 30 min of digestion (Fig. 1). The 1 mM 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane (EGTA)-modified ATPase activity (not shown here) exhibited nearly the same change during tryptic treatment as the EDTA-modified ATPase.

The ITPase activities of reconstituted actomyosin preparations were also altered by trypsin treatment (Fig. 3). When ITPase assays were done at 10 mM KCl,

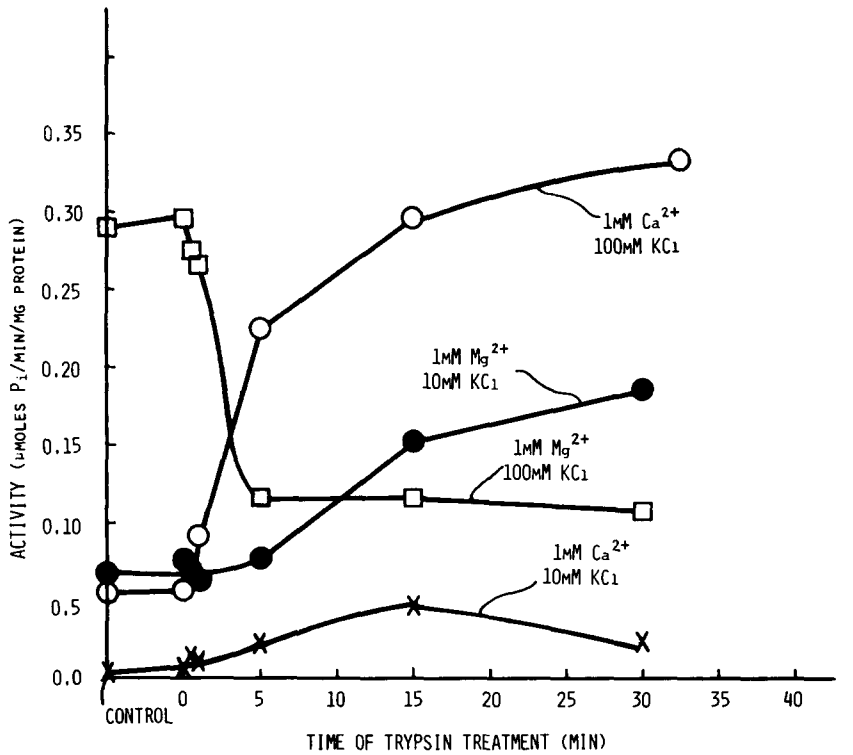

Fig. 3. Effect of trypsin on the ITPase activity of reconstituted actomyosin suspensions. Conditions for trypsin treatment and ITPase assay were identical to those described in the legend to Fig. 1 except that 1 mM ITP was substituted for 1 mM ATP. KCl concentration and activators are indicated specifically by the labels in the figure.

the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ITPase increased nearly 2-fold and the 1 mM Ca^{2+}-modified ITPase activity increased slightly. The very low specific activity of the 1 mM Ca^{2+}-modified ITPase at 10 mM KCl made it difficult to quantitatively estimate the magnitude of the trypsin-induced increase in activity. When ITPase assays were done at 100 mM KCl, the 1 mM Ca^{2+}-modified ITPase increased markedly with increasing time of trypsin treatment to a specific activity almost 10-fold the initial activity (Fig. 3). The 1 mM Mg^{2+} + 0.05 mM Ca^{2+} -modified ITPase activity, however, decreased with increasing time of trypsin treatment at 100 mM KCl, until after 30 min of treatment, the specific activity was only 35–40% as high as the initial activity. Tryptic digestion of myofibrils or myosin B produced changes in ITPase activities identical to those shown here for reconstituted actomyosin.

To prove that the tryptic-induced changes in NTPase activities of actomyosin preparations reflect an alteration in the actin–myosin interaction, it is necessary to demonstrate that trypsin does not cause identical changes in the NTPase activity of myosin in the absence of actin. The results in Figs. 4 and 5 show that the tryptic-induced increase in 1 mM Ca^{2+}-modified ATPase activity at 10 mM KCl occurs during digestion of pure myosin as well as during digestion of actomyosin (*cf.* Figs. 1 and 4). However, none of the other tryptic-induced changes in actomyosin NTPase activity

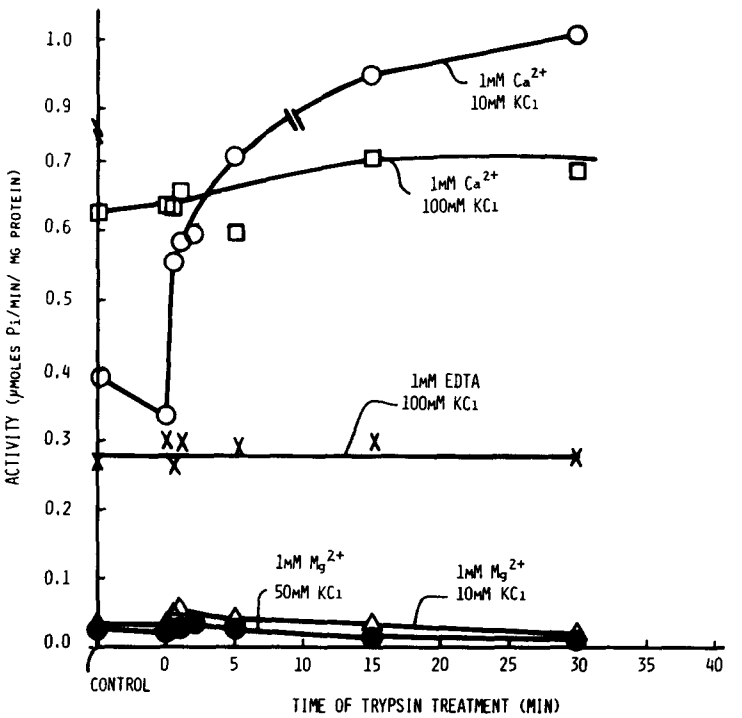

Fig. 4. Effect of trypsin on the ATPase activity of myosin. Myosin was incubated with trypsin (1 part of trypsin to 500 parts of myosin, by weight) for the times specified on the abscissa and the reaction was then stopped by a 4-fold addition of soybean trypsin inhibitor. Control and zero-time samples were prepared as described in legend to Fig. 1. Conditions for trypsin treatment: 2.0 mg myosin/ml, 100 mM KCl, 50 mM Tris–HCl (pH 7.6), 25°. Conditions for ATPase assay: 0.2 mg myosin per ml, 20 mM Tris–HCl (pH 7.6) 1 mM ATP, 25°, KCl and activators as indicated specifically by labels in the figure.

that are shown in Figs. 1–3 occur when myosin is substituted for actomyosin. Therefore with the exception of the 1 mM Ca^{2+}-modified ATPase activity, the tryptic-induced changes in NTPase activity recorded in Figs. 1–3 occur only when myosin NTPase activity is modified by interaction with actin. It follows then that these changes in NTPase activity reflect an effect of trypsin on the actin–myosin interaction.

When myofibrils are treated with trypsin at low ionic strength, the myofibrils can be sedimented out of the incubation mixture, and the supernatant analyzed for soluble protein released by tryptic hydrolysis. Since heavy meromyosin is water soluble, such an experiment affords an estimate of the amount of heavy meromyosin produced by tryptic hydrolysis under our experimental conditions. This estimate is a maximal estimate of heavy meromyosin production since it has been shown that 1–2 min of trypsin digestion under the conditions used in this study removes both the Z-line[38,44] and troponin[45] from myofibrils. However, since heavy meromyosin is enzymatically active, ATPase assays of the supernatant protein may be used in conjunction with amount of soluble protein released to provide a reliable indication of heavy meromyosin release, even if troponin and Z-line protein are being solubilized at the same time. Such an experiment shows that about 8% of total myofibrillar protein is solubilized after 1 min, about 22% after 5 min, and about 58% after 15 min of incubation with trypsin at trypsin to myofibrillar protein ratios of 1 to 100, but that the Ca^{2+}-modified, ATPase activity of the supernatant protein does not increase markedly until after

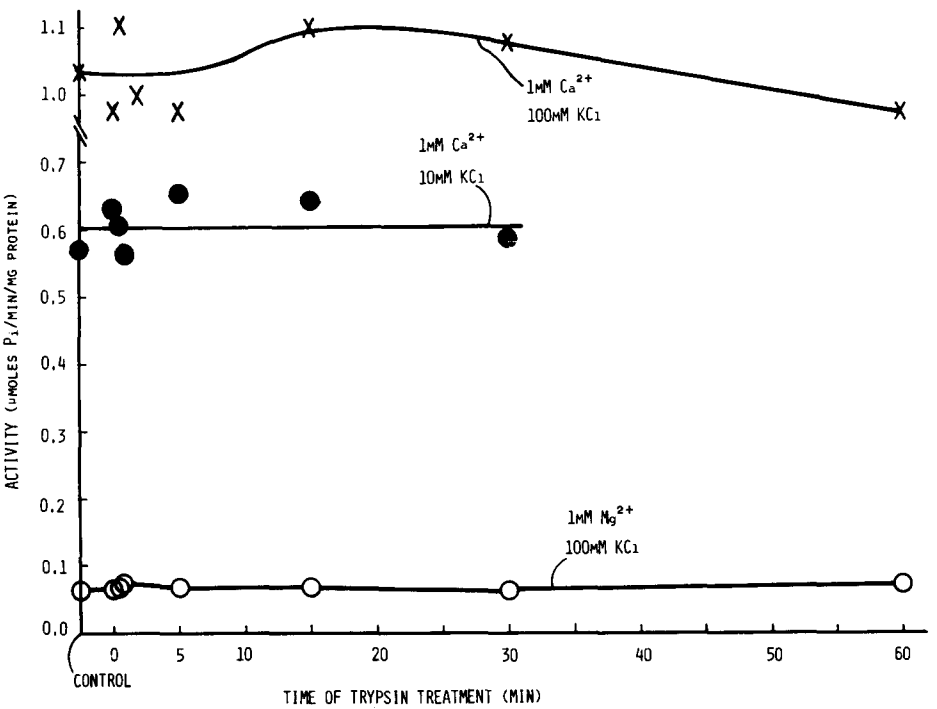

Fig. 5. Effect of trypsin on the ITPase activity of myosin. Conditions for trypsin treatment and ITPase assay were identical to those described in the legend to Fig. 4 except that 1 mM ITP was substituted for 1 mM ATP. KCl concentrations and activators are indicated specifically by the labels in the figure.

TABLE II

AMOUNT AND ENZYMIC ACTIVITY OF SOLUBLE PROTEIN RELEASED BY TRYPTIC TREATMENT OF MYOFIBRILS

Myofibrils were treated with trypsin (1 part of trypsin to 100 parts of myofibrillar protein (w/w) at 25° for the indicated times. Conditions for trypsin treatment: 110 mM KCl, 110 mM Tris–HCl (pH 7.6), 2.80 mg myofibrillar protein per ml. After treatment, the myofibrils were diluted with 150 mM KCl, 30 mM Tris–HCl (pH 7.6) to a concentration of 1.0 mg/ml and then sedimented at 1000 × *g* for 2 min. The supernatant solution was analyzed for protein concentration by biuret analysis and for ATPase activity. Conditions of ATPase assay: 110 mM KCl, 50 mM Tris–HCl (pH 7.6), 0.80 mg myofibrillar protein per ml, 5 mM ATP, activators as shown.

Time of trypsin treatment (min)	*Protein concentration in supernatant (mg/ml)*	*ATPase activity* (μmoles P_i/min per mg of supernatant protein)*	
		5 mM Ca^{2+}	*5 mM Mg^{2+} 2 mM EGTA*
0	0.04	0.139 ± 0.013**	0.127 ± 0.010
1	0.12	0.115 ± 0.017	0.041 ± 0.008
5	0.26	0.144 ± 0.012	0.032 ± 0.006
15	0.62	0.280 ± 0.001	0.010 ± 0.000
30	0.60	0.285 ± 0.014	0.010 ± 0.001

* Since supernatant protein concentration of zero-time samples was very low, small differences between duplicate samples resulted in large relative errors and in considerable uncertainty in specific activities calculated from these protein concentrations.

** Means *plus* or *minus* standard errors of four determinations.

5 min of tryptic digestion (Table II). Although the results in Table II indicate that no further release of soluble protein occurs between 15 and 30 min of tryptic hydrolysis, it is probable that additional proteolytic release of soluble fragments is offset by degradation of the previously solubilized protein into fragments too small to be detected by the biuret reaction. Under the conditions used in Table II, heavy meromyosin or other enzymatically active fragments of myosin are activated by Ca^{2+} but inhibited by Mg^{2+} in the absence of Ca^{2+}. Therefore, an increase in Ca^{2+}-modified ATPase activity or a decrease in Mg^{2+} + EGTA-modified ATPase activity indicates a release of heavy meromyosin or other enzymatically active proteolytic fragments of myosin. The data in Table II indicate that most of the protein solubilized during the first 5 min of tryptic digestion originates from the Z-line or other nonenzymatically active parts of the myofibril, and that under our conditions, extensive heavy meromyosin production from myofibrils does not occur until after 5 min of tryptic digestion. This conclusion is supported by phase micrographs of trypsin-treated myofibrils (Fig. 6), showing that the Z-line is removed within the first min of tryptic digestion. Since the increase in 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase activity occurs in the first min of tryptic digestion (*cf.* Fig. 2), it is clear that the tryptic modification responsible for this increase in specific activity occurs more rapidly than heavy meromyosin production.

Turbidity experiments

The second line of evidence that trypsin modifies the actin–myosin interaction involves tryptic-induced changes in the ability of actomyosin suspensions to give a turbidity response upon addition of ATP. The turbidity response has been interpreted

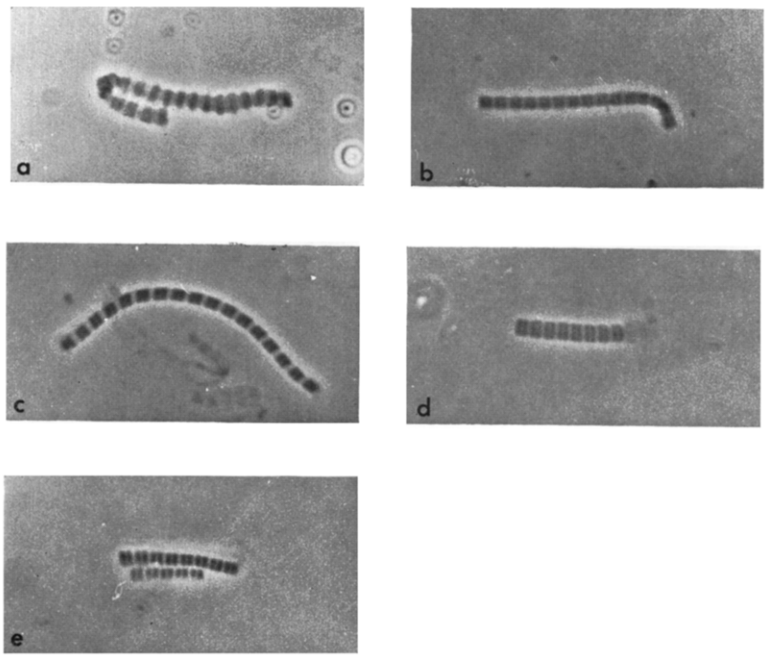

Fig. 6. Phase micrographs of myofibrils after different times of trypsin treatment. Conditions for trypsin treatment: 2.8 mg myofibrillar protein per ml, 110 mM KCl 110 mM Tris–HCl (pH 7.6), 1 part of trypsin to 100 parts of myofibrillar protein by weight, 25°; reaction was stopped by a 4-fold addition of soybean trypsin inhibitor. (a) myofibril before trypsin treatment. (b) Myofibril after 1 min of trypsin treatment. (c) Myofibril after 5 min of trypsin treatment. (d) Myofibril after 15 min of trypsin treatment. (e) Myofibril after 30 min of trypsin treatment. Magnification for all myofibrils 1150×.

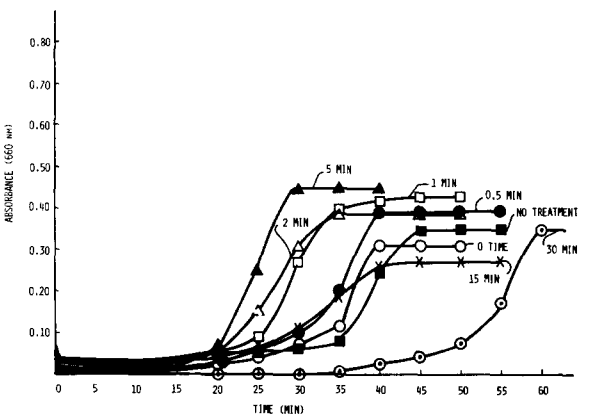

Fig. 7. Effect of trypsin on the superprecipitation of reconstituted actomyosin. Control and zero-time samples prepared as described in the legend to Fig. 1. Conditions for trypsin treatment: 6.00 mg reconstituted actomyosin per ml, 100 mM KCl, 50 mM Tris–HCl, 1 part of trypsin to 3000 parts of reconstituted actomyosin, by weight, 25°, time of trypsin treatment indicated. Conditions for superprecipitation assay: 0.4 mg reconstituted actomyosin per ml, 100 mM KCl, 20 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM ATP.

as a measure of contraction in actomyosin suspensions[46], although the quantitative significance of such experiments remains unclear[47]. Nonetheless, turbidity assays are easily repeatable and while their exact relationship to contraction must await further analysis, they are very useful for studying relative changes in the actin–myosin interaction.

As in the NTPase assays of tryptic-treated actomyosin, it was necessary to do the turbidity experiments directly on the trypsin digests immediately after trypsin treatment. Results of such turbidity assays show that the effect of trypsin on the turbidity response of reconstituted actomyosin suspensions parallels the effect of trypsin on the 1 mM Mg^{2+} + 0.05 mM Ca^{2+} ATPase activity (*cf.* Figs. 2 and 7). Tryptic digestion rapidly causes an initial increase in rate of turbidity development followed by a gradual decrease in rate of turbidity response. The trypsin to reconstituted actomyosin ratio for the experiment shown in Fig. 7 was 1 to 3000, considerably less than the 1 to 500 or 1 to 100 ratios for the experiments shown in Fig. 2. Therefore, the rate of tryptic modification of the turbidity assay shown in Fig. 7 is slower than the rate of modification of the ATPase activities shown in Fig. 2. However, a number of other experiments have shown that rate of modification of the turbidity response is identical to rate of modification of the 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase activity when tryptic digestion is done at identical trypsin to actomyosin ratios. The precautions listed in the preceding section as necessary to consistently observe the tryptic-induced increase in 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase activity were also found necessary to detect the tryptic-induced increase in rate of turbidity response shown in Fig. 7. Since it is impossible to elicit clear turbidity responses in the absence of added Mg^{2+} (*cf.* ref. 15), the turbidity experiments were limited to the conditions shown in Fig. 7.

Ultracentrifugal studies were done on the same actomyosin preparations used in the exeriment shown in Fig. 7 by adding enough 3 M KCl to the trypsin-treated suspensions immediately after treatment to raise the final KCl concentration to 0.6 M, and then stirring briefly to dissolve the protein. These studies demonstrated that very little light meromyosin or heavy meromyosin was present in these preparations after 15 min of trypsin digestion, and that even after 60 min of trypsin treatment, approx. 50% of the myosin remained intact (Fig. 8). Since it might be possible for heavy meromyosin to interact with actin to form a product ultracentrifugally indistinguishable from actomyosin itself, 6 mM Mg^{2+}-ATP was added to each of the trypsin-treated solutions to dissociate the actomyosin and permit inspection of myosin alone (Fig. 8, wedge cell patterns). Such experiments confirmed the conclusion that when tryptic digestion is done at trypsin to actomyosin ratios of 1 to 3000, light meromyosin and heavy meromyosin do not constitute an appreciable proportion of total protein present until after 30 min of digestion. This is well after the tryptic-induced increase in rate of turbidity development has occurred. Thus, these experiments demonstrate that the tryptic modification responsible for the increase in rate of turbidity development occurs more rapidly than heavy meromyosin production.

A second important point that may be obtained from the turbidity and ultracentrifugal experiments described in this section is that a considerable amount of ultracentrifugally normal actomyosin was present in the 60-min trypsin-treated samples even though ability of this actomyosin to give a turbidity response was greatly reduced (the 60-min sample did not give a turbidity response after 120 min under the

conditions shown in Fig. 7). Consequently, it may be concluded that trypsin had reduced the ability of this ultracentrifugally normal actomyosin to give a turbidity response. This reduced ability to produce a turbidity response, occurring subsequent to the trypsin-induced increase in rate of turbidity development, suggests that trypsin has at least two separate effects on the actin–myosin interaction. These same two effects can also be seen in the biphasic response of 1 mM Mg^{2+} + 0.05 mM Ca^{2+}-modified ATPase activity to trypsin (Fig. 2).

"Relaxation" experiments

The third line of evidence that trypsin modifies the actin–myosin interaction concerns the ability of brief tryptic digestion to lengthen contracted myofibrils in

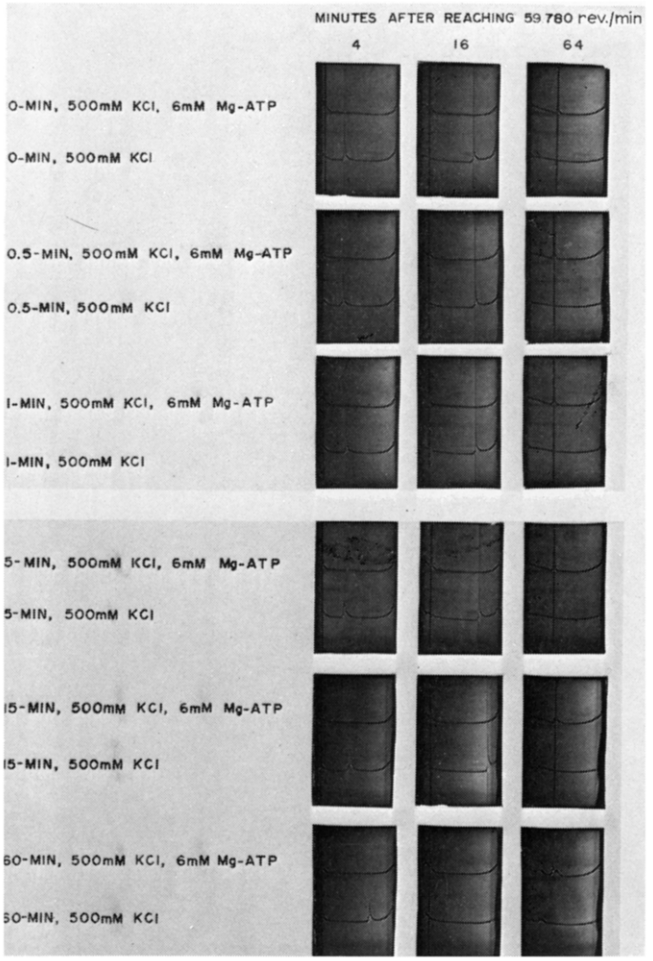

Fig. 8. Sedimentation of the same trypsin-treated reconstituted actomyosin shown in Fig. 7. Conditions of trypsin treatment, therefore, are the same as described in the legend to Fig. 7. Protein concentration in all cases is 4.00 mg/ml. Actomyosin is dissolved in 500 mM KCl, 40 mM Tris–HCl (pH 7.6), 6 mM $MgCl_2$, 6 mM ATP, or in 500 mM KCl, 40 mM Tris–HCl (pH 7.6), as indicated. Times of trypsin treatment indicated. For all runs: phase plate angle, 65°; temperature, 20.0°.

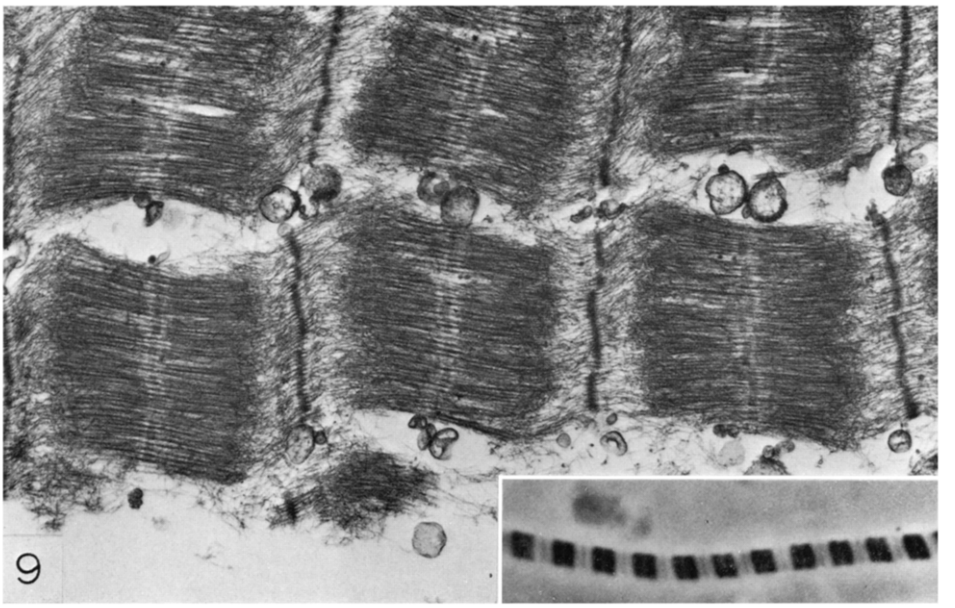

Fig. 9. Micrographs of untreated rabbit myofibrils. Myofibrils prepared by a modification of the method of PERRY AND COTTERILL[53] were suspended in 100 mM KCl, 20 mM Tris–HCl (pH 7.6), 0.01 mM $CaCl_2$, 0.05 mM $MgCl_2$. Electron micrograph of a section through the myofibril pellet shows the relaxed appearance which is also seen in the phase microscope (inset). 20500 × ; inset 2000 ×.

the absence of ATP or other interaction inhibitors[48]. Since sarcomere shortening or lengthening cannot be measured in myosin B or reconstituted actomyosin systems, the experiments described in this section were done only with myofibrils. STROMER *et al.*[38] reported that trypsin treatment of rigor-shortened bovine myofibrils caused relative movement among thick and thin filaments and that this movement resulted in an increase in sarcomere length. Consequently, an experiment was designed to test whether ATP-contracted myofibrils could be lengthened by short-time trypsin treatment.

Myofibrils in 100 mM KCl, 20 mM Tris–HCl (pH 7.6) before the addition of ATP exhibited the banding pattern typical of relaxed muscle (Fig. 9) As measured in the phase microscope, sarcomere lengths of these control myofibrils averaged 2.1 μ. Phase microscopy showed that addition of ATP to a final concentration of 0.1 mM caused severe contraction of 90% or more of the sarcomeres in a myofibril preparation. The only structure visible in the phase microscope after ATP addition was a pattern of alternating light and dark bands (Fig. 10, inset). This appearance is due to either a crumpling of thick filaments against or the protrusion of thick filaments through the Z-line (Fig. 10). Sarcomere lengths after ATP addition (1.0 μ) were shorter than thick filament lengths (1.2–1.4 μ), so distortion of the A-band and complete absence of the I-band are not unexpected.

The severely shortened myofibrils were sedimented, resuspended in fresh 120 mM KCl, 50 mM Tris–HCl (pH 7.6), 0.1 mM $CaCl_2$, and treated with trypsin at 25° and trypsin to myofibrillar protein ratios of 1 to 200 for varying periods of time. Trypsin hydrolysis was stopped by addition of a 4-fold excess of soybean inhibitor, and the treated myofibrils again examined in the phase and electron microscope.

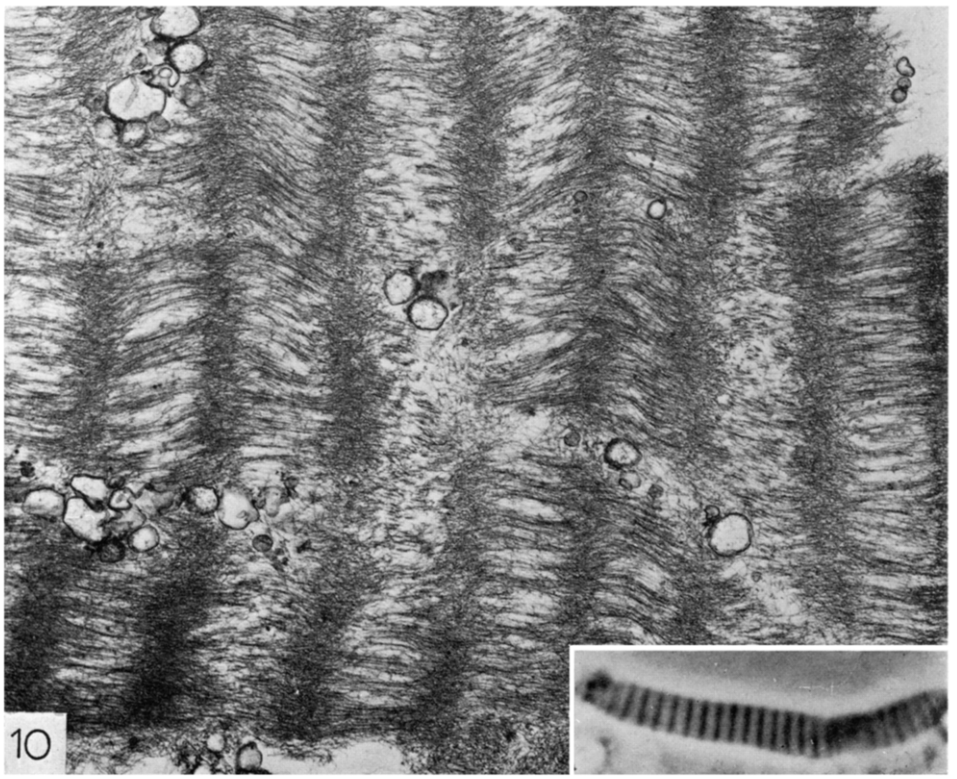

Fig. 10. Micrographs of contracted rabbit myofibrils. Myofibrils were prepared and suspended in the same solution as in Fig. 9, but containing 0.1 mM ATP in addition to the other constituents. The ATP was added last and 2 min after ATP addition, the myofibrils were sedimented at 2000 × *g* for 1 min and resuspended in 120 mM KCl, 50 mM Tris–HCl (pH 7.6). After this treatment, the myofibrils are severely shortened as is evident both in the electron micrograph (sarcomere lengths, 0.95 μ) of the sectioned pellet and in the phase microscope (inset). 17 500 ×; inset, 2000 ×.

Trypsin treatment caused the return of a distinct A-band, and a narrow I-band and H-zone (Figs. 11a and 11b). After 1 min of trypsin treatment, about 20% of the sarcomeres displayed this "relaxed" banding pattern, and if trypsin treatment was continued for 4 min, about 80% of the sarcomeres were "relaxed". Average length of the "relaxed" sarcomeres was 1.7 μ, about 20–25% shorter than the average length of control sarcomeres before ATP contraction. Longer trypsin treatment caused no further increase in sarcomere length. Instead, it appeared that length of trypsin treatment was related to the proportion of "relaxed" sarcomeres, and even those sarcomeres that were "relaxed" after 1 min of trypsin treatment had average sarcomere lengths of 1.7 μ. Control samples, subjected to this same procedure, but omitting the trypsin, remained supercontracted; this confirmed that the observed "relaxation" was indeed due to trypsin. The electron micrographs in Figs. 11a and 11b demonstrate that register and straightness of the thick filaments improves after trypsin treatment (*cf.* Figs. 10 and 11). Thin filaments are also clearly seen protruding from both sides of the A-band after trypsin treatment (Figs. 11a and 11b). Since the thin filaments were completely drawn into the A-band during ATP shortening (Fig. 10), their appearance

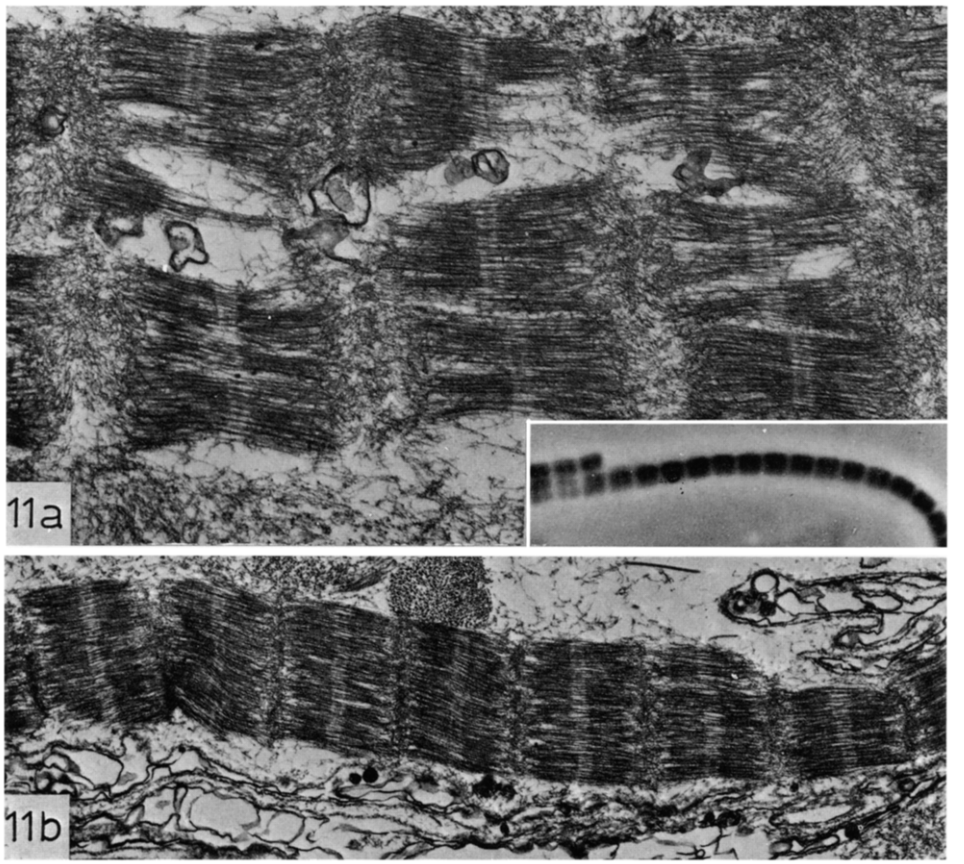

Fig. 11. Micrographs of rabbit myofibrils after trypsin "relaxation". The contracted myofibrils shown in Fig. 10 were treated for 4 min with 1 part of trypsin to 200 parts of myofibrillar protein under the following conditions: 120 mM KCl, 50 mM Tris–HCl (pH 7.6), 0.1 mM $CaCl_2$, 0.007 mM $MgCl_2$, 5.5 mg myofibrillar protein per ml. Trypsin digestion was stopped by a 4-fold addition of soybean trypsin inhibitor. Although Z-line density has been greatly reduced, a distinct A-band and a narrow I-band and H-zone are very evident in the phase and electron microscope (sarcomere length is 1.7 μ in a). a, 21 400 ×; inset, 2000 ×. 12 500 ×.

after trypsin treatment indicates that some relative movement between the thick and thin filaments has occurred. Removal of the Z-line does not appear to be the primary cause of tryptic "relaxation" because occasionally a supercontracted fibril without Z-lines is observed.

In order to estimate the proportion of myosin that had been cleaved to light and heavy meromyosin during trypsin "relaxation", some of the "relaxed" myofibrils in Fig. 11 were dissolved in 500 mM KCl, 50 mM Tris–HCl (pH 7.6), 0.05 mM $CaCl_2$ and 5 mM ATP by gentle stirring for 14–16 h at 2°. After clarification, the resulting actomyosin was examined in the analytical ultracentrifuge (Fig. 12). As in the turbidity experiments (*cf.* Fig. 8), the actomysin solution from "trypsin-relaxed" myofibrils was sedimented both in 500 mM KCl, 50 mM Tris–HCl (pH 7.6), and in this solution *plus* 10 mM Mg^{2+}ATP to permit clear identification of any light and heavy meromyosin formed during the "relaxation" process. As determined by measuring areas under the

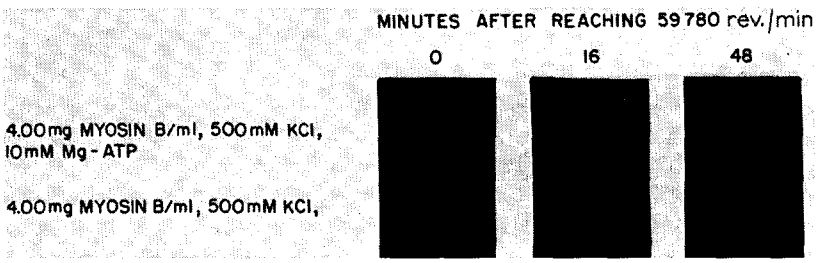

Fig. 12. Sedimentation of "trypsin-relaxed" myofibrils shown in Fig. 11. After "relaxation" by 4 min of trypsin treatment as described in Fig. 11, the myofibrils were sedimented at 2000 × *g* for 10 min, and enough 2 M KCl and 0.1 M ATP were added to bring the final concentrations to 0.5 M and 5 mM, respectively. The myofibrils were dissolved by stirring gently for 14–16 h in 500 mM KCl, 50 mM Tris–HCl (pH 7.6), 0.05 mM $CaCl_2$ and 5 mM ATP at 2°. This suspension was dialyzed against several changes of 500 mM KCl for 24 h, then clarified at 15000 × *g* for 20 min, and the supernatant examined in the ultracentrifuge. Upper (wedge) cell: 4.00 mg of actomyosin from "trypsin-relaxed" myofibrils per ml in 500 mM KCl, 50 mM Tris–acetate (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP; lower cell: 4.00 mg of actomyosin from "trypsin-relaxed" myofibrils per ml in 500 mM KCl, 50 mM Tris–acetate (pH 7.5). Phase plate angle, 65°; temperature, 20.0°.

Schlieren diagrams[49], less than 35% of the myosin from 4-min "trypsin-relaxed" myofibrils had been converted to light and heavy meromyosin, even though approx. 80% of the sarcomeres are "relaxed" by 4 min of trypsin treatment. We have consistently observed in these experiments that during the first 4–5 min of trypsin treatment, trypsin-relaxed myofibrils are present in quantitatively greater proportions than heavy meromyosin. It is also evident from Fig. 12 that "trypsin-relaxed" myofibrils possess considerable amounts of ultracentrifugally normal actomyosin.

DISCUSSION

Together, the three lines of evidence presented in this paper show that trypsin modifies the actin–myosin interaction in at least two ways: (1) a very rapid initial modification that for purposes of the discussion here will be termed a "strengthening" of the interaction, since it is manifested by an increase in the Mg^{2+} + Ca^{2+}-modified ATPase activity and an increase in the rate of turbidity response; (2) a subsequent, slower modification that will be called a "weakening" of the interaction since it is seen as a decrease in the Mg^{2+} + Ca^{2+}-modified ATPase activity, a decrease in the rate of turbidity development, and a lengthening of shortened myofibrils in the absence of ATP. The terms "strengthening" and "weakening" should not be interpreted in a strictly literal sense and are used here only for purposes of convenience.

It is clear that our results are due to an effect of trypsin on the actin–myosin interaction and not solely to a tryptic modification of the active center of myosin NTPase. First, purified myosin does not give a turbidity response, and it is generally accepted that the turbidity increase reflects some property of the actin–myosin interaction[46,47]. Therefore, that trypsin modifies the turbidity response of actomyosin suspensions shows that trypsin is modifying the actin–myosin interaction. Second, although we have confirmed previous studies[50,51] that trypsin can change the Ca^{2+} and EDTA-modified ATPase activities of myosin alone when assayed under selected conditions, we have also shown that trypsin causes large changes in certain NTPase activities of actomyosin without having any effect on the same NTPase activities

of myosin alone. This effect is particularly noticeable for the Ca^{2+}-modified ITPase activity, where 30 min of trypsin treatment causes an 8-fold increase in the actomyosin activity but has no effect on the myosin activity, and for the $Mg^{2+} + Ca^{2+}$-modified ATPase activity, where trypsin causes a biphasic response in the actomyosin activity but has no effect on the myosin activity. Hence, although trypsin causes some changes in NTPase activity of myosin alone, it also causes other changes in NTPase activity that are detected only when actin is present and that therefore must reflect alterations in the actin–myosin interaction. Since trypsin is able to alter certain NTPase activities of actomyosin without altering the corresponding NTPase activities of myosin alone, our results confirm earlier findings[52,53] that the actin-binding site and the NTPase site are located in different parts of the myosin molecule. MARUYAMA AND NAGASHIMA[54] and CHAPLAIN[55] have recently reported that trypsin changed some ATPase activities of myofibril or myosin B systems; however, these results were complicated by tryptic digestion of troponin and could not be interpreted as an effect of trypsin solely on the actin–myosin interaction. The use of reconstituted actomyosin in our study circumvented this problem.

It is also well known that trypsin will cleave myosin into two subfragments, light meromyosin and heavy meromyosin. Our ultracentrifugal patterns demonstrate, however, that under the conditions used in this study only a small proportion of the total myosin is converted to light and heavy meromyosin during the first 4–5 min of trypsin treatment at trypsin to actomyosin ratios between 1 to 300 and 1 to 500. Yet, our NTPase and turbidity results show that the "strengthening" modification occurs within 1–2 min of trypsin treatment under these conditions, and our experiments with trypsin "relaxation" of contracted myofibrils show that weakening of the actin–myosin interaction can be detected before any substantial light and heavy meromyosin production has occurred. Consequently, it is clear that tryptic modification of the actin–myosin interaction is not due simply to cleavage of myosin to light and heavy meromyosin, but instead that tryptic modification of the actin–myosin interaction normally occurs before cleavage to light and heavy meromyosin. Indeed, our ultracentrifugal diagrams of trypsin-modified actomyosin indicate the presence of a trypsin-modified but ultracentrifugally normal myosin.

Although a detailed mechanism for the two tryptic modifications that we propose here must await additional study, it may be suggested that the "strengthening" modification, at least, originates from a proteolytic cleavage in the "head" or subfragment 1 region of myosin. This suggestion is based on the observation that the "strengthening" modification is most easily detected when trypsin digestion is done at ionic strengths below 0.15 and in the presence of Ca^{2+}. BIRO *et al.*[25], GRAF *et al.*[26] and BRIO AND BALINT[56] have recently reported that trypsin cleavage of myosin into light and heavy meromyosin is suppressed at low ionic strength or in the presence of Ca^{2+}, and that under these conditions, hydrolysis in the "head" or subfragment 1 region of myosin is favored. These observations indicate that conditions favoring the "strengthening" modification also favor proteolysis in the "head" region of myosin. Moreover, myosin is a rod-shaped molecule and both the light meromyosin portion and the light and heavy meromyosin cleavage site are physically separated from the actin-combining site in the "head" region of the molecule by 300 Å or more. This is a considerable distance in molecular terms. It is easier to envision that a trypsin-induced change in the actin-combining site would be caused by a direct effect of trypsin on a region

that is physically close to the actin-combining site than it is to suggest that such a change is caused by an effect of trypsin on light meromyosin or the light meromyosin–heavy meromyosin cleavage region, both of which are a considerable distance from the actin-combining site.

Since tryptic modification of myosin occurs before its conversion into light and heavy meromyosin, all heavy meromyosin preparations will be modified. Therefore, our results provide a simple explanation for the reports[29–34] that the interaction of heavy meromyosin with actin differs from the interaction of actin with myosin in several respects. It is also possible that tryptic modifications of the kind we propose may account, at least in part, for several recent observations that interaction of myosin with ATP produces no, or only a very small, conformational change in myosin[57–59], whereas interaction of heavy meromyosin with ATP causes measurable changes in protein conformation[58–60]. Consequently, caution should be exercised in extrapolating experimental findings on trypsin-treated actomyosin or heavy meromyosin directly to the untreated actomyosin or intact myosin systems.

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REFERENCES

1 W. F. HARRINGTON, P. H. VON HIPPEL AND E. MIHALYI, *Biochim. Biophys. Acta*, 32 (1959) 303.
2 E. MIHALYI AND W. F. HARRINGTON, *Biochim. Biophys. Acta*, 36 (1959) 447.
3 P. H. VON HIPPEL AND W. F. HARRINGTON, *Biochim. Biophys. Acta*, 36 (1959) 426.
4 J. D. YOUNG AND F. H. CARPENTER, *J. Biol. Chem.*, 236 (1961) 743.
5 S. LOWEY AND C. COHEN, *J. Mol. Biol.*, 4 (1962) 293.
6 S. LOWEY, *Science*, 145 (1964) 597.
7 D. M. YOUNG, S. HIMMELFARB AND W. F. HARRINGTON, *J. Biol. Chem.*, 239 (1964) 2822.
8 D. M. YOUNG, S. HIMMELFARB AND W. F. HARRINGTON, *J. Biol. Chem.*, 240 (1965) 2428.
9 D. M. SEGAL, S. HIMMELFARB AND W. F. HARRINGTON, *J. Biol. Chem.*, 242 (1967) 1241.
10 S. LOWEY, L. GOLDSTEIN, C. COHEN AND S. M. LUCK, *J. Mol. Biol.*, 23 (1967) 287.
11 J. GERGELY, *Federation Proc.*, 9 (1950) 176.
12 S. V. PERRY, *Biochem. J.*, 48 (1951) 257.
13 J. GERGELY, *J. Biol. Chem.*, 200 (1953) 543.
14 E. MIHALYI AND A. G. SZENT-GYORGYI, *J. Biol. Chem.*, 201 (1953) 189.
15 S. LOWEY, H. S. SLAYTER, A. G. WEEDS AND H. BAKER, *J. Mol. Biol.*, 42 (1969) 1.
16 H. MUELLER AND S. V. PERRY, *Biochem. J.*, 80 (1961) 217.
17 A. G. SZENT-GYORGYI, *Arch. Biochem. Biophys.*, 42 (1953) 305.
18 H. E. HUXLEY, *J. Mol. Biol.*, 7 (1963) 281.
19 R. V. RICE, in J. GERGELY, *Biochemistry of Muscle Contraction*, Little Brown, Boston, 1964, p. 41.
20 H. S. SLAYTER AND S. LOWEY, *Proc. Natl. Acad. Sci U.S.*, 58 (1967) 1611.
21 H. MUELLER AND S. V. PERRY, *Biochim. Biophys. Acta*, 50 (1961) 599.
22 D. R. KOMINZ, F. R. MITCHELL, T. NIHEI AND C. M. KAY, *Biochemistry*, 4 (1965) 2373.
23 T. NIHEI AND C. M. KAY, *Biochim. Biophys. Acta*. 160 (1968) 46.
24 H. OPPENHEIMER, K. BARANY AND J. FENTON, *Arch. Biochem. Biophys.*, 132 (1969) 355.
25 N. A. BIRO, M. BALINT AND L. GRAF, *Acta Biochim. Biophys. Hung.*, 1 (1966) 115.
26 L. GRAF, N. A. BIRO AND P. KOVACS, *Acta Biochim. Biophys. Hung*,. 3 (1968) 239.

27 A. HOLTZER AND S. LOWEY, *J. Am. Chem. Soc.*, 81 (1959) 1370.
28 E. F. WOODS, S. HIMMELFARB AND W. F. HARRINGTON, *J. Biol. Chem.*, 238 (1963) 2374.
29 K. YAGI, T. NAKATA AND I. SAKAKIBARA, *J. Biochem.*, 58 (1965) 236.
30 E. EISENBERG AND C. MOOS, *J. Biol. Chem.*, 242 (1967) 2945.
31 L. LEADBEATER AND S. V. PERRY, *Biochem. J.*, 87 (1963) 233.
32 S. V. PERRY AND J. COTTERILL, *Nature*, 206 (1965) 161.
33 S. V. PERRY AND J. COTTERILL, *Biochem. J.*, 96 (1965) 224.
34 K. MARUYAMA, Y. ISHIKAWA AND S. EBASHI, *J. Biochem.*, 55 (1964) 581.
35 N. ARAKAWA, R. M. ROBSON AND D. E. GOLL, *Biochim. Biophys. Acta*, 200 (1970) 284.
36 K. SERAYDARIAN, E. J. BRISKEY AND W. F. H. M. MOMMAERTS, *Biochim. Biophys. Acta*, 133 (1967) 399.
37 R. M. ROBSON, D. E. GOLL AND M. J. MAIN, *J. Food Sci.*, 32 (1967) 544.
38 M. H. STROMER, D. E. GOLL AND L. E. ROTH, *J. Cell. Biol.*, 34 (1967) 431.
39 S. V. PERRY AND T. C. GREY, *Biochem. J.*, 64 (1956) 184.
40 A. G. GORNALL, C. T. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
41 R. M. ROBSON, D. E. GOLL AND M. J. TEMPLE, *Anal. Biochem.*, 24 (1968) 339.
42 1969 Product Catalog, Worthington Biochemical Company, Freehold, N.J.
43 D. E. GOLL AND R. M. ROBSON, *J. Food Sci.*, 32 (1967) 323.
44 D. E. GOLL, W. F. H. M. MOMMAERTS, M. K. REEDY AND K. SERAYDARIAN, *Biochim. Biophys. Acta*, 175 (1969) 174.
45 S. EBASHI AND A. KODAMA, *J. Biochem.*, 59 (1966) 425.
46 S. EBASHI, *J. Biochem.*, 50 (1961) 236.
47 E. J. BRISKEY, K. SERAYDARIAN AND W. F. H. M. MOMMAERTS, *Biochim. Biophys. Acta*, 133 (1967) 412.
48 M. BARANY AND F. JAISLE, *Biochim. Biophys. Acta*, 41 (9160) 192.
49 N. ARAKAWA, D. E. GOLL AND M. J. TEMPLE, *J. Food Sci.*, in the press.
50 M. BARANY, K. BARANY, E. GAETJENS AND G. BAILIN, *Arch. Biochim. Biophys.*, 113 (1966) 205.
51 J. GERGELY, D. PRAGAY, A. F. SCHOLZ, J. C. SEIDEL, F. A. SRETER AND M. M. THOMPSON, in S. EBASHI, F. OOSAWA, T. SEKINE AND Y. TONOMURA, *Molecular Biology of Muscular Contraction*, Elsevier, Amsterdam, 1965, p. 145.
52 M. BARANY AND K. BARANY, *Biochim. Biophys. Acta*, 35 (1959) 293.
53 S. V. PERRY AND J. COTTERILL, *Biochim. J.*, 92 (1964) 603.
54 K. MARUYAMA AND S. NAGASHIMA, *J. Biochem.*, 62 (1967) 392.
55 R. A. CHAPLAIN, *Arch. Biochem. Biophys.*, 115 (1966) 450.
56 N. A. BIRO AND M. BALINT, *Acta Biochim. Biophys. Hung.*, 1 (1966) 13.
57 W. G. GRATZER AND S. LOWEY, *J. Biol. Chem.*, 244 (1969) 22.
58 W. D. MCCUBBIN, C. M. KAY AND K. OIKAWA, *Biopolymers*, 5 (1967) 236.
59 M. R. IYENGAR, S. C. GLAUSER AND R. E. DAVIES, *Biochem. Biophys. Res. Commun.*, 16 (1964) 379.
60 F. MORITA AND K. YAGI, *Biochem. Biophys. Res. Commun.*, 22 (1960) 297.