Degradation of smooth-muscle myosin by trypsin-like serine proteinases

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1. Hydrolysis of the myosins from smooth and from skeletal muscle by a rat trypsin-like serine proteinase and by bovine trypsin at pH 7 is compared. 2. Proteolysis of the heavy chains of both myosins by the rat enzyme proceeds at rates approx. 20 times faster than those obtained with bovine trypsin. Whereas cleavage of skeletal-muscle myosin heavy chain by both enzymes results in the generation of conventional products i.e. heavy meromyosin and light meromyosin, the heavy chain of smooth-muscle myosin is degraded into a fragment of mol.wt. 150 000. This is dissimilar from heavy meromyosin and cannot be converted into heavy meromyosin. It is shown that proteolysis of the heavy chain takes place in the head region. 3. The 'regulatory' light chain (20 kDa) of smooth-muscle myosin is degraded very rapidly by the rat proteinase. 4. The ability of smooth-muscle myosin to have its ATPase activity activated by actin in the presence of a crude tropomyosin fraction on introduction of Ca^{2+} is diminished progressively during exposure to the rat proteinase. The rate of loss of the Ca^{2+} -activated actomyosin ATPase activity is very similar to the rate observed for proteolysis of the heavy chain and 3–4 times slower than the rate of removal of the so-called 'regulatory' light chain. 5. The significance of these findings in terms of the functional organization of the smooth muscle myosin molecule is discussed. 6. Since the degraded myosin obtained after exposure to very small amounts of the rat proteinase is no longer able to respond to Ca^{2+} , i.e. the functional activity of the molecule has been removed, the implications of a similar type of proteolysis operating *in vivo* are considered for myofibrillar protein turnover in general, but particularly with regard to the initiation of myosin degradation, which is known to take place outside the lysosome (i.e. at neutral pH).

Almost all of the proteins in the body are continually being degraded and replaced at characteristic rates. Extracellular molecules such as serum proteins, peptide hormones and connective-tissue matrix components are taken up by endocytosis and degraded predominantly within the lysosomes of the cells involved (Kay, 1980a). Studies with lysosomotropic agents and specific proteinase inhibitors have indicated that lysosomes are also involved in the catabolism of intracellular proteins (Dean, 1979; Shaw & Dean, 1980; Grinde & Seglen, 1980). However, it has become clear that degradation within lysosomes can account for only a fraction of the endogenous protein breakdown within cells (Knowles & Ballard, 1976; Poole *et al.*, 1977), and it has been suggested that, in addition to the lysosomal pathway, most cells have a second extralysosomal route for degrading their intracellular proteins [for a review, see Kay (1978)].

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The quantitative importance of this second route in tissues such as muscle with a relatively low lysosomal content is as yet unclear, but its significance was emphasized when it was shown that whereas 'general' protein turnover in isolated hearts (Wildenthal *et al.*, 1980) and skeletal-muscle cells in culture (Riebow & Young, 1980) was inhibited by chloroquine and leupeptin, the degradation of myosin was not affected by these inhibitors of lysosomal proteolysis. Thus it would appear that at least the initial stages in the degradation of myosin (and presumably, therefore, other myofibrillar proteins) take place outside the lysosomes in muscle.

Many investigations have been carried out into muscle proteolytic enzymes operative at neutral pH [for reviews, see Pennington (1977) and Katunuma & Kominami (1977)]. However, a number of these enzymes have now been shown to be derived from non-muscle cells, so that two remain as perhaps the most likely neutral proteinases that might be responsible for the non-lysosomal breakdown of muscle protein *in vivo*. The Ca^{2+} -activated thiol

proteinase was first isolated from skeletal muscle (Dayton *et al.*, 1976a), but it has now been detected in a variety of tissues such as brain, platelets, chicken oviduct, cardiac and smooth muscles (see Szpacenko *et al.*, 1981). However, it has little effect *in vitro* on most of the contractile proteins, including myosin. Its major influence appears to be disruption of the ultrastructure of the myofibrils by solubilization of α -actinin with consequent disappearance of the Z-discs (Dayton *et al.*, 1976b). The other neutral muscle proteinase was first identified in rat intestinal smooth muscle and was shown to be a trypsin-like serine proteinase (Beynon & Kay, 1978). Although hydrolysis *in vitro* of peptide substrates or denatured proteins by this enzyme proceeded at rates similar to those observed with other neutral proteinases, the muscle proteinase was approx. 100 times more effective in its attack on globular proteins in their native conformation at neutral pH (Beynon & Kay, 1978; Carney *et al.*, 1978). The tissue distribution of this type of proteinase is not yet known, but an enzyme with this type of trypsin-like activity has been isolated from rat pancreas (J. Kay, C. R. Elliss & J. A. Greweling, unpublished work), and increased amounts of a trypsin-like serine proteinase have been measured in whole homogenates of diseased human skeletal muscle (e.g. in dystrophy and polymyositis) (Kar & Pearson, 1980). The concentration of the trypsin-like proteinase in rat intestinal muscle is very low (Kay, 1980b), so that it is impossible to prepare the enzyme from this tissue in a homogeneous form in sufficient quantities for experimental purposes. However, since it was considered of interest to examine *in vitro* the susceptibility of myosin (and, in particular, smooth-muscle myosin) to digestion at neutral pH by this type of proteolytic activity, the enzyme from rat pancreas was used for this model study, since it can be prepared in a homogeneous form. In addition, since limited proteolysis has already been used extensively as a tool to investigate structure-function relationships of muscle proteins, the proposed studies might also produce some insight into the topography and functional organization of the myosin molecule from smooth muscle.

Materials and methods

Phenylmethanesulphonyl fluoride, ATP, leupeptin, antipain, pepstatin, soya-bean trypsin inhibitor, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'), pig heart isocitrate dehydrogenase, chymotrypsin, bovine pancreatic trypsin (Type III) and papain were obtained from Sigma, St. Louis, MO, U.S.A. Papain was preincubated for 30 min at 37°C with 50 mM-L-cysteine to ensure full activation before use. Pig heart malate dehydrogenase was a gift from Miles Laboratories, Stoke Poges.

Slough, Berks., U.K., and rabbit muscle lactate dehydrogenase and yeast glucose 6-phosphate dehydrogenase were obtained from Boehringer, Lewes, East Sussex, U.K. All other buffers, salts and chemical reagents were of analytical quality.

The trypsin-like serine proteinase was purified to homogeneity from rat pancreas by a procedure which, briefly, involved homogenization of the tissue (3 vol./g) in 20 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-CaCl₂, and centrifugation at 100 000 g for 20 min. The supernatant was incubated at 37°C for about 4 h before chromatography on DEAE-cellulose in the above buffer. The trypsin-like activity that was eluted with a linear (0–1 M) NaCl gradient was subjected directly to affinity chromatography on benzamidine-agarose in the above buffer. The enzyme was desorbed from this matrix by elution at room temperature with the above buffer containing 0.2 M-benzamidine. The activity of this preparation (after dialysis to remove the benzamidine) towards all substrates could be stopped completely and instantaneously at any stage by the addition of equivalent amounts of soya-bean trypsin inhibitor (see the Results section).

Smooth-muscle myosin was extracted from chicken gizzards and purified to homogeneity (>97%) by a procedure (carried out at 2°C in the presence of 0.4 mM-dithiothreitol) which, briefly, involved washing of a homogenate of the tissue by repeated centrifugation/resuspension in 0.075 M-KCl/0.01 M-potassium phosphate buffer, pH 7.6. Crude actomyosin was extracted from the washed pellets with 5 mM-ATP/2 mM-EDTA and the extract was centrifuged at 35 000 g for 17 h in the presence of 0.1 M-MgCl₂/15 mM-ATP, pH 7.0, in order to sediment actin (Ebashi, 1976). Crude myosin was precipitated from the supernatant by dialysis against 50 mM-KCl/5 mM-Mops (4-morpholinepropanesulphonic acid) buffer, pH 6.8, and resuspended in 0.2 M-MgCl₂/5 mM-Mops buffer, pH 6.5, containing 10 mM-ATP and 1 mM-EGTA (Ikebe *et al.*, 1978). Actin and tropomyosin were sedimented by centrifugation at 100 000 g for 150 min and the supernatant was salted out with (NH₄)₂SO₄. The myosin was precipitated between 50 and 55% saturation (Groschel-Stewart, 1971) and this preparation was entirely free from actin and tropomyosin. Actin and myosin were prepared from rabbit skeletal muscle by the method of Spudich & Watt (1971) and Siemankowski & Dreizen (1978) respectively.

A crude tropomyosin fraction was prepared from chicken gizzard by a modification (Aksoy *et al.*, 1976) of the procedure for isolation of 'native tropomyosin' (Ebashi *et al.*, 1966). The fraction of 'native tropomyosin' that was precipitated between 37 and 55% saturation with (NH₄)₂SO₄ was used without further purification (Aksoy *et al.*, 1976).

This crude tropomyosin fraction contains approx. 50% (w/w) tropomyosin, high activity of Ca^{2+} -sensitive myosin-light-chain kinase and is a potent activator of smooth-muscle actomyosin Mg^{2+} -ATPase activity (Gorecka *et al.*, 1976).

Conversion of the heavy chain of smooth-muscle myosin into the heavy meromyosin derivative was carried out by digestion with chymotrypsin, essentially as described by Seidel (1978). Long rods were prepared by digestion of the myosin with papain [substrate/proteinase ratio of 500:1 (w/w)] for 8 min at 25°C in 0.12 M-NaCl/5 mM-sodium phosphate buffer (pH 7.0)/2 mM- MgCl_2 (Yamamoto & Sekine, 1979). The long rods were purified from the digestion mixture by ethanol fractionation as described by Szent-Gyorgyi *et al.* (1960) and finally redissolved in 0.5 M-KCl/5 mM-potassium phosphate buffer, pH 7.0. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 5 below, track 1) indicated that the major protein component (>95%) in this preparation was long rod, having a subunit mol.wt. of 110 000 (Zobel, 1978). Long rods from rabbit skeletal-muscle myosin were prepared by digestion with chymotrypsin or with papain and isolated in the same fashion.

Proteolysis of smooth-muscle and skeletal-muscle myosins and of their respective long rods by the rat trypsin-like proteinase and by bovine trypsin was carried out at 25°C in 5 mM-potassium phosphate buffer, pH 7.0, usually containing 0.25 M-KCl. Samples for analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were removed at appropriate times during the proteolytic reactions and added to sufficient soya-bean trypsin inhibitor (in water) to inactivate the proteinase instantaneously. Portions (40 μl) from each of these samples were then mixed with 10 μl of a loading solution consisting of 5% (w/v) sodium dodecyl sulphate, 1.4 M-glycerol, 140 mM-mercaptoethanol and 25 mM-sodium phosphate buffer, pH 7.0. After boiling for 5 min, Pyronine Y was added to each sample as a tracking dye and four separate portions (usually containing 2, 4, 25 and 50 μg of protein) from each sample were applied to the gels.

Gradient (5–17%) polyacrylamide gels were prepared using the reagents described by Siemankowski & Dreizen (1978), and electrophoresis of samples was carried out at 100 V in 0.075 M-Tris/acetate buffer, pH 7.9, containing 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol until the dye had migrated to the bottom of the gel. Gels were removed and stained overnight by the method of Fairbanks *et al.* (1971). After extensive destaining, each gel was scanned at 620 nm with a Zeiss Spektralphotometer PM6 fitted with a Spektraphysiks peak integrator.

When quantitative analyses were required, the densitometric data obtained from each set of four

gels (loaded with increasing amounts of protein) were expressed in a plot of band area versus total protein loaded. From this, the amount of each protein species present was calculated (on a percentage basis) and expressed as mol of each species present per half mol of myosin as described previously (Siemankowski & Zobel, 1976), assuming that all of the protein bands present in the mixture together comprised 1 mol of half-myosin (i.e. with a mol.wt. of 234 000). The molecular weight of each protein band was determined by comparison with the mobilities of the individual subunits of rabbit skeletal-muscle myofibrils as marker proteins (Shapiro *et al.*, 1967).

The Mg^{2+} -ATPase activity of myosin was assayed by the rate of phosphate release. Assays were carried out at 25°C in 33 mM-KCl, 25 mM-Tris/HCl buffer, pH 7.4, 4 mM- MgCl_2 , 1 mM-ATP, 5 mM-phosphoenolpyruvate, 1 mM-dithiothreitol, pyruvate kinase (3 units) and either CaCl_2 (25 μM) or EGTA (1 mM). Myosin was added to a final concentration of 2.0 mg of protein/ml when assayed alone and 0.4 mg/ml when assayed in the presence of F-actin. When F-actin was included, it was added to a final concentration of 2.0 mg/ml.

The crude tropomyosin fraction was added to some assay mixtures in amounts that have been shown previously, by using a ^{32}P -incorporation assay system (Mrwa & Hartshorne, 1980), to be sufficient to ensure complete phosphorylation of myosin in the presence of Ca^{2+} within the first 1 min of incubation under the above conditions. Incubation times were varied to ensure the production of between 100 and 500 nmol of P_i per assay. Liberation of phosphate was directly proportional to incubation time over the entire range used. Each measurement was carried out in duplicate along with an enzyme blank and the reactions were stopped by the addition of 0.33 vol. of 13.3% sodium dodecyl sulphate/0.12 M-EDTA (Hegyvary *et al.*, 1979). Phosphate release was measured by the method of Taussky & Shorr (1953).

Results

The most significant feature of the trypsin-like proteinase from rat intestinal smooth muscle was its ability to inactivate enzymes in their native conformation (Beynon & Kay, 1978). The effect of the rat pancreatic proteinase preparation on these substrates is shown in Table 1. Several known modifiers of proteolytic enzymes were tested for their ability to affect the activity of the rat pancreatic enzyme. Complete inhibition was obtained with phenylmethanesulphonyl fluoride (5 mM), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (100 μM), soya-bean trypsin inhibitor, leupeptin and antipain (all 5 μM), whereas pepstatin (0.4 mM), EDTA

Table 1. *Inactivation of native enzymes by the rat pancreatic proteinase*

Each enzyme (0.2 mg/ml) was incubated at pH 7.5, 30°C with the pancreatic proteinase (0.2–2 µg). Samples were removed at various times for determination of the residual activity of the substrate enzymes. Hydrolysis of azocasein was measured by the increase in A_{340} resulting from trichloroacetic acid-soluble dye-containing peptides.

Substrate	Initial rate of inactivation (% of initial activity lost/min per pmol) by:		
	Rat pancreatic proteinase	Rat muscle proteinase†	Bovine trypsin†
Lactate dehydrogenase	0.08	0.11	0.0002
Glucose 6-phosphate dehydrogenase	0.54	0.65	0.004
Isocitrate dehydrogenase	3.9	4.8	0.055
Malate dehydrogenase	2.65	2.4	0.008
Azocasein	Initial rate of hydrolysis (ΔA_{340} /min per nmol)		
	Rat pancreatic proteinase	Rat muscle proteinase†	Bovine trypsin†
	0.13	0.18	0.14

† Data from Beynon & Kay (1978).

Fig. 1. *A schematic representation of the heavy chains of the myosin molecule*

Cleavage of the heavy chains of skeletal-muscle myosin by trypsin, chymotrypsin or papain under appropriate conditions gives the fragments indicated. In smooth-muscle myosin each heavy chain (200 kDa) combines in the subfragment 1 region with two light chains (one of 17 kDa and the 'regulatory' light chain of 20 kDa). Abbreviations used: LMM, light meromyosin; HMM, heavy meromyosin; SF₁ and SF₂, subfragments 1 and 2.

(10 mM), mercaptoethanol (15 mM) and Ca²⁺ (10 mM) had no effect on the enzyme. The enzyme from rat pancreas was found to migrate in an identical manner to bovine trypsin on electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. Thus it would appear that the trypsin-like proteinase isolated from rat pancreas is very similar to the activity measured in crude proteinase preparations from rat intestinal muscle described previously. The pancreatic proteinase was used in this model study as representative of this type of activity.

Limited proteolysis of skeletal-muscle myosin by bovine trypsin converts the heavy-chain species into heavy meromyosin and light meromyosin (Szent-Gyorgyi *et al.*, 1960), whereas chymotrypsin or papain can cleave at several locations in the heavy

chain to produce various fragments, depending on the conditions used (see Fig. 1). Previous investigations into the effect of bovine pancreatic trypsin on smooth-muscle myosin have concentrated on the breakdown of the regulatory (20 kDa) light chain with little analysis of the fate of the (200 kDa) heavy chain (Okamoto & Sekine, 1978).

Gizzard myosin was incubated with the rat trypsin-like proteinase at a myosin/proteinase ratio (w/w) of 20 000:1 and portions were removed at appropriate times for gel electrophoresis and for measurement of the residual Mg²⁺ ATPase activity.

A typical time course of digestion is shown in Fig. 2. The gels from several such digestions were scanned as described in the Materials and methods section and the densitometric data were used to obtain a quantitative measure for every band. The

Fig. 2. The extent of digestion of gizzard myosin by the rat trypsin-like proteinase

Smooth-muscle myosin (250 mg in 42 ml of 0.25 M-KCl/5 mM-potassium phosphate buffer, pH 7.0) was incubated with rat trypsin-like proteinase (12.5 μ g) at 25°C and samples (8 ml) were removed at the indicated times and added to an excess of soya-bean trypsin inhibitor (40 μ l; 5 mg/ml in water). Portions (50 μ l) were taken from each and loadings corresponding to 25 μ g (a) and 2 μ g (b) of original myosin protein were applied to sodium dodecyl sulphate/polyacrylamide gels. A zero-time control sample was treated identically except that the soya-bean trypsin inhibitor was added before the myosin. Smooth-muscle myosin is composed of heavy chain (200 kDa) and two light chains (20 kDa and 17 kDa).

Fig. 3. Effect of digestion by the rat proteinase on the individual subunits and ATPase activity of smooth-muscle myosin

Gizzard myosin was incubated with rat proteinase and samples were prepared for gel electrophoresis as described in the legend to Fig. 2. Densitometric analysis of each protein subunit or fragment band was carried out as described in the Materials and methods section. The values obtained are plotted directly, except for those of the digestion products (150 kDa; 18.4 kDa) which accumulated. These are expressed in terms of (1.00 - value obtained). (a) ●, 17 kDa light chain; ▲, regulatory (20 kDa) light chain; ■, 18.4 kDa appearance. (b) ●, 200 kDa heavy chain alone; □, sum of 200 kDa + 175 kDa + 163 kDa; ▲, 150 kDa appearance. (c) Actomyosin Mg^{2+} -ATPase activity measured in the presence of tropomyosin fraction and Ca^{2+} .

resultant data (expressed as mol of subunit or fragment per half-mol of myosin) are shown (on a semilogarithmic basis) in Fig. 3.

It can be seen (Fig. 2a) that the 17 kDa light chain of smooth-muscle myosin appears to be unaffected by exposure to the rat proteinase, since it was maintained at a constant value (Fig. 3a). By contrast, the regulatory (20 kDa) light chain was

degraded very rapidly (Fig. 2a) and a pseudo-first-order rate constant of $0.33 \pm 0.04 \text{ min}^{-1}$ was calculated (Fig. 3a) under these conditions. A new fragment appeared with an apparent mol.wt. of

Table 2. *Effect of digestion by the rat proteinase on the Mg^{2+} ATPase activity of smooth-muscle myosin*

Gizzard myosin was incubated with the rat proteinase as described in the legend to Fig. 2 and samples were removed at various times. After taking portions (50 μ l) from each for gel electrophoresis, the remainder of each was adjusted to 55% saturation with $(NH_4)_2SO_4$ and centrifuged at 25 000 g for 15 min. The supernatants contained no protein and were discarded. Each pellet was redissolved in 0.5 M-KCl/2 mM-Mops (4-morpholinepropanesulphonic acid) buffer, pH 6.9, containing 0.2 mM-dithiothreitol and dialysed against this buffer before determination of the Mg^{2+} ATPase activity remaining. Assays were carried out by measurement of phosphate released. F-actin, crude tropomyosin fraction and Ca^{2+} (or EGTA) were added to the assay mixtures as appropriate.

Additions to assay			After digestion for (min) . . .	Specific activity (nmol of P_i /min per mg)			
Actin	Tropomyosin fraction	Ca^{2+}		0	1	7	10
—	—	—		2.1	2.2	4.4	6.8
—	—	+		2.2	2.2	4.3	7.0
—	+	—		1.7	1.8	3.6	5.6
—	+	+		8.7	6.8	7.6	9.5
+	—	—		6.9	6.5	12.3	17.0
+	—	+		9.9	8.5	13.7	19.3
+	+	—		3.0	3.4	8.0	11.1
+	+	+		241	227	114	78

18400 (Fig. 2a). This was produced initially at a very rapid rate (Fig. 3a) that diminished greatly after about 1–2 min. Although it was not possible to obtain an accurate rate constant for the initial phase from the current data, it is nevertheless evident that the initial rate of production of the 18.4 kDa fragment appears to be considerably faster than that at which the 20 kDa subunit was being degraded. Thus it would seem that the protein band that is generated with a mol.wt. of 18400 is not derived from the regulatory (20 kDa) subunit and by difference, therefore, is probably a small fragment from the myosin heavy chain.

Degradation of the heavy (200 kDa) chain (Fig. 2b) was accompanied by the generation of fragments of apparent mol.wts. of 175 000, 163 000 and 150 000 (as determined by comparison with marker proteins) and complete conversion of the heavy chain into the 150 kDa fragment was achieved after approx. 14 min. The degradation of the 200 kDa chain followed distinctly biphasic kinetics (Fig. 3b): the very rapid first phase was complete within 30 s and in this approx. 20% of the total 200 kDa subunit was removed. The second, slower, phase followed pseudo-first-order kinetics with $k_{obs.} = 0.11 \pm 0.04 \text{ min}^{-1}$.

The appearance of the 150 kDa degradation fragment also followed biphasic kinetics (Fig. 3b): 0.2 mol per half-mol of myosin were generated within 30 s and thereafter the appearance of more 150 kDa fragment was much slower with an apparent pseudo-first-order rate constant of $0.036 \pm 0.011 \text{ min}^{-1}$. During the first 30 s incubation of smooth-muscle myosin with the rat proteinase there was very little change in the Mg^{2+} ATPase

activity of the myosin (Table 2; see below). It might then be considered that the myosin preparations used were heterogeneous with respect to the species of myosin that they contained; 20% of the myosin molecules were in an inactive conformation and their heavy (200 kDa) chains were converted rapidly and directly to 150 kDa fragments. The remaining 80% had retained their ATPase activity and the 200 kDa subunits underwent a progressive proteolysis: 200 kDa $\rightarrow$ 175 kDa $\rightarrow$ 163 kDa $\rightarrow$ 150 kDa. The rate constant obtained (Fig. 3b) for the disappearance of total heavy-chain species (i.e. 200 kDa + 175 kDa + 163 kDa) was $0.035 \pm 0.011 \text{ min}^{-1}$, in reasonable agreement with that calculated above for the appearance of the 150 kDa fragment in the second phase.

These digestions of soluble smooth-muscle myosin (i.e. in 0.25 M-KCl) by the rat proteinase were also carried out with the gizzard myosin in filamentous form (in 0.12 M-KCl) and very similar results were obtained. This would seem to indicate that proteolysis of smooth-muscle myosin proceeds independently of the ionic strength and, therefore, the state of aggregation of the myosin.

A sample of heavy meromyosin was prepared from gizzard myosin by digestion with bovine chymotrypsin. By comparison of the mobility of this subfragment on sodium dodecyl sulphate/polyacrylamide gels, it can be seen (Fig. 4) that all of the heavy-chain fragments produced in the digestion by the rat proteinase were larger than heavy meromyosin. It would thus appear that the initial cleavages of the heavy chain of smooth-muscle myosin by the rat proteinase must have taken place either at the N-terminal end (i.e. in subfragment 1;

Fig. 4. Comparison of heavy meromyosin (HMM) with the heavy-chain fragments obtained from proteolysis of gizzard myosin by the rat proteinase

Samples of gizzard myosin digested with rat proteinase (1) and heavy meromyosin from a digestion with bovine chymotrypsin (2) were analysed separately and as a mixture (3) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Abbreviation used: HMM, heavy meromyosin.

Fig. 5. Extent of digestion of long rods from smooth-muscle myosin by the rat trypsin-like proteinase

Smooth-muscle myosin long rods were incubated at 25°C with rat proteinase at substrate/proteinase ratios of 10000:1 and 680:1. Samples were removed from the former reaction after 0 (1) and 20 min (2) and from the latter at 10, 20 and 40 min (3, 4 and 5 respectively). All digestions were terminated by the addition of an excess of soya-bean trypsin inhibitor. Samples were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Fig. 1) or at the C-terminus (in light meromyosin). To investigate this aspect further, long rods (i.e. heavy chain devoid of subfragment 1) were prepared (by treatment of myosin with papain as described in the Materials and methods section). The purified long rods were incubated with rat proteinase at ratios (w/w) of 10000:1 and 680:1. In the former reaction (twice as much proteinase being used as in the digestion of whole myosin) no significant digestion of the long rods was observed after 20 min (Fig. 5; tracks 1 and 2). The major (long rod) band has been deliberately overloaded to indicate the lack of proteolysis. By contrast, samples taken from the digest at 680:1 after 10, 20 and 40 min (Fig. 5) indicated that some degradation of the long rods had been achieved by using almost 30 times as much proteinase as was used in the whole myosin digest. Thus, under the conditions used to digest myosin, the long rods do not appear to be susceptible to attack by the rat proteinase. It would seem, then, that the cleavage sites in smooth-muscle myosin heavy chain are located in the N-terminal region of subfragment 1 and not in light meromyosin (see Fig. 1).

Since it has been shown (Seidel, 1978; Onishi & Watanabe, 1979) that chymotrypsin will convert smooth-muscle myosin into heavy meromyosin and light meromyosin, the following additional experiment was carried out. A sample of smooth-muscle

myosin was digested with rat proteinase such that most of the heavy chain was converted into 175, 163 and 150 kDa fragments but without complete conversion into the 150 kDa derivative (track 2 of Fig. 6). The reaction was stopped by the addition of soya-bean trypsin inhibitor. After removal of samples for gel electrophoresis, bovine chymotrypsin was added at a myosin protein/chymotrypsin ratio (w/w) of 240:1 and a second digestion was carried out at 25°C for 10 min. Phenylmethanesulphonyl fluoride (100 mM) was added to stop this reaction and samples were taken for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Two control samples of gizzard myosin were digested, one with bovine chymotrypsin as described above and one with bovine pancreatic trypsin at a myosin/proteinase ratio (w/w) of 600:1. Samples taken from the latter digest at appropriate times and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the 150 kDa fragment was produced by bovine trypsin (Fig. 6; track 3) as well as by the rat proteinase (tracks 2 and 4 of Fig. 6). Complete removal of the 200 kDa heavy chain by bovine trypsin was achieved within about 6 min, but the 150 kDa fragment generated was digested further by bovine trypsin to produce a light meromyosin species (track 3) in contrast with the situation observed with the rat

Fig. 6. *Products of digestion of smooth-muscle myosin by the rat proteinase, by bovine trypsin and by chymotrypsin*

Smooth-muscle myosin was incubated at 25°C with each of the three proteinases as described in the text. One-half of the material digested with the rat proteinase was further exposed to chymotrypsin for 10 min. Samples were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 1, Undigested myosin; 2, myosin + rat proteinase; 3, myosin + trypsin; 4, mixture of 2 and 3; 5, myosin + rat proteinase + chymotrypsin; 6, myosin + chymotrypsin; 7, mixture of 5 and 6; 8, heavy meromyosin prepared from smooth-muscle myosin. Abbreviations used: HMM, heavy meromyosin; LMM, light meromyosin.

proteinase (track 2). However, further proteolysis of the products from the rat proteinase digestion with chymotrypsin generated light meromyosin (track 5). No heavy meromyosin was produced, in contrast with the situation observed (track 6) when smooth-muscle myosin was exposed initially to chymotrypsin. The small amount of contaminating 150 kDa fragment (present initially in this preparation of smooth-muscle myosin; track 1) was maintained after digestion with chymotrypsin (track 6), so that it would appear to be relatively resistant to proteolysis by chymotrypsin.

Since it was not possible to produce heavy meromyosin by chymotryptic proteolysis of the mixture of 175, 163 and 150 kDa fragments, but yet light meromyosin was generated, it would appear that the attack on smooth-muscle myosin heavy chain by the rat proteinase must take place at the *N*-terminal end (i.e. in the head region). This is the locus in the molecule that is responsible for (a) light-chain interaction, (b) actin binding and therefore (c) Mg^{2+} ATPase activity, so that we decided to examine how these changes in structure might be reflected in alterations in ATPase activity.

Each sample that was taken during the proteolytic digestions (described in Figs. 2 and 3) was

assayed for residual ATPase activity. Representative data are given in Table 2. For the control ($t = 0$) myosin, its low intrinsic Mg^{2+} ATPase activity was not altered by the addition of Ca^{2+} or the crude tropomyosin fraction individually, whereas both together produced a slight apparent increase in specific activity, probably due to the low Ca^{2+} -sensitive ATPase activity that is present in the crude tropomyosin fraction itself. Addition of actin (without crude tropomyosin) caused a slight activation of the myosin ATPase activity, which was not Ca^{2+} -sensitive to any appreciable extent. In contrast, addition of the tropomyosin fractions and Ca^{2+} to actomyosin resulted in a large (approx. 23-fold) increase in actomyosin ATPase activity and omission of the Ca^{2+} decreased the actomyosin activity to that intrinsic to myosin alone.

As the incubation with the proteinase proceeded, an enhancement was observed (Table 2) in the Mg^{2+} ATPase activity of myosin (with or without Ca^{2+} and crude tropomyosin). Similarly, the actomyosin Mg^{2+} ATPase activity in the absence of crude tropomyosin ($\pm Ca^{2+}$) increased on incubation with the proteinase. Digestion also increased the activity of actomyosin measured in the presence of crude tropomyosin (and EGTA) in parallel with the myosin and actomyosin ATPase activities, thus reflecting merely the activation of myosin ATPase activity by proteolysis, with no alteration in the potency of the crude tropomyosin to inhibit actomyosin activity in the absence of Ca^{2+} . However, the most dramatic effect of proteolysis was the removal of the activating effect of the crude tropomyosin and Ca^{2+} together on actomyosin ATPase activity. This activation by crude tropomyosin + Ca^{2+} was lost in a pseudo-first-order fashion, from which $K_{obs.} = 0.10 \pm 0.02 \text{ min}^{-1}$ was calculated (Fig. 3c). Thus the net effect of proteolysis appears to be a loss of Ca^{2+} -sensitivity of the actomyosin complex, due primarily to loss of the activatability by crude tropomyosin and Ca^{2+} .

Since the heavy chain of smooth-muscle myosin appeared to be susceptible to attack by trypsin-like proteinases (at very low concentrations of proteinase) only in the *N*-terminal region, it was considered worthwhile, for the purposes of the present study, to compare the effects of this type of proteinase on skeletal-muscle myosin.

Rabbit skeletal-muscle myosin was incubated with rat proteinase and with bovine trypsin at ratios (w/w) of 20000:1 and 600:1 respectively. Samples were removed at appropriate times for electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (Fig. 7). As observed previously with smooth-muscle myosin, the regulatory light chain of skeletal myosin was degraded very rapidly by both proteinases (barely visible in Fig. 7, but readily detectable in the gels). However, proteolysis of the

Fig. 7. Extent of digestion of rabbit skeletal muscle myosin by the rat trypsin-like proteinase and by bovine trypsin

Skeletal-muscle myosin was incubated at 25°C with rat proteinase and bovine trypsin at myosin/proteinase ratios (w/w) of 20000:1 and 600:1 respectively. Portions were removed from the former reaction at 0, 3, 10 and 30 min (1, 2, 3 and 4 respectively) and from the latter at 10 and 30 min (5 and 6). All reactions were terminated by the addition of an excess of soya-bean trypsin inhibitor and samples were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Gel 7 is a mixture of samples 4 and 6. Abbreviations used: HMM, heavy meromyosin; LMM, light meromyosin; Reg. L chain, regulatory light chain.

Fig. 8. Extent of digestion of long rods from skeletal-muscle myosin by the rat trypsin-like proteinase

Long rods (prepared from skeletal-muscle myosin by digestion with chymotrypsin and by papain) were incubated with rat proteinase at a substrate/proteinase ratio (w/w) of 1000:1. Samples were removed from the digestion of the chymotrypsin-prepared rods at 0, 10, 20 and 40 min (1, 2, 3 and 4 respectively) and from the digestion of the papain-prepared rods at 0, 20 and 40 min (5, 6 and 7). All reactions were terminated by the addition of an excess of soya-bean trypsin inhibitor. Samples were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

heavy chain of skeletal-muscle myosin by both enzymes appeared to result in direct conversion into heavy meromyosin and light meromyosin, with little evidence of the formation of intermediate species (such as the 175 kDa and 163 kDa fragments from smooth-muscle myosin). By comparison with the mobilities of rabbit myofibril proteins as standards, the apparent molecular weights of the two major breakdown products were calculated to be 140 000 and 70 000 respectively. The products from both digestions co-migrated on electrophoresis (track 7, Fig. 7).

Long rods that had been prepared from rabbit skeletal-muscle myosin by digestion with chymotrypsin or papain were incubated with rat proteinase at a rod/proteinase ratio (w/w) of 1000:1 (Fig. 8). Very rapid breakdown of the rods (prepared by either chymotrypsin or papain digestion) into smaller fragments took place. Digestion was virtually complete within 10 min at 25°C, somewhat faster than was observed previously for the digestion of long rods from smooth-muscle myosin.

Discussion

In previous reports from other laboratories, the susceptibility of myofibrillar proteins to several proteolytic enzymes active in the neutral-alkaline pH range has been investigated (e.g. Yasogawa *et al.*, 1978). These chymotrypsin-like serine proteinases were thought at the time to originate within the muscle, but subsequent immunofluorescence studies have indicated that they were, in fact, located within the mast cells of the tissue (Woodbury *et al.*, 1978a,b) and thus were unlikely to be involved in any myofibrillar protein breakdown under physiological conditions. The Ca^{2+} -activated proteinase has little effect if any on the myosin and actin components of the myofibril (Dayton *et al.*, 1976b) and although its precise cellular location has yet to be determined, it has been suggested recently that this enzyme might be situated on the external face of the plasma membrane (Gerard & Schneider, 1980) and might have a more 'specialized' role to play than the initiation of myofibrillar protein breakdown (Szpacenko *et al.*, 1981), possibly, for example, in the processing of hormone receptors (Vedeckis *et al.*, 1980).

Cathepsin L has been shown to be the most active of the lysosomal proteinases towards protein substrates (Kirschke *et al.*, 1980), but even at the optimum pH of 4, degradation of the myosin heavy chain by cathepsin L was not complete after 2 h at a substrate/proteinase ratio (w/w) of 80:1 (Okitani *et al.*, 1980). In any case, it seems unlikely that whole myosin would be presented as a substrate for digestion within the lysosomal compartment since inhibitors of lysosomal proteolysis do not diminish

the rate of myosin turnover (Riebow & Young, 1980; Wildenthal *et al.*, 1980).

However, irrespective of whether these various proteinases might have accessibility to native myosin *in vivo*, it seems that very low myosin/proteinase ratios are necessary to obtain significant digestion of the myosin within a reasonable time *in vitro*. By contrast, with the rat trypsin-like serine proteinase using smooth-muscle myosin/proteinase ratios as high as 20000:1, degradation of the myosin into a functionally inactive derivative was achieved within minutes. Since myosin was so sensitive to this type of activity, the proteinase from rat pancreas was used for these studies *in vitro* rather than the enzyme from intestinal muscle tissue, which can only be obtained as a crude preparation, partially purified to an $(\text{NH}_4)_2\text{SO}_4$ -fractionation stage (Beynon & Kay, 1978). The cellular location of the trypsin-like proteinase(s) has also yet to be determined in muscle and pancreatic tissue, so that the availability of a specific antiserum to the homogeneous pancreatic proteinase should not only facilitate testing of identity between the enzymes from the different tissues but might also permit immunocytochemical location of the proteinases within the cell. Nevertheless, this type of activity would appear to have a great potential for the degradation of myosin and other myofibrillar proteins, provided that ready access to the substrate proteins is available.

Proteolysis of rabbit skeletal-muscle myosin by the rat trypsin-like proteinase took place in a conventional fashion, i.e. to produce heavy meromyosin and light meromyosin. In this respect, as might perhaps be expected of a trypsin-like proteinase, it behaved very similarly to bovine pancreatic trypsin (Szent-Gyorgyi *et al.*, 1960), apart from the fact that the rate of heavy-chain breakdown obtained with the rat pancreatic proteinase was approx. 15 times faster than that for bovine pancreatic trypsin. With gizzard smooth-muscle myosin, when a concentration of rat proteinase was used that was 30 times lower than that used in the parallel experiment with bovine trypsin (as determined by active-site titration), the rate of proteolysis of the heavy chain was approx. one-half of that measured for trypsin. Thus the heavy chain of smooth-muscle myosin appears to be degraded by the rat proteinase approx. 15–20 times more rapidly than by bovine trypsin. However, the products of both digestions were very similar. The heavy chain of gizzard myosin was converted by both proteinases into a fragment of 150 kDa. Since bovine trypsin was included at a concentration 30 times higher than that used with the rat proteinase, further digestion of this 150 kDa fragment into light meromyosin was observed with bovine trypsin, whereas the rat proteinase was apparently not added at high enough

concentrations to be able to achieve this under the conditions used. The 150 kDa fragment was larger in size than heavy meromyosin, but it was not possible to convert this fragment into heavy meromyosin. This, together with the other evidence cited, appears to indicate that proteolysis of smooth-muscle myosin heavy chain by trypsin-like serine proteinases takes place at the *N*-terminal end of the molecule within subfragment 1.

The biphasic kinetics observed for the digestion of the myosin heavy chain are consistent with a very rapid burst in which around 20% of the heavy chains were converted directly into the 150 kDa fragment (probably accompanied by the release of an 18.4 kDa fragment and other smaller peptides) followed by a slower reaction in which the proteolysis of the 200 kDa heavy chain took place at a rate (0.11 min^{-1}) very similar to that measured for the loss of the Ca^{2+} -activated actomyosin ATPase activity (0.10 min^{-1}).

Primary control of the regulation of muscle contraction is achieved through the intracellular Ca^{2+} concentration, and in smooth muscle it has been suggested that the Ca^{2+} -sensitive mechanism resides in the myosin molecule through phosphorylation of one (20 kDa) of its light chains [see, e.g., the review by Hartshorne & Gorecka (1980)]. Introduction of Ca^{2+} results in its binding to calmodulin, which in turn binds to and activates the myosin-light-chain kinase. Phosphorylation of the 20 kDa light chain by the kinase was thought to be correlated with activation of the myosin ATPase activity by actin (Dabrowska *et al.*, 1977) thus initiating cross-bridge cycling and muscle contraction.

However, it is clear from the results of the present study that the 20 kDa ('regulatory') light chain is degraded at a rate 3–4 times faster than that in which the activation of actomyosin ATPase activity by crude tropomyosin is lost. The present data do not exclude the possibility that a small oligopeptide-sized fragment of this light chain might have remained bound in the digested myosin and thereby still have been involved functionally in regulation of the myosin activity. Although this seems unlikely, further investigation is necessary. Nevertheless, from the current data it would appear that irrespective of the extent of phosphorylation of the 20 kDa light chain of smooth-muscle myosin, its involvement in maintaining the Ca^{2+} -sensitivity of the actomyosin complex seems uncertain. Indeed, it has been suggested that phosphorylation of this light chain has little or no effect on the modification of actomyosin ATPase activity (Mikawa *et al.*, 1977), and more recently, a reinvestigation of the role of the light-chain kinase has confirmed this (Persechini *et al.*, 1981). These findings, together with a previous report (Seidel, 1979), suggest a role for

some as yet unidentified component(s) of the crude tropomyosin fraction (other than tropomyosin itself) in Ca^{2+} regulation. Possible similarities to the leiotonin system (Mikawa *et al.*, 1978) remain to be investigated.

Thus the rat trypsin-like proteinase produces a degraded smooth-muscle myosin molecule in which the heavy chain has been converted into a fragment of 150kDa and the 20kDa light chain has been completely destroyed. The ability of the degraded myosin to have its ATPase activity activated by actin in the presence of Ca^{2+} and the crude tropomyosin fraction is diminished finally to the low value that is intrinsic to myosin alone. If a similar effect of proteolysis takes place in the muscle *in vivo*, then the degraded myosin would be unable to be activated by Ca^{2+} released into the sarcoplasm, i.e. the muscle would be permanently relaxed. At the same time, digestion of myosin does not lead to a significant increase in the Mg^{2+} ATPase activity of actomyosin in the presence of the crude tropomyosin fraction (in the absence of Ca^{2+}), so that depletion in the amount of cellular ATP would not appear to present a serious problem for the cell.

The relative ease with which these effects on myosin can be achieved at very low concentrations of the rat trypsin-like proteinase (in comparison with other enzymes) suggests that this type of activity operating *in vivo* could have a profound influence on the metabolic function of the cell, i.e. its ability to contract. The rat trypsin-like proteinase is able to degrade other myofibrillar proteins with equal facility (Kay *et al.*, 1982), so that the activity of this type of proteinase would have to be regulated very carefully within the cell under physiological conditions. A specific inhibitor has been isolated from smooth muscle and characterized (Carney *et al.*, 1980). It is, however, of paramount importance to determine the cellular location of this type of proteinase before its involvement as (one of) the extra-lysosomal proteinase(s) required for the initiation of the degradation of myofibrillar proteins *in vivo* can be considered.

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