

# Effects of Proteolysis on the Adenosinetriphosphatase Activities of Thymus Myosin<sup>†</sup>

Ngoc-Diep Vu and Paul D. Wagner\*

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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**ABSTRACT:** Limited proteolysis was used to identify regions on the heavy chains of calf thymus myosin which may be involved in ATP and actin binding. Assignments of the various proteolytic fragments to different parts of the myosin heavy chain were based on solubility, gel filtration, electron microscopy, and binding of <sup>32</sup>P-labeled regulatory light chains. Chymotrypsin rapidly cleaved within the head of thymus myosin to give a 70 000-dalton N-terminal fragment and a 140 000-dalton C-terminal fragment. These two fragments did not dissociate under nondenaturing conditions. Cleavage within the myosin tail to give heavy meromyosin occurred more slowly. Cleavage at the site 70 000 daltons from the N-terminus of the heavy chain caused about a 30-fold decrease in the actin concentration required to achieve half-maximal stimulation of the magnesium-adenosinetriphosphatase (Mg-ATPase) activity of unphosphorylated thymus myosin. The actin-activated ATPase activity of this digested myosin was only slightly affected by light chain phosphorylation. Actin inhibited the cleavage at this site by chymotrypsin. In the presence of ATP, chymotrypsin rapidly cleaved the thymus myosin heavy chain at an additional site about 4000 daltons from the N-terminus. Cleavage at this site caused a 2-fold increase in the ethylenediaminetetraacetic acid-ATPase activity and 3-fold decreases in the Ca<sup>2+</sup>- and Mg-ATPase activities of thymus myosin. Thus, cleavage at the N-terminus of thymus myosin was affected by ATP, and this cleavage altered ATPase activity. Papain cleaved the thymus myosin heavy chain about 94 000 daltons from the N-terminus to give subfragment 1. Although this subfragment 1 contained intact light chains, its actin-activated ATPase activity was not affected by light chain phosphorylation.

Vertebrate smooth muscle and nonmuscle myosins have similar properties and subunit compositions, being composed of two 200 000-dalton heavy chains and two pairs of light chains, *M<sub>r</sub>* 20 000 and 17 000. The actin-activated adenosinetriphosphatase (ATPase)<sup>1</sup> activities of these myosins are regulated by phosphorylation of their 20 000-dalton light chains (LC20) (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). However, the mechanism of this regulation appears to vary from one myosin to another. Unphosphorylated gizzard smooth muscle myosin appears to be inactive, and phosphorylation causes a large increase in the maximum rate, *V<sub>max</sub>*, of the actin-activated ATPase activity of this myosin (Sobieszek & Small, 1977; Sherry et al., 1978; Onishi & Watanabe, 1979; Sellers et al., 1981, 1982). On the other hand, the actin-activated ATPase activities of unphosphorylated and phosphorylated calf thymus nonmuscle myosins have the same *V<sub>max</sub>*. Phosphorylation regulates the actin-activated ATPase activity of this nonmuscle myosin by causing a decrease in *K<sub>app</sub>*, the actin concentration required to achieve 1/2 *V<sub>max</sub>* (Wagner & George, 1986).

Proteolytic digestions of muscle myosins have been used to make two soluble fragments, heavy meromyosin (HMM) and subfragment 1 (S1). HMM and S1 do not form filaments and have been very useful in the determination of the kinetics of ATP hydrolysis and in the understanding of the mechanisms of regulation of muscle contraction (Adelstein & Eisenberg, 1980). In 0.6 M KCl, chymotrypsin digests the heavy chains of striated and smooth muscle myosins about 140 000 daltons from their N-termini to give HMM and light meromyosin (LMM) (Weeds & Pope, 1977; Seidel, 1980; Onishi & Watanabe, 1979; Sellers et al., 1981). See Figure 1 for a de-

scription of these different proteolytic fragments. LMM contains the C-terminal part of the myosin heavy chain, and it is insoluble at low ionic strength. HMM is composed of the two myosin heads joined by the S2 portion of the myosin rod, is soluble at low ionic strength, and retains the actin-activated ATPase activity of the myosin. Light chain phosphorylation regulates the actin-activated ATPase of gizzard HMM by causing about a 25-fold increase in *V<sub>max</sub>* (Onishi & Watanabe, 1979; Sellers et al., 1982). Papain digests the heavy chain of striated and smooth muscle myosins about 94 000 daltons from their N-termini to give S1 and rod (Margossian & Lowey, 1973; Sobieszek & Small, 1976; Seidel, 1980). S1 is a single myosin head, and it contains the actin and ATP binding sites. While gizzard S1 contains LC20, its actin-activated ATPase activity is not regulated by phosphorylation (Nath et al., 1982; Ikebe & Hartshorne, 1985).

In this paper, we report the digestion of thymus myosin by chymotrypsin and papain. The cleavage pattern of thymus myosin by papain was similar to that of the muscle myosins. However, chymotrypsin rapidly digested the heavy chain of thymus myosin about 70 000 daltons from the N-terminus and then more slowly at the HMM/LMM junction. The actin-activated ATPase of thymus myosin which had been cleaved at the site 70 000 daltons from the N-terminus was only very slightly affected by light chain phosphorylation. The effects of ATP and actin on the chymotryptic digestion were also determined. A preliminary account of this work has been

<sup>1</sup> Abbreviations: ATPase, adenosinetriphosphatase; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; HMM, heavy meromyosin; S1, myosin subfragment 1; LC20, 20 000-dalton myosin light chain; EDTA, ethylenediaminetetraacetic acid; LMM, light meromyosin; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane.

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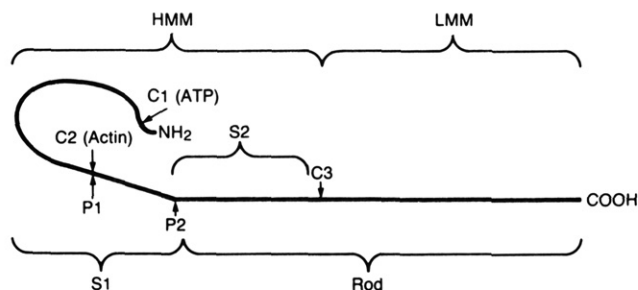


FIGURE 1: Diagram of the various proteolytic subfragments of myosin and of the proteolytic sites on the thymus myosin heavy chain. C1, C2, and C3 are sites of chymotryptic digestion about 4000, 70000, and 125000 daltons from the N-terminus, respectively. Digestion at C1 is affected by ATP, and digestion at C2 is affected by actin. P1 and P2 are sites of papain digestion 70000 and 94000 daltons from the N-terminus, respectively. For simplicity, only a single heavy chain is shown.

presented (Vu & Wagner, 1986).

#### MATERIALS AND METHODS

Skeletal muscle myosin was prepared from rabbit back and hind leg muscles, and actin was prepared from the acetone powder of these muscles (Wagner & Weeds, 1977). Skeletal muscle HMM and S1 were prepared as described by Weeds and Pope (1977). Calf thymus myosin was prepared by using a modification (Wagner et al., 1985) of the "ammonium sulfate procedure of Scholey et al. (1982)". Myosin light chain kinase was isolated from turkey gizzards (Adelstein & Klee, 1981). Bovine testis calmodulin was a gift from Dr. Claude Klee. Papain (type III) and  $\alpha$ -chymotrypsin (type 1-S) were purchased from Sigma.

Thymus myosin, 5 mg/mL, was digested with 0.02 mg/mL chymotrypsin at 25 °C in 0.6 M NaCl, 5 mM  $MgCl_2$ , 0.2 mM DTT, and 10 mM imidazole, pH 7.0, and in the presence and absence of 1 mM ATP. After various lengths of time, the digestions were stopped by the addition of phenylmethanesulfonyl fluoride to 0.5 mM. The products of these digestions were examined by electrophoresis on 7–15% polyacrylamide gels in the presence of NaDodSO<sub>4</sub> and by gel filtration on a 1.6 × 90 cm column of Sepharose 4B (Pharmacia) equilibrated in the digestion buffer. Some of the samples were dialyzed overnight against 100 mM NaCl, 3 mM  $MgCl_2$ , 0.1 mM DTT, 0.1 mM PMSF, and 10 mM imidazole, pH 6.5 at 4 °C, and then centrifuged for 20 min in an airfuge. The supernatants were examined by NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis. Samples were also dialyzed against 150 mM KCl, 10 mM  $MgSO_4$ , 0.5 mM DTT, 0.1 mM PMSF, and 10 mM imidazole, pH 7.0 at 4 °C. These myosin samples were diluted to 0.5 mg/mL, and 10  $\mu$ L was placed on an electron microscope grid. After 10 s, the samples were washed with 5 drops of dialysis buffer and stained with 1% uranyl acetate (Scholey et al., 1982). These negatively stained samples were examined in a Phillips 400 electron microscope.

Thymus myosin, 5 mg/mL, was digested with 0.02 mg/mL papain at 25 °C in 100 mM NaCl, 0.2 mM DTT, 2 mM EDTA, and 10 mM imidazole, pH 7.0. After various lengths of time, the digestions were stopped by the addition of iodoacetic acid to 2 mM. The products of these digestions were examined by NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis, gel filtration, and centrifugation in an airfuge.

Intact and digested thymus myosins were phosphorylated as described previously (Wagner et al., 1985). The level of LC20 phosphorylation was determined by urea/glycerol-polyacrylamide gel electrophoresis (Perrie & Perry, 1970). The actin-activated ATPase activities of both the unphosphorylated

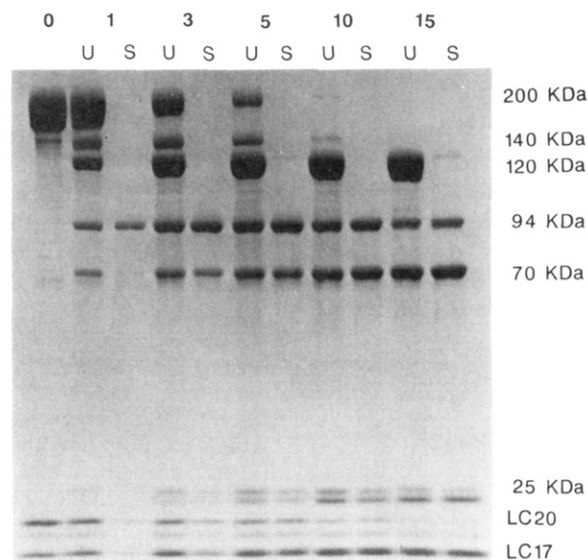


FIGURE 2: Time course of thymus myosin digestion by papain. Thymus myosin (5 mg/mL) was digested at 25 °C by 0.02 mg/mL papain in 100 mM NaCl, 0.2 mM DTT, 2 mM EDTA, and 10 mM imidazole, pH 7.0. After various times, the digestion was stopped by the addition of iodoacetic acid to 2 mM. Part of these samples was centrifuged for 20 min in an airfuge, and the supernatants were removed. These supernatants (S) and samples taken prior to centrifugation (U) were electrophoresed on a NaDodSO<sub>4</sub>-polyacrylamide gel. The times of digestion in minutes are given at the top of each pair of lanes.

and phosphorylated myosins were determined at 37 °C in 50 mM KCl, 10 mM  $MgSO_4$ , 2 mM ATP, 0.5 mM DTT, 1 mM EGTA, and 20 mM imidazole, pH 7.0. The myosin concentrations were 0.3 mg/mL, and the actin concentration was varied from 0 to 100  $\mu$ M. Values for  $V_{max}$  and  $K_{app}$  were determined from plots of  $1/\text{observed velocity}$  vs.  $1/[\text{actin}]$  (Wagner & George, 1986). The EDTA-,  $Ca^{2+}$ -, and  $Mg^{2+}$ -ATPase activities of these myosins were determined at 37 °C in 0.6 M KCl, 20 mM Tris, pH 7.5, 2 mM ATP, and either 2 mM EDTA, 10 mM  $CaCl_2$ , or 10 mM  $MgCl_2$ .

The binding of <sup>32</sup>P-labeled gizzard LC20 to the proteolytic fragments of thymus myosin was performed by the gel overlay procedure described by Sellers and Harvey (1984) for gizzard myosin and its proteolytic fragments.

#### RESULTS

**Digestion of Thymus Myosin with Papain.** Thymus myosin in 100 mM NaCl and 2 mM EDTA was digested with papain for various lengths of time (Figure 2), and the digested samples were centrifuged in an airfuge for 20 min. The supernatants contained 94-, 70-, and 25-kDa heavy chain fragments and the two light chains. Undigested myosin heavy chain and 140- and 120-kDa fragments precipitated. While most of the 70-kDa fragment in the 1-min digest precipitated, most of this fragment in the longer digests remained in the supernatant. The molecular weights of the various proteolytic fragments were based on their mobilities on NaDodSO<sub>4</sub>-polyacrylamide gels. When the supernatant from the 1-min digest was redigested with papain, the 94-kDa fragment was cleaved to give the 70- and 25-kDa fragments. The soluble fractions from these digests bound to actin, hydrolyzed ATP, and eluted from a Sepharose 4B column with skeletal muscle S1 and after skeletal muscle HMM. Thus, papain appeared to digest thymus myosin at the S1/S2 junction to give a 94-kDa S1 fragment. Cleavage of the 94-kDa S1 fragment at a second site within the myosin head gave fragments of 70 and 25 kDa. A similar digestion pattern was obtained in 5 mM  $MgCl_2$

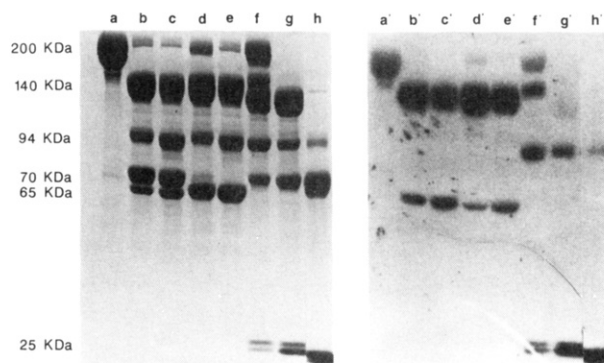


FIGURE 3: Gel overlay of the proteolytic fragments of thymus myosin with  $^{32}\text{P}$ -labeled LC20. (a) Intact myosin; (b) 5-min chymotryptic digestion in the absence of ATP; (c) 10-min chymotryptic digestion in the absence of ATP; (d) 5-min chymotryptic digestion in the presence of ATP; (e) 10-min chymotryptic digestion in the presence of ATP; (f) 3-min papain digestion; (g) 10-min papain digestion; (h) 5-min chymotryptic digestion of thymus myosin subfragment 1. The subfragment 1 was isolated from a papain digestion. These different samples were electrophoresed on a NaDodSO<sub>4</sub>-polyacrylamide gel. The gel was fixed with methanol and acetic acid, washed, and incubated with  $^{32}\text{P}$ -labeled gizzard LC20. Lanes a'-g' are the autoradiogram of this gel showing  $^{32}\text{P}$ -labeled LC20 binding. As noted by Sellers and Harvey (1984), most of the thymus light chains were lost during the washings.

except LC20 was more rapidly digested.

Sellers and Harvey (1984) have found that after NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis, the intact heavy chain of gizzard myosin and fragments of the heavy chain which contain the C-terminal portion of the myosin head can bind  $^{32}\text{P}$ -labeled gizzard LC20. Heavy chain fragments which did not contain this region did not bind  $^{32}\text{P}$ -LC20. Thymus myosin and thymus myosin digested with papain were electrophoresed on a NaDodSO<sub>4</sub>-polyacrylamide gel (Figure 3), and the gel was overlaid with  $^{32}\text{P}$ -labeled gizzard LC20 (Sellers & Harvey, 1984).  $^{32}\text{P}$ -LC20 bound to the intact thymus myosin heavy chain (lane a'), the 94-kDa S1 fragment, and the 25-kDa fragment (lanes f' and g') but not to the 70-kDa fragment. This indicates that the 25-kDa fragment is the C-terminal portion of S1 and that the second site of papain cleavage is about 70 000 daltons from the N-terminus of the heavy chain. The 120-kDa fragment appears to be the myosin rod as it did not bind  $^{32}\text{P}$ -LC20 (Figure 3, lanes f' and g') and it was insoluble in 100 mM NaCl (Figure 2).

The 140-kDa fragment present in the short digests which bound  $^{32}\text{P}$ -LC20 (Figure 3) appears to result from cleavage of the intact heavy chain at the site 70 000 daltons from the N-terminus. Cleavage of the intact heavy chain at this site would give a 70-kDa N-terminal fragment and a fragment which contains the rest of the head (C-terminal portion of S1) and the tail. As this cleavage would be within the myosin head, these two fragments would probably be held together by noncovalent interactions. The 140-kDa fragment and most of the 70-kDa fragment present in the 1-min digest were insoluble in 100 mM NaCl (Figure 2). Subsequent cleavage of the 140-kDa fragment at the S1/S2 junction would give the 120-kDa insoluble rod fragment and S1. This S1 would be composed of the 70-kDa N-terminal and 25-kDa C-terminal fragments. All of the 70-kDa fragment present in the longer digests was soluble, as was the 25-kDa fragment.

Thymus S1 was isolated from a 3-min papain digestion. Most of the 94-kDa S1 heavy chain fragment was intact, and the ratio of LC20 to LC17 was 0.90. The EDTA-,  $\text{Ca}^{2+}$ -, and  $\text{Mg}^{2+}$ -ATPase activities of this S1 were like those of intact

Table I: ATPase Activities of Thymus Myosin<sup>a</sup>

myosin	ATPase act. ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )		
	EDTA	$\text{Ca}^{2+}$	$\text{Mg}^{2+}$
intact	0.78	0.83	0.027
myosin 70/140 <sup>b</sup>	0.73	0.80	0.024
myosin 66/140 <sup>c</sup>	1.34	0.26	0.007
S1 <sup>d</sup>	0.80	0.92	

<sup>a</sup>The ATPase assays were performed at 37 °C in 0.6 M KCl, 20 mM Tris, pH 7.5, 2 mM ATP, and either 2 mM EDTA, 10 mM CaCl<sub>2</sub>, or 10 mM MgCl<sub>2</sub>. <sup>b</sup>Thymus myosin digested with chymotrypsin for 5 min in the absence of ATP. <sup>c</sup>Thymus myosin digested with chymotrypsin for 5 min in the presence of ATP. <sup>d</sup>S1 was isolated from a 3-min papain digestion of thymus myosin.

Table II: Actin-Activated ATPase Activity of Thymus Myosin

myosin	ATPase activity <sup>a</sup>			
	unphosphorylated		phosphorylated	
	$V_{\text{max}}$ (nmol min <sup>-1</sup> mg <sup>-1</sup> )	$K_{\text{app}}$ ( $\mu\text{M}$ )	$V_{\text{max}}$ (nmol min <sup>-1</sup> mg <sup>-1</sup> )	$K_{\text{app}}$ ( $\mu\text{M}$ )
intact	140	70	154	4.8
myosin 70/140 <sup>b</sup>	131	1.5	135	1.0
myosin 66/140 <sup>c</sup>	112	2.7	125	1.7
S1 <sup>d</sup>	200	32	208	39

<sup>a</sup>The ATPase assays for intact myosin, myosin 70/140, and myosin 66/140 were performed at 37 °C in 50 mM KCl, 10 mM MgSO<sub>4</sub>, 2 mM ATP, 0.5 mM DTT, 1 mM EGTA, and 20 mM imidazole, pH 7.0. The ATPase assays for S1 were performed at 37 °C in 7 mM KCl, 4 mM MgCl<sub>2</sub>, 2 mM ATP, 0.3 mM DTT, 0.5 mM EGTA, and 10 mM imidazole, pH 7.0. The level of LC20 phosphorylation was determined by electrophoresis on urea/glycerol-polyacrylamide gels. The unphosphorylated samples contained about 5% phosphorylated LC20, and the phosphorylated samples contained 100% phosphorylated LC20. <sup>b</sup>Thymus myosin digested with chymotrypsin for 5 min in the absence of ATP. <sup>c</sup>Thymus myosin digested with chymotrypsin for 5 min in the presence of ATP. <sup>d</sup>S1 was isolated from a 3-min papain digestion of thymus myosin.

myosin (Table I). However, the actin-activated ATPase activity of this S1 was not regulated by phosphorylation (Table II).

**Digestion of Thymus Myosin with Chymotrypsin.** Thymus myosin was digested with chymotrypsin for various lengths of time in 0.6 M NaCl and 5 mM MgCl<sub>2</sub>, conditions similar to those used to make skeletal and smooth muscle HMMs. In the absence of ATP, chymotrypsin initially cut the myosin heavy chain to give 140- and 70-kDa fragments (Figure 4A). With increasing time of digestion, the 140-kDa fragment was cleaved to give fragments of about 95 and 65 kDa. The 70-kDa fragment was slowly digested to a 68-kDa fragment. The rate of LC20 digestion was comparable to that of the 140-kDa fragment. The addition of ATP caused one major change in the chymotryptic digestion of thymus myosin (Figure 4A). In the presence of ATP, chymotrypsin rapidly digested the 70-kDa fragment to give a 66-kDa fragment. The 70-kDa fragment was present after 1 min of digestion, but after 5 min, it was completely digested to the 66-kDa fragment. This 66-kDa fragment and the 65-kDa fragment generated by digestion of the 140-kDa fragment were not always resolved on NaDodSO<sub>4</sub>-polyacrylamide gels (Figure 4A), but when lower protein loadings were used (Figure 4B), there was a clear separation of these two fragments. The addition of ATP also reduced the rate of LC20 digestion.

The 5-min chymotryptic digests were dialyzed into 100 mM NaCl and then centrifuged for 20 min in an airfuge. All of the 140-kDa fragment and more than 90% of the 70-kDa fragment (66-kDa fragment for the digest performed in ATP) were in the pellet. The insolubility of these fragments suggests that the initial cleavage to give the 140- and 70-kDa fragments



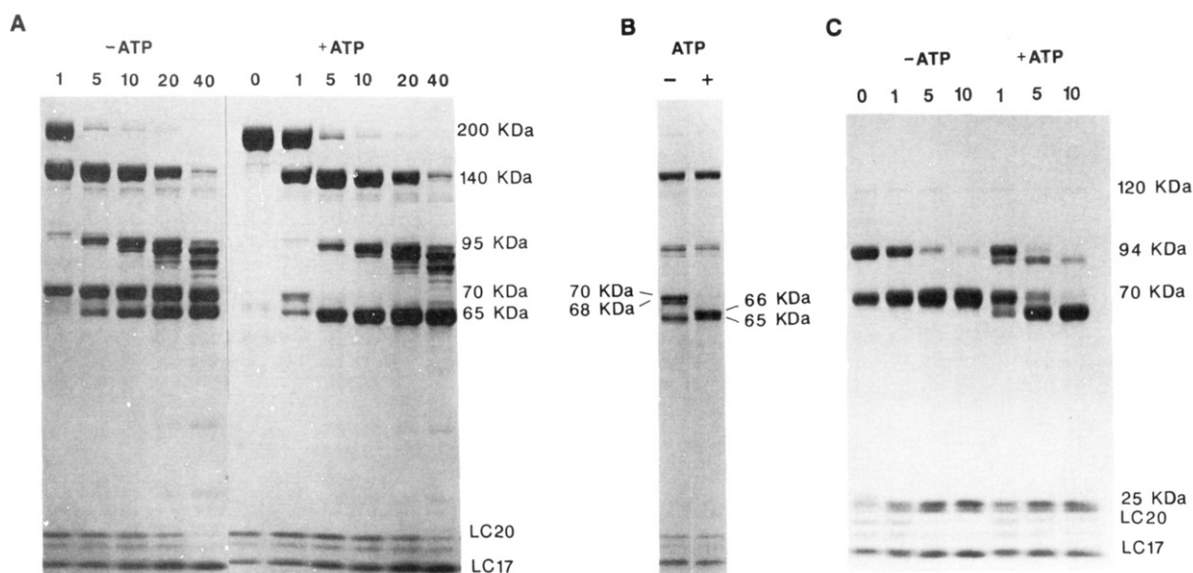


FIGURE 4: Time courses of thymus myosin and S1 digestions by chymotrypsin in the presence and absence of ATP. (A) Thymus myosin (5 mg/mL) was digested at 25 °C with 0.02 mg/mL chymotrypsin in 0.6 M NaCl, 5 mM MgCl<sub>2</sub>, 0.2 mM DTT, and 10 mM imidazole, pH 7.0, in the presence and absence of 1 mM ATP and then electrophoresed on NaDodSO<sub>4</sub>-polyacrylamide gels. The times of digestion in minutes are given at the top of each lane in panel A. In panel B, (-) is a 10-min digestion of myosin in the absence of ATP, and (+) is a 10-min digestion in the presence of ATP. (C) Thymus myosin was digested by papain for 10 min as described in the legend to Figure 2. S1, the low salt supernatant, was digested at 25 °C by 0.02 mg/mL chymotrypsin in 0.6 M NaCl, 5 mM MgCl<sub>2</sub>, and 10 mM imidazole, pH 7.0, in the presence and absence of 1 mM ATP. The times of digestion in minutes are given at the top of each lane.

occurred within the myosin head about 70 000 daltons from the N-terminus. Had the initial cleavage been 140 000 daltons from the N-terminus, HMM and LMM would have been formed, and the 140-kDa fragment (HMM) would probably have remained in the supernatant. The 95-kDa fragment was in the pellet, but most of the 65-kDa fragment was in the supernatant. The 5-min digests were chromatographed on a Sepharose 4B column equilibrated in 0.6 M NaCl. Fractions from this column were examined by NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis. The 140- and 70- (or 66-) kDa fragments eluted with intact myosin and ahead of skeletal muscle HMM. The 140- and 70-kDa fragments also did not separate when chromatographed on DEAE-Sephacryl. Intact myosin and the 5-min digests were dialyzed into 150 mM KCl and 10 mM MgSO<sub>4</sub>, negatively stained, and examined in the electron microscope. The digested samples contained about the same number of bipolar thick filaments as did the intact myosin. The elution of these digested myosins from the Sepharose 4B column and their formation of bipolar thick filaments indicate that the 140- and 70- (or 66-) kDa fragments are held together by noncovalent bonds and that this initial cleavage occurred within the myosin head about 70 000 daltons from the N-terminus of the heavy chain.

After 20 min of digestion, there was a decrease in the amount of 140-kDa fragment and an increase in the amount of 95- and 65-kDa fragments (Figure 4). These digests were dialyzed into 100 mM NaCl and centrifuged. The supernatant contained the 70- (or 66-) and 65-kDa fragments and the two light chains. These soluble fractions were chromatographed on the Sepharose 4B column in 0.6 M NaCl. The 70- (or 66-) and 65-kDa fragments eluted with skeletal muscle HMM and ahead of skeletal muscle S1. Thus, after the initial cleavage within the myosin head to give a 70-kDa N-terminal and 140-kDa C-terminal fragments, chymotrypsin cut within the rod to form HMM and LMM. The 95-kDa LMM fragment was insoluble in 100 mM NaCl. The heavy chain of this HMM appears to be composed of a 70- (or 66-) kDa N-terminal fragment and a 65-kDa C-terminal fragment which contained S2 and part of the head.

The chymotryptic fragments of thymus myosin were also examined by <sup>32</sup>P-LC20 binding (Figure 3, lanes b-e). <sup>32</sup>P-LC20 bound the 140- and 65-kDa fragments but not the 95-, 70-, and 66-kDa fragments (lanes b'-e'). This is consistent with the 70-kDa fragment (the 66-kDa fragment for digestions performed in ATP) being the N-terminal part of the heavy chain and with the 65-kDa fragment containing the C-terminal portion of the head. The inability of the 95-kDa fragment to bind this light chain is consistent with it being LMM. In contrast, the 94-kDa S1 fragment formed by papain digestion (lanes f' and g') bound <sup>32</sup>P-LC20.

Thymus myosin was phosphorylated by myosin light chain kinase and then dialyzed to remove ATP. Electrophoresis on urea/glycerol-polyacrylamide gels showed that the myosin was not dephosphorylated during this dialysis. Phosphorylation of LC20 had no obvious effect on the digestion of thymus myosin heavy chains by chymotrypsin in the presence and absence of ATP. However, phosphorylation of LC20 slowed its digestion by chymotrypsin.

Thymus myosin was also digested at a variety of ionic strengths, in the presence and absence of ATP, and in both EDTA and MgCl<sub>2</sub>. Under all conditions tried, the heavy chain was rapidly cleaved by chymotrypsin at a site 70 000 daltons from the N-terminus. The conditions described above (0.6 M NaCl, 5 mM MgCl<sub>2</sub>, and 1 mM ATP) appeared to give the best protection of LC20 from digestion by chymotrypsin.

**Effect of Proteolysis on ATPase Activities of Thymus Myosin.** Thymus myosin was digested for 5 min by chymotrypsin in the presence and absence of ATP to make two different forms of digested myosin (Figure 4A). These two digested forms of myosin are referred to respectively as myosin 66/140 and myosin 70/140. The ratios of LC20 to LC17 for the intact myosin, myosin 70/140, and myosin 66/140 were, respectively, 1.15, 1.05, and 1.07. Phosphorylation of LC20 caused a 15-fold decrease in the  $K_{app}$  of the actin-activated ATPase activity of intact thymus myosin (Table II). The actin-activated ATPase activities of myosin 70/140 and myosin 66/140 had about the same  $V_{max}$  as the intact myosin, but the  $K_{app}$  values of unphosphorylated myosin 70/140 and myosin

66/140 were about 30-fold less than that of the unphosphorylated intact myosin. Phosphorylation caused less than a 2-fold decrease in the  $K_{app}$  values of the digested myosins (Table II).

Myosin 70/140 had the same EDTA-,  $Ca^{2+}$ -, and  $Mg^{2+}$ -ATPase activities as the intact myosin (Table I), but myosin 66/140 had an EDTA-ATPase activity twice that of intact myosin and  $Ca^{2+}$ - and  $Mg^{2+}$ -ATPase activities about one-third of those of intact myosin. Thus, cleavage of a 4-kDa fragment from the N-terminal 70-kDa fragment altered the ATPase activities of thymus myosin.

**Digestion of Thymus S1 by Chymotrypsin.** When thymus myosin was digested by chymotrypsin in the presence of ATP, the 70-kDa N-terminal fragment was rapidly digested to a 66-kDa fragment (Figure 4A). To determine whether this digestion occurred at the N-terminus or C-terminus of the 70-kDa fragment, thymus S1 was digested by chymotrypsin in 0.6 M NaCl and 5 mM  $MgCl_2$  (Figure 4C). In the absence of ATP, chymotrypsin digested the 94-kDa S1 heavy chain into 70- and 25-kDa fragments. The 25-kDa fragment appears to come from the C-terminus of S1 as this fragment bound  $^{32}P$ -LC20 (Figure 3, lane h'). The 70-kDa fragment did not bind  $^{32}P$ -LC20. Thus, this site of chymotryptic cleavage appears to be the same as that obtained with the intact heavy chain. In the presence of ATP, most of the 94-kDa fragment was digested to give the 70- and 25-kDa fragments, but some of the 94-kDa fragment was digested to give a 90-kDa fragment (Figure 4C). The 70-kDa fragment was rapidly digested to the 66-kDa fragment, and the intensity of the 90-kDa band decreased with longer digestions. As chymotrypsin in the presence of ATP did not digest the intact myosin heavy chain at a site 90 000 daltons from the N-terminus, it appears that the cleavage of the 94-kDa S1 fragment to a 90-kDa fragment results from the removal of a 4-kDa fragment from the N-terminus of the heavy chain. Also, if the 90-kDa fragment had resulted from the cleavage of a 4-kDa fragment from the C-terminus of the 94-kDa fragment, the subsequent cleavage by chymotrypsin at the site 70 000 daltons from the N-terminus would have given a C-terminal fragment about 4000 daltons smaller than that made in the absence of ATP. However, the same C-terminal 25-kDa fragment was made in the presence and absence of ATP (Figure 4C).

In the absence of ATP, the 70-kDa fragment was slowly digested by chymotrypsin to a 68-kDa fragment (Figure 4). The addition of ATP after 20 min of digestion in the absence of ATP resulted in the rapid cleavage of this 68-kDa fragment to a fragment the same size as the 66-kDa fragment obtained by a 5-min chymotryptic digestion of intact myosin in the presence of ATP. This suggests the 68-kDa fragment resulted from the removal of a 2-kDa fragment from the N-terminus of the heavy chain.

**Effect of Actin on the Chymotryptic Digestion of Thymus Myosin.** In an attempt to make HMM with intact heavy chains, thymus myosin was digested with chymotrypsin in the presence of actin. In the absence of actin, almost no intact thymus myosin heavy chains remained after 5 min of digestion with chymotrypsin. However, in the presence of actin, about half of the heavy chains remained intact after 20 min of digestion (Figure 5), and there were decreases in the amounts of the 140- and 70-kDa fragments. Thus, actin binding reduced the rate of cleavage at the site 70 000 daltons from the N-terminus. The rate of cleavage in the rod at the LMM/HMM junction was not affected by actin binding as the rate of formation of the 95-kDa LMM fragment was not affected by actin (Figure 5). Also, in the presence of actin, a 125-kDa

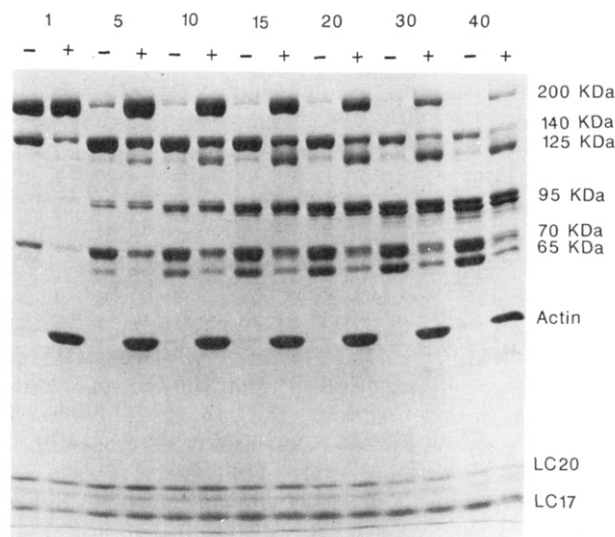


FIGURE 5: Effect of actin on the digestion of thymus myosin by chymotrypsin. Thymus myosin was digested in the absence of ATP as described in Figure 4 in the presence (+) and absence (-) of 45  $\mu$ M actin and electrophoresed on a NaDodSO<sub>4</sub>-polyacrylamide gel. The times of digestion in minutes are given at the top of each pair of lanes.

fragment was formed. When this digest was centrifuged at 100 000g in 0.6 M KCl, the 125-kDa fragment was in the pellet. This pellet was homogenized in 50 mM NaCl, 5 mM  $MgCl_2$ , 2 mM ATP, and 10 mM imidazole, pH 7, and then recentrifuged. The 125-kDa fragment remained in the supernatant. Thus, in the presence of actin, chymotrypsin appears to digest the intact myosin heavy chain at the HMM/LMM junction to give a 125-kDa N-terminal HMM fragment and a 95-kDa LMM fragment. This HMM bound to actin in the absence of ATP but not in its presence. With longer digestions, the 125-kDa HMM fragment was cleaved at the site 70 000 daltons from the N-terminus.

## DISCUSSION

The diagram given in Figure 1 shows what we believe are the major sites on the heavy chain of thymus myosin for digestion by papain and chymotrypsin. These sites of digestion are similar to those found on the heavy chains of muscle myosins, but there are differences in the relative rates of digestion at these sites and in the effects of these digestions on ATPase activity.

Papain appeared to digest the heavy chain of thymus myosin at sites approximately 70 000 daltons (site P1) and 94 000 daltons (site P2) from the N-terminus. The rate of cleavage at site P2 was faster than that at site P1. Cleavage at site P2 produced a 94-kDa S1 fragment and a 120-kDa rod fragment. Subsequent cleavage at site P1 gave S1 with a heavy chain made up of 70- and 25-kDa fragments. Cleavage of the intact heavy chain at site P1 produced a digested form of myosin with 70- and 140-kDa heavy chain fragments. These two fragments were held together by noncovalent interactions as most of the 70-kDa fragment present in the early stages of papain digestion precipitated when the samples were centrifuged in 100 mM NaCl. Gizzard myosin (Nath et al., 1982; Sellers & Harvey, 1984; Marianne-Pepin et al., 1985) and brush border myosin (Citi & Kendrick-Jones, 1986) are also digested by papain at sites about 70 000 and 94 000 daltons from the N-termini of their heavy chains. While the pattern of digestion of brain myosin by papain is more complex than that of thymus myosin, the results appear to be consistent with there being sites of digestion 70 000 and 94 000 daltons from the N-terminus (Matsumura et al., 1985).

Phosphorylation appears to regulate the actin-activated ATPase activities of gizzard and thymus myosins by different mechanisms. Phosphorylation regulates the actin-activated ATPase of gizzard myosin by increasing the  $V_{max}$ , and the two heads of this myosin are regulated in a cooperative manner. On the other hand, phosphorylation regulates the actin-activated ATPase of thymus myosin by increasing its apparent affinity for actin, and the two heads of this myosin appear to be independently regulated. However, as has been observed with gizzard S1 (Nath et al., 1982; Ikebe & Hartshorne, 1985), the actin-activated ATPase activity of thymus S1 was not regulated by LC20 phosphorylation (Table II).

In 0.6 M NaCl, chymotrypsin rapidly cuts the heavy chains of striated and smooth muscle myosins at the HMM/LMM junction (Weeds & Pope, 1977; Onishi & Watanabe, 1979; Seidel, 1980; Sellers et al., 1981). The resulting HMMs have heavy chain fragments of about 140 000 daltons. With longer digestions, the heavy chain fragment of smooth muscle HMM is cut within the head to give two fragments of about 65 000 daltons (Seidel, 1980; Okamoto & Sekine, 1981b; Sellers & Harvey, 1984). Under these conditions, chymotrypsin rapidly digested thymus myosin to give 140- and 70-kDa heavy chain fragments. However, this initial cleavage occurred within the myosin head at site C2 and not at the HMM/LMM junction. The resulting 140-kDa fragment was not HMM; rather, it contained the myosin tail and part of the head. With longer periods of digestion, chymotrypsin did cut at site C3 to produce HMM. The heavy chain of this HMM was composed of a 70-kDa N-terminal fragment and a 65-kDa C-terminal fragment which contained S2 and part of S1. At both high and low ionic strengths, with and without ATP, and in  $MgCl_2$  or in EDTA, the rate of cleavage at site C2 was much faster than at site C3. Chymotrypsin also appears to rapidly digest brain and platelet myosins (Matsumura et al., 1985; Sellers et al., 1985; Barylko et al., 1986) about 70 000 daltons from the N-termini of their heavy chains.

Actin binding to thymus myosin inhibited cleavage at site C2 by chymotrypsin. This inhibition suggests either that the region around site C2 is part of the actin binding site or that actin binding alters the conformation of this part of the myosin heavy chain. Cleavage at this site resulted in a large decrease in the  $K_{app}$  of the actin-activated ATPase of thymus myosin which also suggests that the region around site C2 contributes directly or indirectly to the actin binding site of thymus myosin. The actin-activated ATPase activity of thymus myosin digested at this site was not regulated by light chain phosphorylation (Table II). This suggests the possibility that one effect of phosphorylation is to alter the conformation around this site on the heavy chain. While the effect of this digestion on the actin-activated ATPase of gizzard myosin has not been reported, cleavage of skeletal muscle S1 by trypsin at a site about 70 000 daltons from the N-terminus causes a large increase in the  $K_{app}$  of its actin-activated ATPase activity (Botts et al., 1982). Proteolytic digestions (Yamamoto & Sekine, 1979) and cross-linking experiments (Mornet et al., 1981) have suggested that the actin binding site of skeletal muscle myosin is located in a region about 70 000 daltons from the N-terminus.

Thymus HMM made by chymotryptic digestion in the absence of actin cannot be used to study the interaction of intact myosin heads with actin as the heavy chains of this HMM are cleaved at site C2. While it does appear possible to make HMM with intact heavy chains by performing the digestion in the presence of actin, thus far we have been unable to isolate enough of this HMM to perform kinetic experiments.

One reason for the low yields is that it is necessary to use short digestion times to minimize LC20 digestion. There is also a substantial loss of this HMM during the steps used to separate it from actin.

In the presence of ATP, chymotrypsin rapidly digested the thymus myosin heavy chain at a site 4000 daltons from the N-terminus (site C1). The rate of digestion at site C1 was comparable to that at C2. Digestion at site C1 resulted in about a 2-fold increase in the EDTA-ATPase activity and 3-fold decreases in the  $Ca^{2+}$ - and  $Mg^{2+}$ -ATPase activities of thymus myosin. These changes were not due to digestion at site C2 as thymus myosin digested in the absence of ATP had about the same ATPase activity as the intact myosin. In the absence of ATP, chymotrypsin appears to slowly digest the thymus myosin heavy chain at a site about 2000 daltons from the N-terminus. The rate of digestion at this site was much less than at site C1 in the presence of ATP and was comparable to the rate of digestion of LC20. The effect of cleavage at the site 2000 daltons from the N-terminus was not determined as myosin which had been cleaved at this site also contained digested LC20. In the presence of ATP, chymotrypsin digests the heavy chain of gizzard myosin at a site about 5000 daltons from the N-terminus (Okamoto & Sekine, 1981b). As with thymus myosin, this cleavage results in an increase in the EDTA-ATPase activity and a decrease in the  $Ca^{2+}$ - and  $Mg^{2+}$ -ATPase activities of gizzard myosin. In the absence of ATP, chymotrypsin digests the heavy chain of gizzard myosin at a site about 2000 daltons from the N-terminus. Phosphorylation of gizzard LC20 inhibits this cleavage in the absence of ATP, but cleavage at site C1 in the presence of ATP is not affected by LC20 phosphorylation (Okamoto & Sekine, 1981a). Phosphorylation of LC20 had no obvious effects on the digestion of the thymus myosin heavy chain in the presence and absence of ATP. Thus, while LC20 phosphorylation protects the N-terminus of gizzard myosin from chymotryptic cleavage in the absence of ATP, it did not have a similar effect on the digestion of thymus myosin.

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**Registry No.** ATP, 56-65-5; ATPase, 9004-07-3; chymotrypsin, 9004-07-3; papain, 9001-73-4.

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## Isolation of Products and Intermediates of Pancreatic Prosomatostatin Processing: Use of Fast Atom Bombardment Mass Spectrometry as an Aid in Analysis of Prohormone Processing<sup>†</sup>

P. C. Andrews and Jack E. Dixon\*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

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**ABSTRACT:** Major products and an intermediate in the proteolytic processing pathway of preprosomatostatin I from anglerfish (*Lophius americanus*) were purified and characterized. Proteolytic mapping by fast atom bombardment mass spectrometry was used to rapidly locate regions of the peptides whose masses deviated from those deduced from the cDNA sequence. Amino acid analysis and partial Edman sequencing were also used to confirm the structures. The protein structural data indicate a Glu for Gly substitution at position 83 of preprosomatostatin I (aPPSS-I, numbering from the initiator Met) relative to the cDNA sequence. Two of the peptides isolated, aPPSS-I (26-52) (7.5 nmol·g<sup>-1</sup>) and aPPSS-I (26-92) (49.5 nmol·g<sup>-1</sup>), define signal cleavage as occurring between Cys-25 and Ser-26. A partial sequence was obtained from fragment ions in the mass spectrum of a peptide corresponding to aPPSS-I (94-105) (58 nmol·g<sup>-1</sup>). The 14-residue somatostatin [SS-14 corresponding to aPPSS-I (108-121)] has previously been isolated [Noe, B. D., Spiess, J., Rivier, J. E., & Vale, W. (1979) *Endocrinology (Baltimore)* 105, 1410-1415]. Taken together, these peptides suggest a pathway for prosomatostatin I processing in which the residues corresponding to SS-14 and the immediately preceding 14 residues are cleaved from the prohormone via endoproteolysis (order of cleavage not determined). The fragment aPPSS-I (94-105) was isolated in lower yield than SS-14 and may represent a secondary site of cleavage. Subsequent cleavage at arginine-53 results in the minor peptide aPPSS-I (26-52). The terminal basic amino acids generated by endoproteolytic processing were not found for any of the peptides isolated. The peptides described were identified as products of aPPSS-I processing in radiolabeling studies using intact anglerfish islets [Noe, B. D., Andrews, P. C., Dixon, J. E., & Spiess, J. (1986) *J. Cell Biol.* 103, 1205-1211].

**P**rotein sequence data are now being deduced from cDNA sequences at rates that exceed the capacity of protein chemists to analyze posttranslational processing events. Proteins may undergo a large number of posttranslational modifications

including glycosylation, phosphorylation, proteolytic processing, and disulfide bond formation among many others (Wold, 1981). At present, it is not possible to predict, with accuracy, which posttranslational modifications of a particular protein sequence might occur. Principal factors determining post-translational events include primary structure, but higher orders of structure also appear to be important (Oroszlan & Copeland, 1985). Tissue-specific expression of the processing enzyme is also important (Marshak & Yamada, 1984; Pra-

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