

# Qualitative Analysis of Skeletal Myosin as Substrate of $\text{Ca}^{2+}$ -activated Neutral Protease: Comparison of Filamentous and Soluble, Native, and L2-Deficient Myosin

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**ABSTRACT**  $\text{Ca}^{2+}$ -activated neutral protease (CAF) was capable of degrading myosin over a 200-fold range of protease concentrations. CAF selected the heavy chain of myosin, although either prolonged exposure to or high concentrations of the protease degraded the L1, but not the L2 or L3, light chains of myosin. The following results indicated that during the first hour of digestion, under conditions where native myosin was the substrate, CAF selected for the "head" region of the myosin heavy chain: (a) large heavy chain fragments of identical molecular weight were produced from filamentous and from soluble myosin; (b) light meromyosin was not a substrate; (c) agents known to bind to the head of myosin (actin, MgATP, and L2) had both a qualitative and quantitative effect on degradation; and (d) similar cleavage sites could be demonstrated for myosin and for heavy meromyosin (HMM) despite the fact that HMM was a much poorer substrate than myosin. This observation is interpreted as an indication that the conformation of myosin heavy chain is altered in the preparation of HMM. The principal cleavage sites on the heavy chain of myosin were 20,000, 35,000 and 50,000 D from the N-terminus, producing large fragments with molecular weights of 180,000, 165,000, and 150,000 which comprised a "nicked" species of myosin. This nicked species retained both normal solubility properties and normal hydrolytic activities. For this reason, it is concluded that "nicked myosin" is an important pathophysiological species.

It is generally accepted that various conformations of skeletal myosin are fine tuners of troponin's calcium switch. The calcium affinity of troponin is increased when the thin filament is saturated with nucleotide-free myosin (1) or *N*-ethylmaleimide-modified myosin (2, 3), and is decreased when skeletal myosin is deficient in the L2 light chain (4). In fact, the L2 light chain is an important direct determinant of myosin conformation and an indirect determinant of thin filament cooperativity. Loss of L2 exposes a protease-sensitive segment of the subfragment two (S-2)<sup>1</sup> region of the myosin heavy chain (5), and decreases both the mechanical  $V_{\max}$  of skinned fibers (6) and the hydrolytic  $V_{\max}$  of the *in vitro* actomyosin system (7, 8). Phosphorylation of L2 protects the heavy chain of myosin from protease digestion (9, 10) and

increases the affinity of myosin for actin (11, 12), which may be expressed in the fiber as either a decrease in both the rate of cross-bridge cycling (13) and energy utilization (14) or an increase in isometric twitch tension after tetany (15).

We deduced that a biochemical feedback mechanism exists between various conformational states of myosin and the  $\text{Ca}^{2+}$ -affinity of troponin. Some of these alterations of myosin are physiological, such as phosphorylation. Others seem to be of pathophysiological origin: nucleotide-free myosin (ischemia); L2-deficient myosin (cardiomyopathy) (16); or heavy chain degraded myosin (dystrophy) (17). It, therefore, became important to determine whether pathophysiological proteases, such as  $\text{Ca}^{2+}$ -activated neutral protease (CAF), were capable of exerting an influence on the population distribution of various myosin species. It is known that both isolated (18, 19, 20) and myofibrillar myosin (21) are substrates for exogenous CAF. We have observed that dystrophic, but not normal myosin preparations, possess considerable endoge-

<sup>1</sup> Abbreviations used in this paper: CAF,  $\text{Ca}^{2+}$ -activated neutral protease; HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment one.

nous CAF activity. As a result, the heavy chain of unphosphorylated dystrophic myosin is extensively degraded, and the actin concentration at half-maximal velocity of actomyosin in ATPase of actin for myosin is increased, without a substantial impairment of the ATP site (17). For these reasons, we hypothesized that certain species of partially degraded myosin have important functions in the diseased state. As a first step toward proving this hypothesis, it was necessary to undertake a qualitative kinetic analysis of unphosphorylated normal myosin as substrate for CAF. The present investigation establishes the conditions for myosin degradation and localizes the regions on the native myosin molecule accessible to CAF. There have been preliminary reports of some of these findings (20, 22).

## MATERIALS AND METHODS

**Materials:** ATP, disodium salt, vanadium-free, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, and  $\alpha$ -casein (C-7891) were obtained from Sigma Chemical Co., St. Louis, MO. Ammonium sulfate was designated ultra-pure by Schwarz/Mann Biochemical Co., Cambridge, MA. DEAE-cellulose (52) and phenyl-sepharose were obtained from Whatman Chemical Separation Inc., Clifton, NJ and Pharmacia Fine Chemicals, Piscataway, NJ. All gel electrophoresis supplies, including  $M_r$  standards, were obtained from Bio-Rad Laboratories, Richmond, CA. Leupeptin was a gift of Dr. A. Stracher (State University of New York, Downstate Medical Center, Brooklyn, NY).

**Protein Preparations:** Unphosphorylated skeletal myosin was extracted from the back and leg muscles of rabbits as described previously (4). Substituting rabbit skeletal myosin for chicken skeletal myosin has no effect on the results reported herein. Purified  $\text{Ca}^{2+}$ -activated neutral protease was prepared from chicken pectoralis major muscle as described by Dayton et al. (23), with the following exceptions or modifications. There was no pH 6.2 precipitate; protease activity was detected in fractions from 0 to 80% saturation of  $(\text{NH}_4)_2\text{SO}_4$ . Therefore, fractions from the 0 to 40% ( $P_{0-40}$ ) and 40 to 80% ( $P_{40-80}$ )  $(\text{NH}_4)_2\text{SO}_4$  precipitations were pooled (700 mg from 5 kg of ground muscle) and loaded on a DEAE-cellulose column ( $2.5 \times 40$  cm) equilibrated in 20 mM Tris-acetate (pH 7.5) [4 $^\circ$ ], 1 mM  $\text{K}_2\text{EDTA}$ , 0.1%  $\beta$ -mercaptoethanol, 1 mM  $\text{NaN}_3$ . A salt gradient (0–0.6 M KCl in 1,800 ml) isolated the active fraction between 225 and 260 mM KCl. This fraction (10–14 mg) was applied to a  $2.5 \times 20$  cm phenyl-Sepharose column equilibrated in 0.25 M KCl, 20 mM Tris-acetate (pH 7.5) [4 $^\circ$ ], 0.1%  $\beta$ -mercaptoethanol, 1 mM  $\text{K}_2\text{EDTA}$ , 1 mM  $\text{NaN}_3$ . A reverse gradient (0.25 to 0 M KCl in 200 ml) followed by a 1-mM EDTA wash eluted the purified active enzyme (0.5–1 mg/5 kg of ground muscle).

**Assay for Protease Activity:** CAF-mediated degradation of myosin produced only negligible amounts of trichloroacetic acid-soluble material. For this reason, CAF activity was defined as the  $\text{Ca}^{2+}$ -dependent degradation of myosin that was observable on SDS polyacrylamide gels. Unless otherwise indicated, column fractions (50 to 1 wt ratio of myosin to presumptive protease) or purified protease extracts (1,000 to 1 wt ratio) were incubated for 1 h at 25 $^\circ\text{C}$  in 0.25 ml of the following medium: 100 mM NaCl, 100 mM Tris-acetate (pH 7.5) [25 $^\circ\text{C}$ ], 1.25 mg insoluble (filamentous) myosin, 5 mM  $\text{Ca}^{2+}$ , 10 mM  $\beta$ -mercaptoethanol, 1 mM  $\text{NaN}_3$ . In addition, there were two controls: an endogenous protease (plus  $\text{Ca}^{2+}$ , no CAF), and a  $\text{Ca}^{2+}$  (plus CAF, no  $\text{Ca}^{2+}$ ) control. Digestion was terminated by addition of  $\text{K}_2\text{EGTA}$  to a free EGTA of 2 mM and SDS and  $\beta$ -mercaptoethanol to final values of 1%.

Various inhibitors of proteases were assayed with CAF. Leupeptin (1.25 mg/ml), phenylmethylsulfonyl fluoride (10 mM), soybean trypsin inhibitor (11.6  $\mu\text{M}$ ) or potassium tetrathionate (1 mM) were preincubated with CAF (50  $\mu\text{g}$ /ml) 10 min at 25 $^\circ\text{C}$ , followed by the addition of myosin (1,000 to 1 wt ratio of myosin to CAF) (5  $\mu\text{g}$ /ml) and incubation for 1 h at 25 $^\circ\text{C}$ . In the case of potassium tetrathionate, excess reagent was removed by dialysis before the myosin assay, which was run in the presence and absence of  $\beta$ -mercaptoethanol.

**Calcium Concentration:** By varying the ratio of  $\text{K}_2\text{EGTA}$ / $\text{CaEGTA}$  at a constant ionic strength assuming a  $K_d$  of 0.19  $\mu\text{M}$  at pH 7 for  $\text{Ca}^{2+}$  and EGTA (24), we regulated calcium concentration. Stock solutions of CAF contained 5 mM  $\text{K}_2\text{EDTA}$  to inhibit autodigestion. For this reason, it was necessary to adjust the  $\text{Ca}^{2+}$  equation for competition between  $\text{K}_2\text{EDTA}$  ( $4 \times 10^{-7}\text{M}$  in the assay) and  $\text{K}_2\text{EGTA}$  (1 mM) by a modification of the method employed by Bremel and Weber (25) to correct for the competition between free ATP and EGTA for  $\text{Ca}^{2+}$  in their experiments. Above  $10^{-4}\text{M}$ , calcium was essentially unbuffered.

**Protease Concentration:** We based protease concentration on a

molecular weight estimate of 110,000 for CAF (23) and on the Lowry standard curve for myosin which was calibrated as described previously (4). The  $\text{Mg}$ -,  $\text{Ca}$ -, and  $\text{K-EDTA-ATPase}$  activities of myosin were assayed as described by this laboratory (4, 11). Treatment of myosin with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs $_2$  formerly termed DTNB; Nbs $_2$ -myosin = L2-deficient myosin) followed the procedure of Gazith et al. (26). Chymotryptic light meromyosin (LMM) was purified from myosin rods and tails by alcohol fractionation (5). Heavy meromyosin (HMM) was prepared by chymotryptic digestion of soluble myosin in the presence of 4 mM  $\text{MgCl}_2$  to preserve L2 (5), followed by chromatography on DEAE-cellulose to remove contaminant S-1 and heavy chain fragments (27). The front two-thirds of the HMM peak was salted out upon addition of 43–55% saturated ammonium sulfate. This species of HMM was L2-deficient (11) (see Fig. 9).

**Electrophoresis techniques:** SDS electrophoresis was performed on slab gels of 7, 10, or 12% acrylamide essentially as described by Maizel (28). The gels were run at 10 mA until the tracking dye entered the separating gel, and then at 15 mA for 10 cm. Estimations of molecular weight were based upon the mobilities of the following protein standards: myosin heavy chain (200,000); beta-galactosidase (116,000); phosphorylase b (92,500); bovine serum albumin (66,000); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); and lysozyme (14,400). As a precaution against nonspecific fragmentation of the heavy chain (see reference 29), all samples, including controls, were made 5 mM to  $\text{K}_2\text{EGTA}$  before boiling for 3 min. The amount of heavy chain and heavy chain fragments were estimated by measuring the distance in millimeters between inflection points on the integration line produced by the internal integrator of a LKB Ultrosan Laser Densitometer (LKB Instruments, Inc., Gaithersburg, MD).

## RESULTS

Throughout purification of CAF, it was possible to probe for the presence of this protease by monitoring the ability of various fractions to degrade the heavy chain of myosin. No  $\text{Ca}^{2+}$ -insensitive proteolytic activity was detectable with myosin as substrate.  $\text{Ca}^{2+}$ -sensitive proteolytic activity with myosin as substrate was first observed in the pH 4.9 precipitate, and was not separated further by ammonium sulfate fractionation. The former precipitate was, therefore solubilized, clarified, and concentrated before chromatography on DEAE-cellulose. Of the fractions from the DEAE-cellulose column, only fraction VI was capable of degrading the heavy chain of myosin and only in the presence of calcium (Fig. 1). Interestingly, fraction V contained primarily an 85,000-mol-wt component which was devoid of proteolytic activity (with either casein or myosin as substrates) and did not coelectrophore with the 80,000-mol-wt subunit of CAF (Fig. 2).

The purification procedure, although not unique to this point (23), is reported here to assist other investigators in studies in which CAF purity may not be crucial, since a relatively impure fraction of CAF (i.e., well P in Gel A of Fig. 2) can be employed to degrade myosin without risk of contamination by other myosin-cleaving proteases. In the present study, however, CAF purity was essential. Additional purification involving chromatography on phenyl-Sepharose improved the purity of CAF to >90%. This CAF fraction existed as a dimer composed on one 80,000- and one 28,000–30,000-mol-wt subunit (Fig. 2). This extensively purified CAF fraction was used in all the experiments reported herein.

With native myosin as a substrate, the requirements for CAF activity were similar to those reported for other substrates (30, 31). Myosin heavy chain degradation was optimal at pH 7 and inhibited by leupeptin, but not by phenylmethylsulfonyl fluoride or soybean trypsin inhibitor. CAF contained essential thiol groups as determined by reversible inhibition with potassium tetrathionate.

The  $\text{Ca}^{2+}$ -sensitivity of myosin degradation was a property of CAF. With either casein or myosin as substrate, negligible proteolytic activity was observable  $<0.5$  mM  $\text{Ca}^{2+}$ , and the profile of fragments for each substrate was similar between

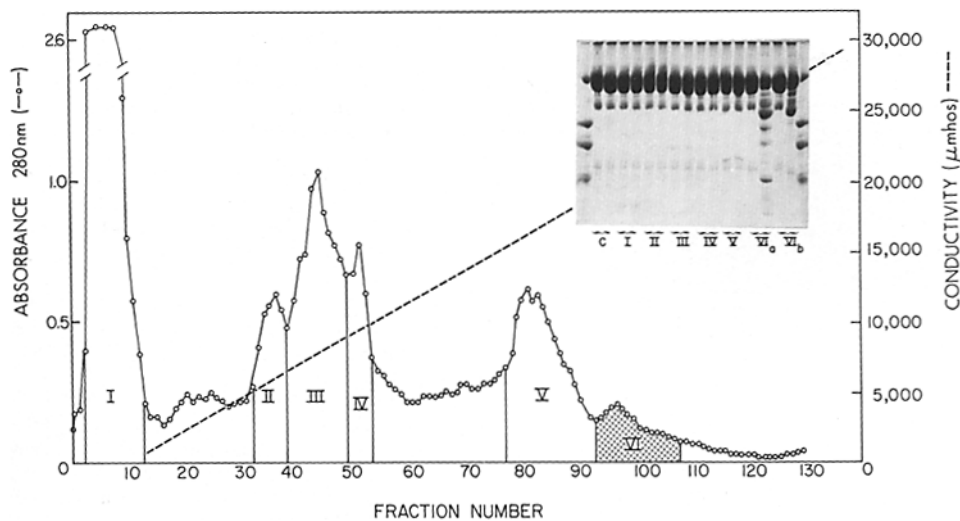


FIGURE 1 Test of the ability of crude extracts of CAF to degrade myosin. All proteolytic activity was  $\text{Ca}^{2+}$ -sensitive and localized in one fraction from a DEAE-cellulose (52) column. 10-ml column fractions were pooled as indicated, concentrated, and tested for the ability to degrade myosin. (Inset) Gel profile (7% gel) of myosin incubated alone (c) or with fractions I–VI in the absence (left lane) or presence (right lane) of  $\text{Ca}^{2+}$  (5 mM); VI<sub>a</sub> and VI<sub>b</sub> refer to two preparations of CAF. For further details see Materials and Methods section.

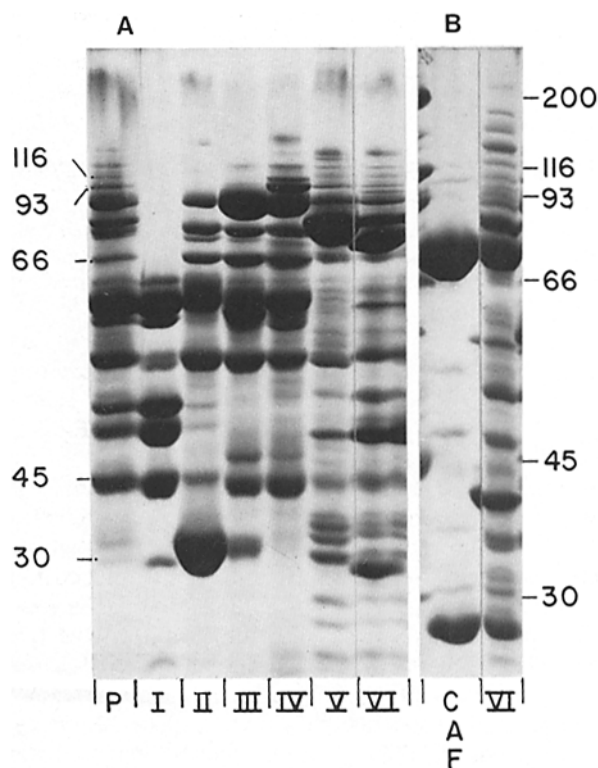


FIGURE 2 CAF fractions at various stages of purification. (Gel A) P, P<sub>0-80</sub> fraction; fractions I–VI from the DEAE-cellulose column (Fig. 1). (Gel B) (Left lane) CAF, 90% pure from the phenyl-Sepharose column; (right lane) fraction VI from the DEAE-cellulose column rerun for comparison. Two 10% gels run at different times. 25  $\mu\text{g}$ /well.

0.5 and 5 mM  $\text{Ca}^{2+}$  (not shown).

The first series of experiments demonstrated that CAF was capable of degrading the heavy chain of myosin over a wide range of protease concentrations (Fig. 3, A and B). At a 1,000 to 1 mg ratio of myosin to protease, the principal degradation products of the myosin heavy chain had approximate molecular weights of 180,000 (site 1), 165,000 (site 2), and 150,000 (site 3). As the concentration of CAF increased, the amount of undegraded heavy chain decreased along with a noticeable increase in the 150,000-mol-wt fragment and fragments of intermediate molecular weights (50,000, 70,000, 95,000,

110,000 and 136,000). At a 50 to 1 ratio of myosin to CAF, two of the corresponding lower molecular weight fragments, one at 20,000 and the other at 15,000, were discernible: at site 1 (200,000 – 180,000 = 20,000), and at site 2 (180,000 – 165,000 = 15,000) or 3 (165,000 – 150,000 = 15,000). These designations (site 1, 2, and 3) refer only to the three major heavy chain products in order of decreasing molecular weight and should not be interpreted as a quantitative indication of the sequence or the rate of degradation. High concentrations of CAF (>25  $\mu\text{g}/\text{ml}$ ) degraded L1 without appreciably affecting L2 or L3 (Fig. 3B). However, the 20,000–21,000-mol-wt fragment migrating below L1 was derived from the heavy chain, since its appearance in time course experiments coincided with formation of the 180,000-mol-wt fragment and not with degradation of L1 (not shown). This experiment indicated the maximum number of cleavage sites on myosin accessible to CAF in 1 h at 25°, when native myosin was in filaments. Unless otherwise indicated, subsequent experiments were performed at the lowest concentration of CAF where myosin degradation was readily apparent, in order to ensure that the principal substrate was native myosin (at a 1,000 to 1 mg ratio of myosin to CAF). Under these conditions, ~50% of the heavy chain was degraded with an increase in the amount of 180,000- (50%), 165,000- (30%), and 150,000-mol-wt (271%) fragments. Molecular weight estimations of the three principal heavy chain fragments were verified by calibration curves of degraded myosin run on 7 and 12% gels (Fig. 4).

A time course profile (Figs. 5 and 6) of myosin degradation at low concentrations of CAF indicated that (a) myosin degradation increased steadily for 2 h with a net loss of 34% of the heavy chain, then remained constant from 2 to 6 h, and finally increased an additional 16% from 6 to 12 h; (b) the 180,000-mol-wt fragment increased more rapidly than the 150,000- and the 165,000-mol-wt fragments (Fig. 5); (c) fragments with molecular weights of 21,000 and 30,000 were discernible after 5 min (gel not shown).

Densitometric estimation of the initial rate of accumulation of the 180,000-, 165,000-, and 150,000-mol-wt components (sites 1–3, respectively) indicated that site 1 was the preferred site of degradation, the rate being at least 3.5 times greater than that at site 2, but only 30% greater than the rate at site 3 (Fig. 6). If all myosin molecules had been sequentially degraded (i.e., attack at site 1 → 2 → 3), the rates of degra-

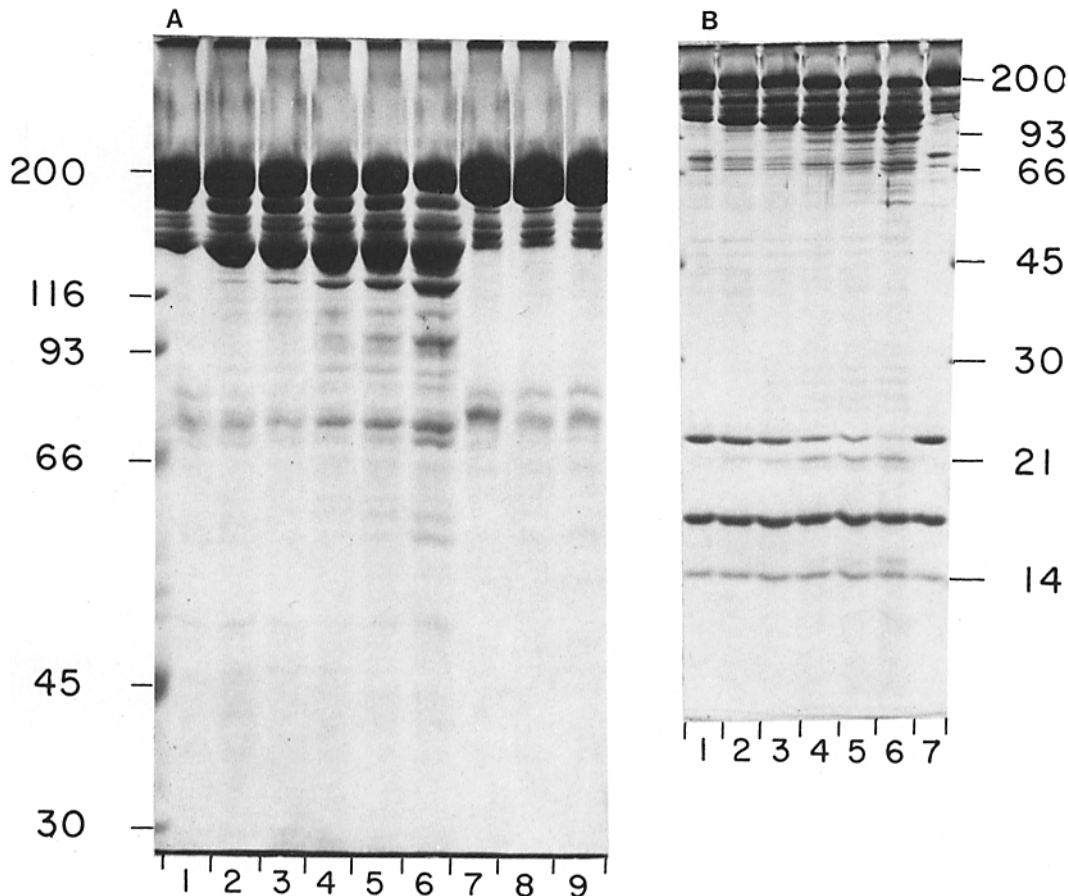


FIGURE 3 The ability of CAF to degrade myosin over a wide range of CAF concentrations. (A) 7% gel, lanes 1-6, decreasing milligram ratios of myosin to CAF (the molar ratios of myosin heavy chain to CAF are given in parenthesis): (1) 10,000:1 (4,400:1); (2) 1,000:1 (440:1); (3) 500:1 (220:1); (4) 200:1 (88:1); (5) 100:1 (44:1); (6) 50:1 (22:1). Lanes 7-9, myosin controls: (7) + CAF, no Ca<sup>2+</sup>; (8) + Ca<sup>2+</sup>, no CAF; (9) no Ca<sup>2+</sup>, no CAF. For further details see Materials and Methods. (B) 12% gel, samples 1-6 from A; sample 7, control, no CAF, no Ca<sup>2+</sup>. 25  $\mu$ g protein based on zero time calculations per lane. (A and B) Gels reduced 10%.

dation at the three sites would have agreed with this sequence and only 21,000- and 15,000-mol-wt components would have been produced. Neither was the case. 30,000-mol-wt and 50,000-mol-wt fragments (180,000 - 150,000 and 200,000 - 150,000, respectively) were discernible on overloaded 7 (Fig. 5) and 12% (not shown) gels. After 10 min of digestion, the gel profiles indicated that the initial point of attack could be either site 1 or site 3. Therefore, the kinetics of myosin degradation favored two sequences: site 1  $\rightarrow$  3  $\rightarrow$  2 or site 3  $\rightarrow$  1  $\rightarrow$  2. A time course analysis in the presteady state of the appearance of the low molecular weight fragments verified the relative reaction rates at the three sites. Thus, the 21,000-mol-wt fragment was the first to accumulate, followed by the 30,000-mol-wt fragment at 5 min and the 50,000-mol-wt fragment between 5 and 10 min. After 10 min; secondary degradation of the low molecular weight fragments (from 21,000 to 14,000, and 50,000 to 35,000) prevented further analysis.

Under the conditions of the present study, the rate of formation of the 165,000-, 150,000-, or 15,000-mol-wt fragments did not correspond to the rate of decrease of the 180,000-mol-wt fragment. This was because (a) undegraded myosin was the principal substrate for the duration of the experiment; (b) myosin was in great excess over CAF and, therefore, after the first 10 min, the reaction approximated the steady state with respect to the 180,000-, 165,000-, and

the 150,000-mol-wt fragments; and (c) site 1 was only slightly preferred over site 3 as the initial point of attack (Fig. 6).

The rate of myosin heavy chain degradation was limited by autodegradation of CAF. An incubation profile of CAF in the absence of myosin (Fig. 7) confirmed that the protease could be autodegraded to produce fragments with molecular weights of 59,000, 54,000, 42,000, 37,000, 24,000, 22,000, and 19,000. Several laboratories have reported similar findings (30, 32). Since the same preparation of CAF is represented in Fig. 2 and the control wells of Fig. 7, limited autodegradation of CAF (at 54,000, 35,000, and 24,000) occurred in the absence of Ca<sup>2+</sup> when 10 mM EDTA (as in Fig. 2) was replaced by 5 mM EGTA (0- and 6-h incubation controls in Fig. 7). This amounted to a 5% decrease in the density of the 80,000-mol-wt subunit and a 30% decrease in the density of the 30,000-mol-wt subunit. Fortunately, an intact 30,000-mol-wt subunit did not appear to be necessary for protease activity since it was completely absent from some preparations and rapidly degraded without loss of protease activity in other preparations. Ca<sup>2+</sup>-insensitive proteolytic activity was minimal and restricted to CAF. Degradation of casein (not shown) and myosin required Ca<sup>2+</sup> (Figs. 1, 3, and 5). In the presence of Ca<sup>2+</sup>, gradual disappearance of the 80,000-mol-wt subunit of CAF (2-4 h) paralleled a decrease in the rate of myosin heavy chain degradation. After 2 h, only 10% of the 80,000-mol-wt subunit remained and this corresponded to a general

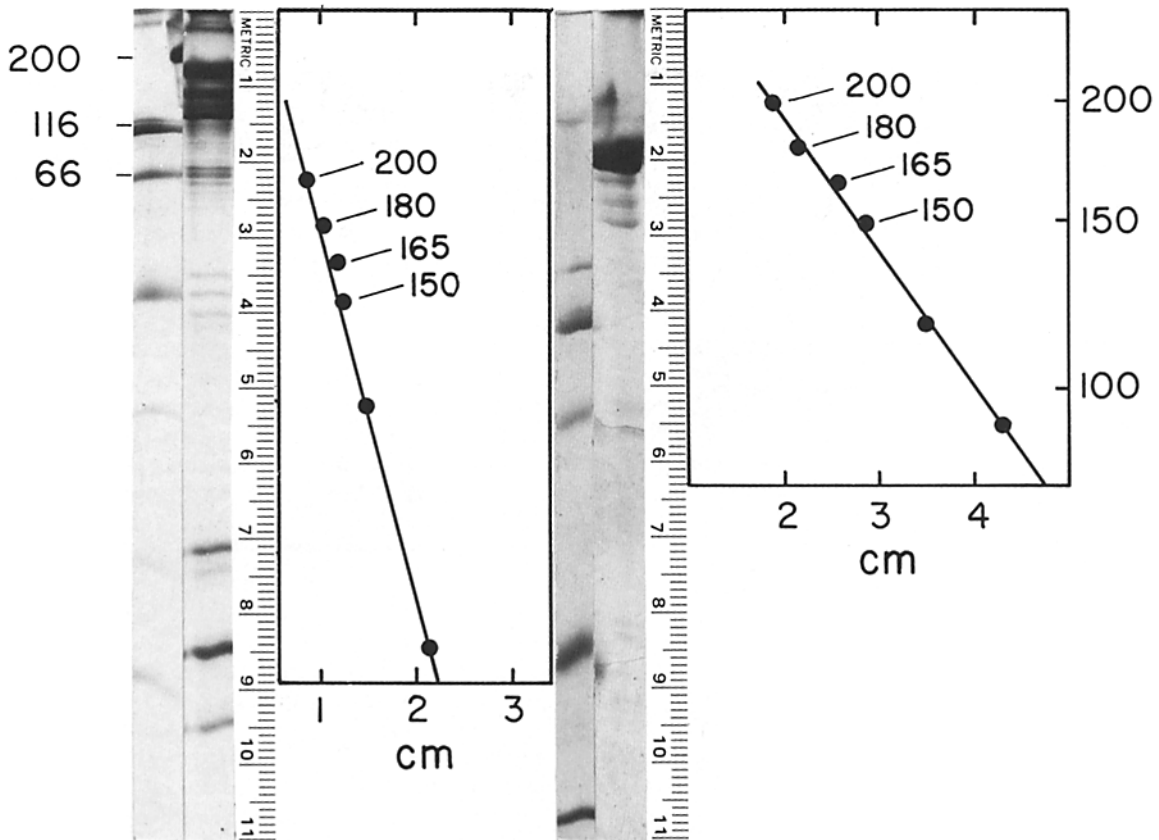


FIGURE 4 Calibration curves in centimeters (cm) of degraded myosin (10  $\mu$ g) run on 12 (left) and 7% (right) gels to indicate the approximate  $M_r$  of the three principal heavy chain fragments. The  $M_r$  of the myosin heavy chain was calibrated from the top of the heavy chain band. Gel reduced 10%.

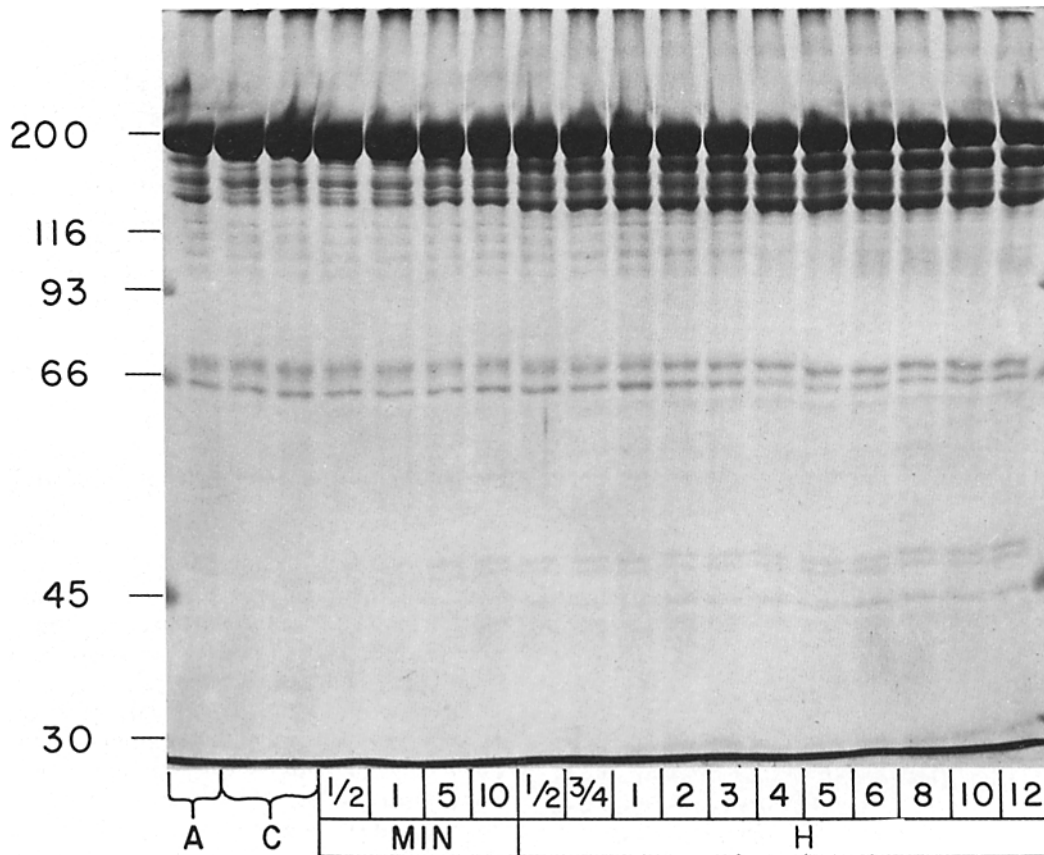


FIGURE 5 Time course at 25°C of degradation of myosin heavy chain by CAF (1,000 to 1 mg ratio of myosin to CAF). (Lane A) 5  $\mu$ g each of 45 min sample and CAF control (no  $\text{Ca}^{2+}$ ) indicating that control samples contain small amounts of heavy chain fragments that co-electrophorese with similar fragments produced by CAF. (Lane C) Control (+ CAF, no  $\text{Ca}^{2+}$ ) at zero time (left lane) and after 12 h (right lane). Note the three principal heavy chain fragments. See text for further details. 25  $\mu$ g protein based on zero time calculations per lane; 7% gel. Gel reduced 10%.

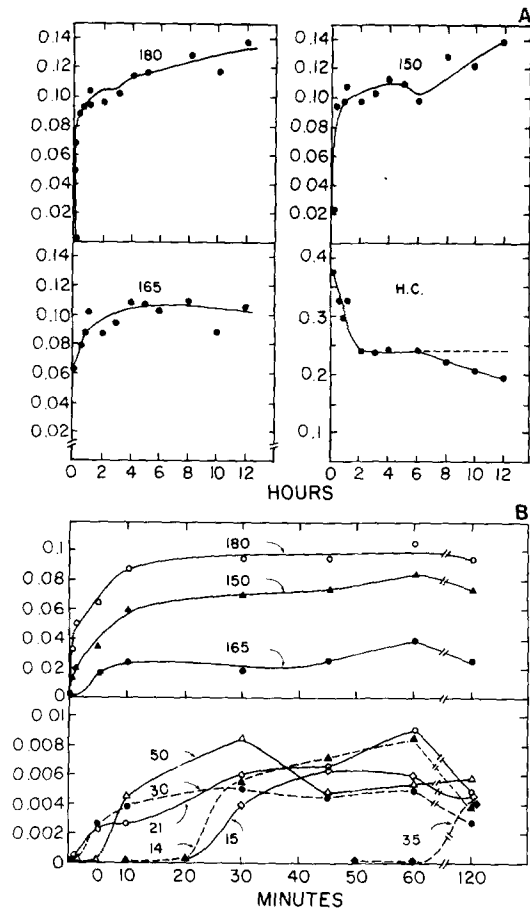


FIGURE 6 Densitometric estimation (concentration expressed in equivalent arbitrary values) of band intensities during degradation of filamentous myosin by CAF. The gel on which these calculations were based is presented in Fig. 5. The graph at the bottom was calculated from a 12% gel (not shown). See text for further details.

cessation of heavy chain degradation from 2 to 6 h (Figs. 5 and 6). There was, however, from 6 to 12 h an unexplained increase (16%) in the rate of heavy chain degradation and 150,000-mol-wt accumulation (Fig. 6). Since our experiments were limited to the first 2 h of digestion, autodegradation of the 80,000-mol-wt subunit of CAF appeared to be limiting the extent of myosin degradation.

It was possible to test this hypothesis by both direct and indirect analyses. For technical reasons, the time course experiment of CAF autodegradation described above was performed in the absence of myosin and at higher concentrations of CAF (4  $\mu$ M) than that normally employed (0.04  $\mu$ M) with myosin as substrate. Direct confirmation of CAF autodegradation was also obtained at 0.4  $\mu$ M CAF. The kinetics of autodegradation was not inhibited by a 10-fold decrease in CAF concentration (not shown). This is in agreement with recent results from the laboratory of Mellgren et al. (33) who found the rate of autodegradation to be unaffected by protease concentration from 5 to 275  $\mu$ g CAF/ml; this is the precise range of the present investigation. Dayton (30) and Mellgren et al. (33) reported rapid inactivation of CAF by autodegradation even in the presence of large excesses of the substrates, casein and succinylated lysozyme, respectively. Therefore, we concluded that CAF autodegradation followed zero order kinetics and that both CAF and myosin were substrates in the present experiments. This hypothesis was verified by indirect analysis:

(a) pre-incubation of a very low concentration of CAF (0.04  $\mu$ M) with  $\text{Ca}^{2+}$  for 6 h completely inhibited myosin degradation (Fig. 8A, lane e); and (b) many short-term treatments with a low concentration of CAF (0.04  $\mu$ M) or a single treatment with a high concentration of CAF (0.9  $\mu$ M) resulted in almost complete disappearance of the myosin heavy chain (Fig. 8, A and B). The extent of myosin heavy chain degradation was limited by autodegradation of CAF during the time frame of the present study (6 h) (Fig. 8A, lane e). There was no evidence that the plateau observed between 2 and 6 h for heavy chain degradation (Figs. 5 and 6) was due to a low affinity of myosin heavy chain for CAF. Nor was there any evidence for a population of myosin heavy chain completely resistant to CAF (Fig. 8A, lane a, and Fig. 8B).

It should be emphasized that either multitreatments with low concentrations of CAF (Fig. 8B) or one treatment with a high concentration of CAF (Figs. 3B and 8A) degraded L1. In these instances, L1 was more resistant to degradation when myosin was soluble rather than insoluble, presumably because soluble myosin had a greater number of cleavage sites on the heavy chain which competed successfully with sites on L1 (Fig. 8B). These results indicated that the rate of degradation of L1 was either slower than that of the heavy chain or that CAF selected for L1 bound to heavy chain fragments. In either case, the initial and preferred cleavage sites were located on the myosin heavy chain. It was not possible to determine whether CAF preferred a particular isozyme of myosin (e.g., native heavy chain associated with L1).

During the first hour of digestion, CAF was unable to distinguish between filamentous and soluble myosin. Increasing the ionic strength from 0.1 to 0.6 only slightly increased

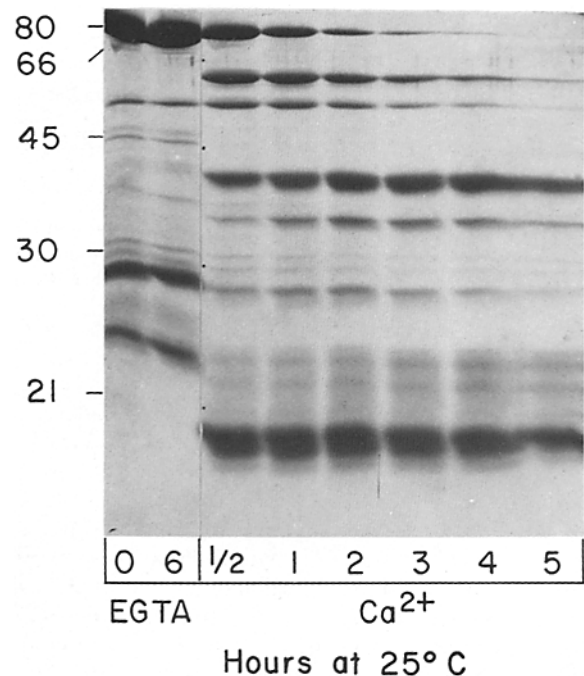


FIGURE 7 Autodegradation of CAF (4  $\mu$ M) at 25°C in the presence of 5 mM  $\text{Ca}^{2+}$ . The 80,000-mol-wt subunit of CAF was more resistant to autodegradation than the 30,000-mol-wt subunit; 15  $\mu$ g/well; 12% gel. This is the same preparation of CAF depicted in Fig. 2B, indicating that very limited autodegradation of CAF occurs upon storage at 4°C, even under sterile conditions and in the absence of  $\text{Ca}^{2+}$  (See text for further details). Degradation of myosin, however, requires  $\text{Ca}^{2+}$  (Figs. 1, 3A, and 5).



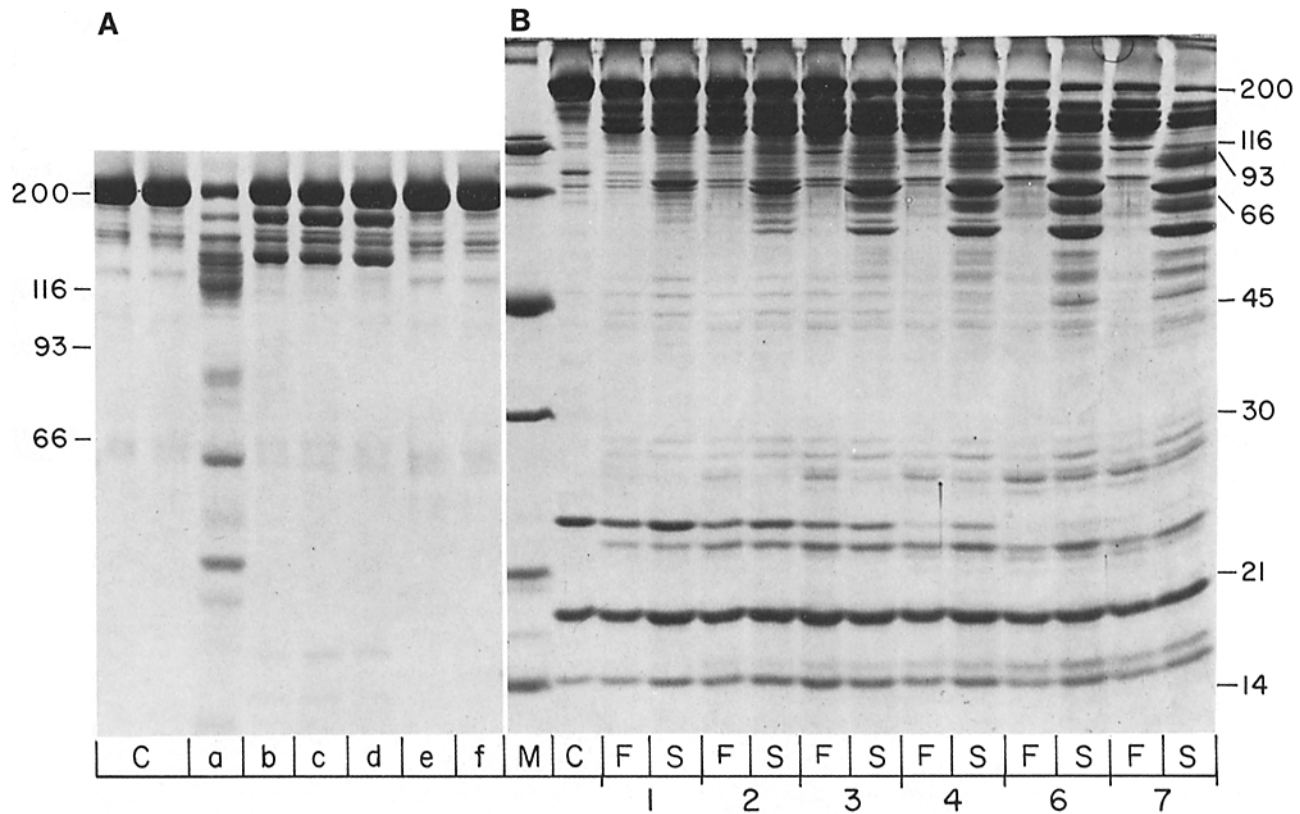


FIGURE 8 (A) The limiting factor for myosin heavy chain degradation was autodegradation of CAF. (lane C) Controls: (no  $\text{Ca}^{2+}$ , no CAF for 2 h (left); +  $\text{Ca}^{2+}$ , no CAF for 8 h [right]). Myosin to CAF wt ratio of 50 to 1 (lane a) or 1,000 to 1 (lane b), +  $\text{Ca}^{2+}$  for 2 h. Effect of pre-incubation of CAF (0.04  $\mu\text{M}$ ) in the absence (lane c) or presence of 5 mM  $\text{Ca}^{2+}$  for 30 min (lane d); 6 (lane e) or 8 h (lane f), on the degradation of myosin (1,000 to 1 mg ratio of myosin CAF, CAF at 0.04  $\mu\text{M}$ ). Pre-incubation of low concentrations of CAF in 5 mM  $\text{Ca}^{2+}$  in lane e or lane f completely inhibited degradation of myosin; this time interval coincided with the disappearance of an intact 80,000-mol-wt subunit of CAF (Fig. 7). High concentrations of CAF (as in lane a) degraded >90% of the myosin heavy chain in 2 h. 25  $\mu\text{g}$ /well; 7% gel. (B) Evidence that myosin is a good substrate for CAF when correction is made for autodegradation of CAF. Multitreatments of filamentous (lanes marked F) or soluble (lanes marked S) myosin with CAF (0.04  $\mu\text{M}$ ) resulted in 90% reduction of undegraded myosin heavy chain after 12 h (seven treatments), whereas only 40% of the myosin heavy chain was degraded after a single treatment with CAF (0.04  $\mu\text{M}$ ) for 12 h (Fig. 5). Lane M,  $M_r$  standards ( $\times 10^{-3}$ ) lane C, control, + CAF, no  $\text{Ca}^{2+}$  for 2 h). 1-7 refer to the number of consecutive 2 h treatments with 0.04  $\mu\text{M}$  CAF. Ionic strength: 0.1 for filamentous; 0.3 for soluble myosin. In 2 h at 25°C and 5 mM  $\text{Ca}^{2+}$ , 20% of the 80,000-mol-wt subunit of CAF remained (Fig. 7). This decreases the ratio of myosin to CAF only slightly from 1,000 to 1 at one treatment to 800 to 1 after seven treatments. Note that the conditions of this experiment are unique to all others in this study (i.e. 2-h incubations/treatments with CAF for a total of seven treatments (12 h). There is a qualitative difference here between filamentous and soluble myosin, and the principal substrate is no longer undegraded myosin. See Fig. 9 for proof that CAF cannot distinguish between filamentous and soluble undegraded myosin during the first hour of digestion. 15  $\mu\text{g}$ /well, 12% gel. (A and B) Gels reduced 10%.

the amount of degradation and had no qualitative effect on the size distribution of the fragments (Fig. 9A). Exceptions to this observation occurred during prolonged incubations (2 h) or multitreatments with CAF (Fig. 8B), where there was an increased probability of competing substrates (myosin and heavy chain fragments). These results support the hypothesis that CAF attacks the "head" of myosin. Presumably, if CAF had attacked the "tail" (LMM) region of myosin, degradation would have been inhibited at low ionic strength during which myosin is in filaments and the tail region is inaccessible to proteolytic attack (5). Furthermore, when extensively degraded myosin (four treatments with CAF, 6 h apart at a 1,000 to 1 mg ratio of myosin to CAF) was centrifuged (10,000 g for 15 min) at physiological ionic strength, >98% of the 180,000-, 165,000-, and 150,000-mol-wt fragments co-precipitated with myosin. The supernatant represented a mere 2% of the total protein. Therefore, either intact LMM was contained in the 180,000-, 165,000-, and 150,000-mol-wt frag-

ments and/or the quaternary structure of myosin had remained intact during limited digestion of the myosin heavy chain.

The latter conclusion was substantiated by activity assays. Both filamentous and soluble degraded myosin retained comparable hydrolytic activities. Heavy chain degradation had little effect on the  $\text{Mg}^-$  (0.052 to 0.063  $\text{s}^{-1}$ ) or  $\text{Ca}$ -ATPase (2.09-2.16  $\text{s}^{-1}$ ) activity, but decreased by 50% (from 14 to 6  $\text{s}^{-1}$ ) the  $\text{K}^+/\text{EDTA}$  ATPase activity. Even so, all values remained in the normal range (34, 35).

Although CAF could not distinguish between filamentous and soluble myosin, CAF could distinguish between native and L2-deficient myosin. For this experiment, 50% of the heads were made L2-deficient by  $\text{Nbs}_2$  treatment. Two new heavy chain fragments appeared at a 1,000 to 1 ratio of L2-deficient myosin to CAF with molecular weights of 146,000 and 95,000 (Fig. 9B). Only the 95,000-mol-wt fragment was soluble. In a typical time course experiment (not shown),

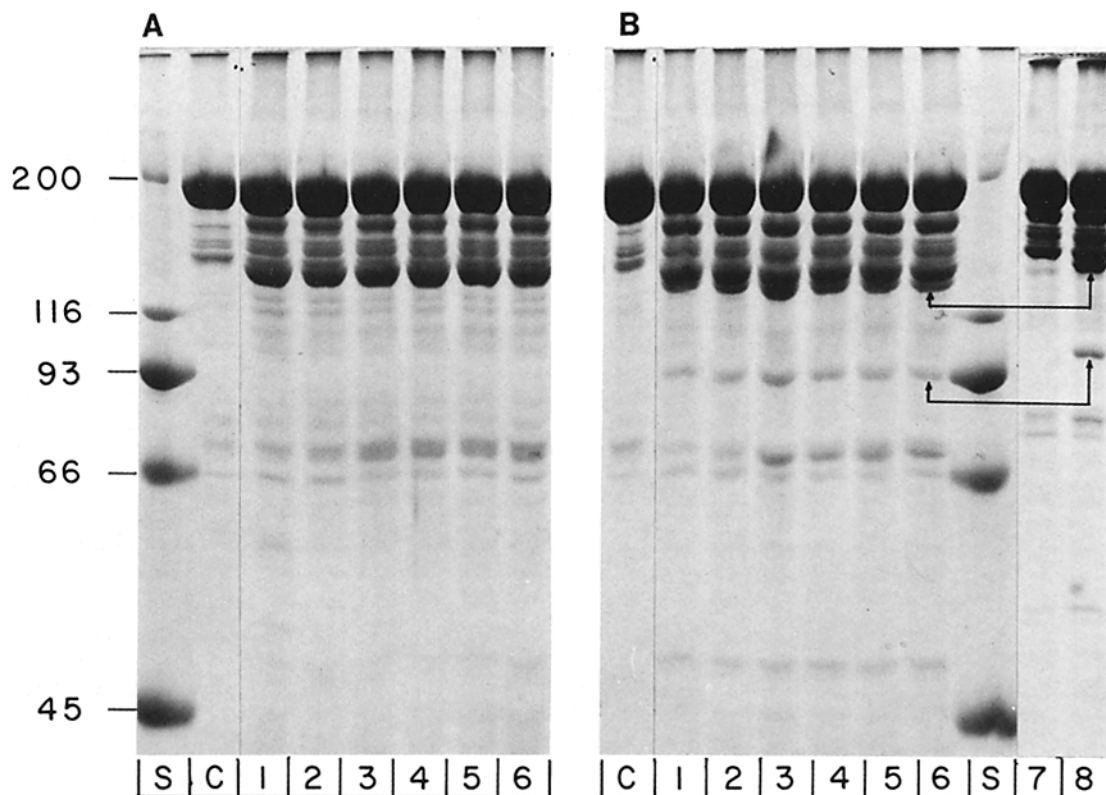


FIGURE 9 (A) During the first h of degradation, CAF could not distinguish between filamentous and soluble myosin, but could distinguish between native (2 mol L2/mol) and L2-deficient (1 mol L2/mol) myosin. (A) Native myosin. Lane S,  $M_r$  standards  $\times 10^{-3}$ ; lane C, control (+ CAF, no  $\text{Ca}^{2+}$ ); lanes 1-6 refer to ionic strength conditions of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6, respectively, during incubation (45 min) at 1,000 to 1 mg ratio of myosin to CAF. (B) L2-deficient myosin (1 mol L2/mol). Lanes 1-6, the same as in A; lanes 7 (insoluble native myosin) and 8 (insoluble L2-deficient myosin), + CAF for 1/2 h. Loss of L2 resulted in two additional heavy chain fragments, one at the 146,000 and the other at the 95,000 band (indicated by arrows). The time conditions of this experiment are the same as those in every figure except Fig. 8. 15  $\mu\text{g}/\text{well}$ , 7% gel. (A and B) Gel reduced 10%.

these new heavy chain fragments were discernible after 5 min (not shown), increased from 5 to 30 min (Fig. 9B, well 8), and did not co-electrophorese with fragments of native myosin (2 mol L2/mol) formed at similar CAF concentrations (not shown). However, the 95,000-mol-wt fragment did co-electrophorese with a minor fragment present at much higher concentrations of CAF (50 to 1 ratio). Therefore, loss of L2 greatly increased the rate of cleavage at this site. Similar to the situation for native myosin, the gel pattern of heavy chain degradation for L2-deficient myosin did not exhibit a salt-sensitivity during the first hour of digestion (Figure 9B). These results support the hypothesis that CAF degrades the head region of the myosin heavy chain. These results should not be interpreted as part of the hypothesis advanced by some investigators (8, 36) that loss of L2 alters the filamentous structure of myosin. Differential proteolysis by CAF of native as compared to L2-deficient myosin was qualitatively unaffected by the state of aggregation of myosin (Fig. 9, A and B).

Additional evidence that CAF attacked the head region of myosin was obtained by comparing the chymotryptic fragments LMM and HMM as substrates of CAF. LMM appeared as two major and three minor molecular weight bands between 60,000 and 70,000. This population of LMM was almost completely resistant to degradation by CAF both at high and low salt, even at ratios of LMM to CAF as low as 15 to 1 (43  $\mu\text{M}$  LMM heavy chain). No LMM fragments at 32,000-36,000 D or 20,000 D were apparent (Fig. 10). It is unlikely, therefore, that CAF attacked the LMM region of intact myosin.

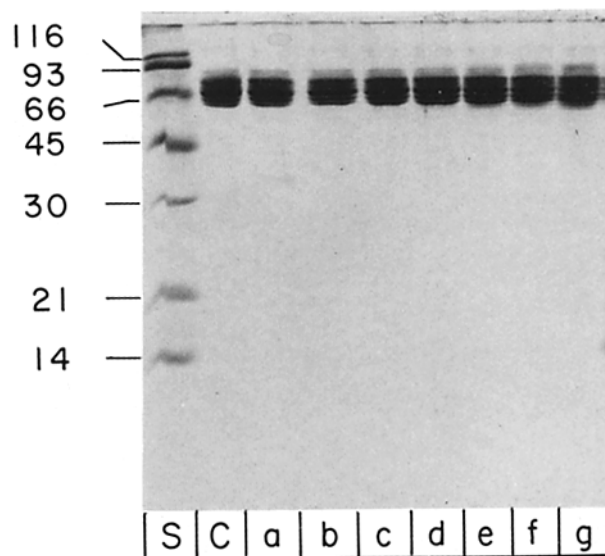


FIGURE 10 LMM was not a substrate of CAF. Lane S,  $M_r$  standards ( $\times 10^{-3}$ ); lane C, control (no  $\text{Ca}^{2+}$ , no CAF). Lanes a-g show decreasing milligram ratios of LMM to CAF. (a) 1,000:1; (b) 500:1; (c) 200:1; (d) 100:1; (e) 50:1; (f) 25:1; (g) 15:1. LMM at 44  $\mu\text{M}$  heavy chain and 0.5 M salt, 1-h incubation at 25°C. 15  $\mu\text{g}/\text{well}$ , 15% gel.

Surprisingly, HMM was not as good a substrate as native (Figs. 3 and 5) or L2-deficient myosin (Figs. 9B and 11). Unchromatographed HMM contained a small amount of 95,000-mol-wt S-1 heavy chain as a contaminant. This frag-



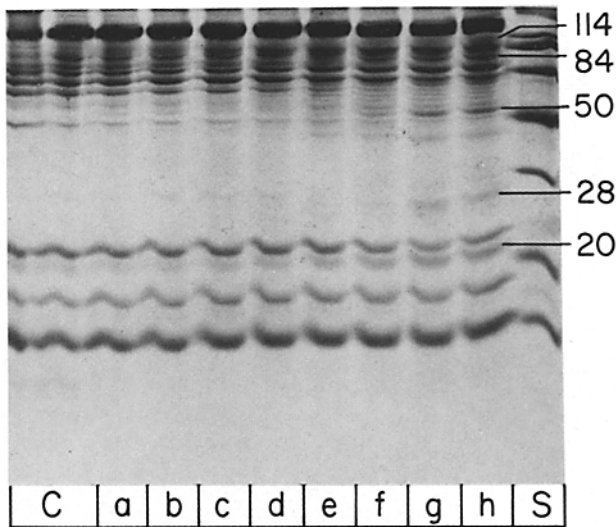


FIGURE 11 HMM was a poor substrate of CAF. Lane *S*, *M*, standards; lane *C*, chromatographed HMM at 0 time (*left*), and after 1 h at 25°C and no  $\text{Ca}^{2+}$  (*right*). HMM heavy chains, 30  $\mu\text{M}$ . Lanes *a*–*g* show decreasing milligram ratios of HMM to CAF as in Fig. 10. Lane *h* is a duplicate of lane *g*. Because HMM preparations are L2-deficient, the 95,000-mol-wt fragment is present, in addition to three cleavage sites in common with native myosin. For HMM, cleavage at site 1 is indicated by 114,000- and 20,000-mol-wt bands; at site 3 by 84,000- and 50,000-mol-wt bands. Cleavage at site 2 is barely observable as a 28,000-mol-wt fragment. 15  $\mu\text{g}$  per lane, 15% gel. See text for further details.

ment was removed completely after brief treatment (30 s) with a low concentration of CAF. No additional degradation was apparent over the next 2 h (not shown). Contaminant S-1 heavy chain, therefore, was not competing with the HMM heavy chain as substrate for CAF. In fact, the heavy chain of HMM was completely resistant to degradation by CAF until addition of a 50 to 1 ratio of HMM to CAF. Therefore, quantitatively, myosin was the preferred substrate. Qualitatively, at 10 to 20 times the CAF concentration needed to observe heavy chain degradation of myosin, it was possible to demonstrate similar cleavage sites on the HMM heavy chain. Cleavage at site 1 produced the 180,000- and 20,000-mol-wt myosin fragments and the 114,000- and 20,000-mol-wt HMM fragments. Cleavage at site 2 produced the 165,000- and the 32,000–35,000-mol-wt heavy chain fragments of myosin, and was barely distinguishable in the case of HMM. In fact, the 28,000-mol-wt HMM fragment was observable because the rate of attack at site 2 was much slower than the rate of attack at sites 1 and 3. Cleavage at site 3 produced 84,000- and 50,000-mol-wt HMM fragments and 150,000- and 50,000-mol-wt myosin fragments (Fig. 3, *A* and *B*, and 11). As was the case with myosin, only the L1 light chain was susceptible to degradation by CAF. Because most preparations of chymotryptic HMM prepared from unphosphorylated myosin are L2-deficient (11), both HMM and L2-deficient myosin (Figure 9*B*) exhibited a 95,000-mol-wt fragment when incubated with CAF. For HMM, this was the preferred point of attack. The same was not true for L2-deficient myosin (Fig. 9*B*). We concluded that although myosin and HMM held three cleavage sites in common, the kinetics of heavy chain degradation by CAF were quite different for HMM and myosin. To our knowledge, this may be the first indication of a conformational alteration in the S-1 and/or the S-2 regions

of the myosin heavy chain during preparation of HMM.

At this stage, it was of interest to determine if either the substrate MgATP, or the co-factor, actin, would have a qualitative effect on the three major regions of the myosin heavy chain accessible to CAF. At a 50 to 1 ratio of myosin to CAF additional cleavage sites were apparent (Figs. 3*A* and 12). For native myosin (2 mol L2/mol), actin and MgATP, when tested separately, inhibited cleavage at site 1, and MgATP, but not actin, inhibited cleavage at site 2 and at two minor sites (the 136,000- and 95,000-mol-wt fragments). Therefore, CAF could distinguish among (*a*) nucleotide-free myosin; (*b*) rigor complexes between actin and nucleotide-free myosin; and (*c*) the principal myosin intermediate during steady state hydrolysis of MgATP ( $\text{M}^{**}\text{ADP.P}$ ). Furthermore, in the presence of either actin or MgATP, a doublet was apparent at the 150,000-mol-wt band (site 3) similar to what had been observed with L2-deficient myosin (Fig. 9*B*). The latter observation that L2 removal, MgATP-, and actin-binding had a qualitative effect upon degradation at site 3 indicated that the L2-binding domain is near the ATP and the actin sites on myosin. Since substances that were known to bind to the head of myosin altered the heavy chain sites accessible to CAF, whereas filament formation was without effect, CAF attacked the head region of myosin.

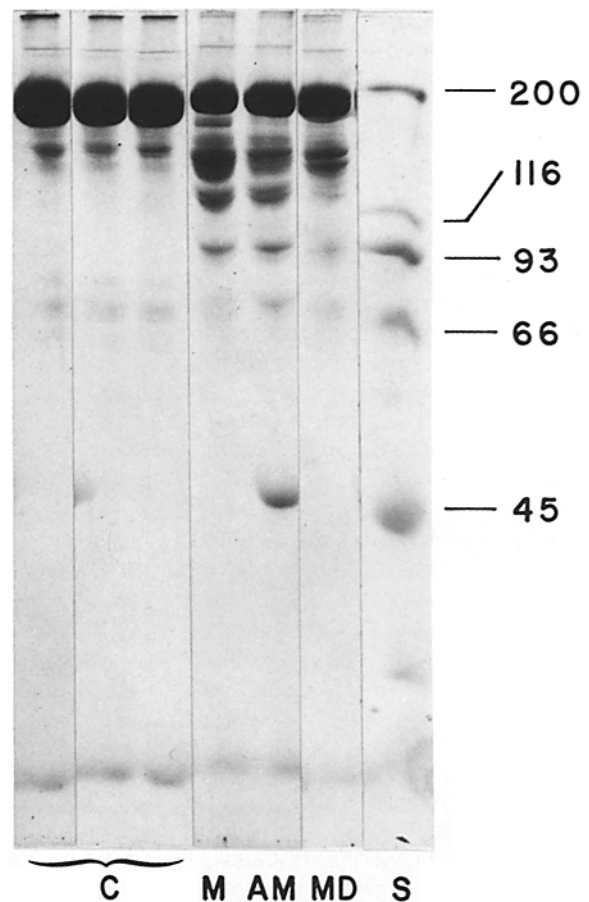


FIGURE 12 CAF can distinguish between nucleotide-free myosin (lane *M*), rigor-like complexes between actin and nucleotide-free myosin (lane *AM*), and the myosin intermediate during steady state hydrolysis of MgATP,  $\text{M}^{**}\text{ADP.P}$  (lane *MD*). Lanes *C*, controls for the three myosin conformations (+CAF, no  $\text{Ca}^{2+}$ ); lane *S*, *M*, standards. Lanes *M*, *AM*, and *MD* were incubated for 1 h at 25°C at a 50/1 ratio of myosin/CAF. 25  $\mu\text{g}$ /well; 7% gel. (10% enlargement.)

## DISCUSSION

The present study characterized myosin as a substrate of CAF. The preferred sites of cleavage were located on the myosin heavy chain. Under *in vitro* conditions, the limiting factor in determining the extent of myosin heavy chain degradation was autodegradation of the 80,000-mol-wt subunit of CAF, the rate of which appeared to be independent of both protease and myosin (substrate) concentrations. Similar results were reported for the rabbit dimeric protease (34). Our protocol for purifying CAF selected for the high  $\text{Ca}^{2+}$ -requiring form of the enzyme since autodegradation (37) was inhibited by  $\text{Ca}^{2+}$ -free buffers. The reported low yield of peak I protease, a low  $\text{Ca}^{2+}$ -requiring form of CAF (38) precluded its detection in the protein fractions from the DEAE-cellulose column (Fig. 1). Therefore, the present study simulated the pathophysiological state (i.e., Duchenne Muscular Dystrophy, or following acute myocardial infarction) in which the free calcium concentration in the sarcoplasm reaches millimolar levels (39). It is generally accepted that the yield of CAF from normal muscle is poor and that normal myosin lacks endogenous CAF activity. The same is not true of dystrophic muscle. This laboratory has reported that CAF copurifies with dystrophic chicken myosin when EGTA is omitted from the extraction buffers (17). These results suggest altered levels, activity or binding profiles, of CAF in the diseased state.

The present study indicated that CAF can exist in the dimeric form in chicken muscle. Since the 30,000-mol-wt subunit was not necessary for activity and was rapidly degraded at high  $\text{Ca}^{2+}$  (see results and reference 33), it is suggested that the experimental protocol determines whether CAF is purified in the dimeric or monomeric form. Either brief exposure to  $\text{Ca}^{2+}$ , or to organo-mercurials (33, 40) will dissociate the 80,000- and 30,000-mol-wt subunits.

During the initial stages of myosin degradation, there was little evidence that bound light chains were substrates of CAF (Fig. 3B). For technical reasons it was not possible to determine whether CAF selected the L<sub>1</sub> homodimer (41). (a) The quaternary structure of myosin remained intact; therefore, no additional bands would be apparent on nondenaturing gels. (b) The low protein concentrations run on nondenaturing gels made it very difficult to resolve 30% heavy chain fragmenta-

tion when SDS PAGE was applied in the second dimension.

The preferred cleavage sites on myosin are presented in Fig. 13. All cleavage sites are localized in the head region. Data in support of this conclusion were (a) during the first hour of digestion large heavy chain fragments of identical molecular weight accumulated for both degraded filamentous and degraded soluble myosin (Fig. 9A); (b) LMM was not a substrate (Fig. 10); (c) agents known to bind to the head of myosin (actin, MgATP, and L2) had both a qualitative and quantitative effect on heavy chain degradation (Fig. 12).

At high (1,000 to 1 mg) ratios of myosin to CAF, the initial and slightly preferred cleavage site was ~20,000 D from the N-terminus of the heavy chain. This conclusion was based on a densitometric analysis of the time course experiment (Fig. 5). This cleavage site on myosin is common to CAF and to trypsin (42). The second cleavage site was another 13,000–15,000 D along the primary sequence from the N-terminus. As a result, fragments at the 165,000-mol-wt band and below the L3 light chain were often apparent. The third cleavage site was 50,000 D from the N-terminus. Differential degradation (different rates) at all three sites produced molecular weight fragments at 180,000, 165,000, 150,000, 50,000–52,000, 30,000, 21,000, 15,000, and 14,000. Thus, the model presented in Fig. 13 indicates only the regions on the myosin heavy chain which were accessible to CAF and should not be interpreted as an indication of an ordered reaction (site 1 → 2 → 3) which produces a homogeneous population of degraded myosin molecules. Although the principal fragments were at 180,000-, 165,000-, and 150,000-mol-wt bands increasing the concentration of CAF produced additional fragments at 136,000-, 110,000-, and 95,000-mol-wt bands. In the latter case, it was not possible to determine whether the substrate was native heavy chains or the three principal fragments (Figure 3B). Sites 2 and 3 appeared to be unique to CAF.

It was not unusual that at millimolar concentrations of calcium, ionic strength, and thus the filamentous structure of myosin, had no qualitative effect on the fragmentation pattern of the myosin heavy chain (Fig. 9, A and B). Similar results had been obtained with chymotrypsin (42, 43). Surprisingly, the transition from filamentous to soluble myosin did not substantially increase the extent of degradation during the

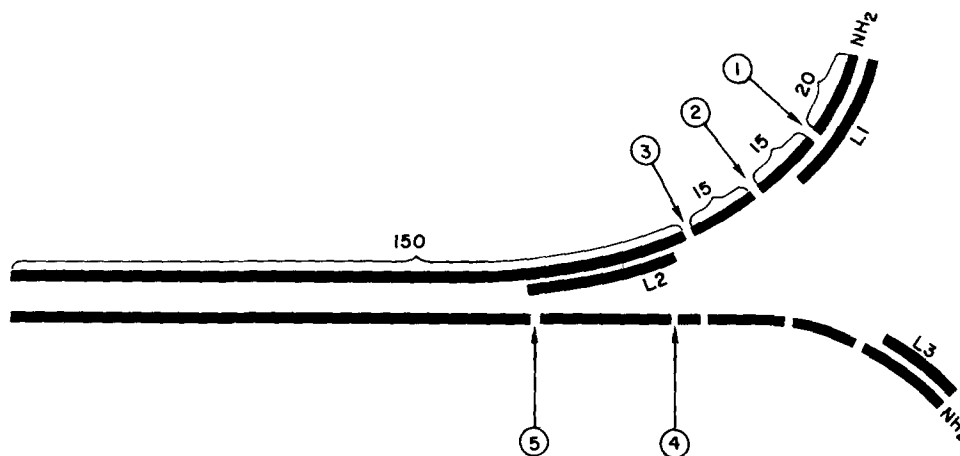


FIGURE 13 Diagrammatic representation of the principal CAF degradation points on the native myosin heavy chain in the presence (sites 1–3) and in the absence of L2 (sites 1–5). The light chains are positioned to depict only the region of the heavy chain influenced by the presence of the light chains, not the relative juxtaposition of the light chains and the heavy chains. Thus, cleavage at heavy chain site 1 eventually results in L<sub>1</sub> but not L<sub>3</sub> degradation; L<sub>2</sub> removal exposes heavy chain sites 4 and 5 to degradation at very low concentrations of CAF (1,000 to 1 mg ratio of myosin to CAF). See text for further details.

first h of digestion (Fig. 9). A 35–60% increase had been reported with chymotrypsin, due to increased exposure of the protease-sensitive region in the myosin rod to produce HMM heavy chain (5). This region was resistant to attack by CAF. Therefore, one characteristic property of CAF was that all the preferred cleavage sites were located on the heavy chain in the vicinity of the light chains. Cations, when in the presence of L2, protected the head region of myosin from attack by chymotrypsin (5, 43), but were unable to protect this region from attack by CAF. In this regard, CAF was similar to trypsin (42) and to papain (44).

Loss of L2 exposed two new regions of the myosin heavy chain to attack by low concentrations of CAF (Figs. 9B and 11). One site (4), ~54,000 D along the primary sequence from the N-terminus, was very close to site 3 at 50,000 D from the N-terminus. The other site (5), ~95,000 D from the N-terminus, was also easily attacked by trypsin, chymotrypsin, and papain (45). This means that a 41,000-D stretch (95,000 – 54,000) of the S-2 region of the myosin heavy chain is in the vicinity of L2 (molecular weight, 18,000). These observations support the asymmetrical ellipsoidal model for the structure of L2 (46).

Under certain conditions L1 was degraded by CAF, trypsin, and papain (see Results and references 45, 47), whereas L1 was resistant to attack by chymotrypsin (5, 47). This might explain why chymotryptic S-1 (A-1) and S-1 (A-2) could be separated readily on ion exchange columns (48), while only partial separation of these isozymes was reported with tryptic S-1s (49).

It was surprising that myosin was a much better substrate than HMM. The CAF recognition site on myosin may be located near the HMM-LMM junction, or the conformation of the heavy chain in the head region may not be equivalent for HMM and for myosin. At present, the second explanation is favored because during the first hour of digestion, CAF could not distinguish (qualitatively nor quantitatively) between filamentous myosin, on which the HMM-LMM junction is at least partially buried, and soluble myosin, on which this region is more readily accessible. There is information in the literature to support an altered conformation of the heavy chain of S-1. For example, tryptic digestion of myosin produces a 20,000-D N-terminal peptide (42), whereas a 27,000-D N-terminal peptide is produced when S-1 is the substrate (50, 51). The observation that MgATP inhibited degradation at site 1 (the 20,000-D N-terminal peptide) substantiated previous reports which localized the reactive lysines, and a region of the ATP-binding site in the 27,000-D N-terminal peptide (51–53). However, the observation that actin (in the absence of MgADP) also inhibited degradation at site 1 was unexpected since a similar effect was not observed with tryptic S-1 and the 27,000-D fragment (51). Interestingly, neither actin nor MgATP inhibited degradation at site 3, although both induced a qualitative effect on CAF recognition similar to that observed upon L2 removal. The last observation supports previous *in vitro* studies from this laboratory which indicated that L2 modulates actin-myosin interaction in skeletal muscle (4, 11).

Interpretation of the present results, in light of differential proteolysis studies with trypsin, suggest the following conclusions: (a) The conformation of the 20,000–27,000-D N-terminal peptide is different for myosin, HMM, and S-1. (b) Substrates and actin influence CAF-mediated degradation of myosin heavy chain over a 50,000-D segment of the N-

terminus. (c) The region of the S-1 heavy chain that is essential for the actin-activated MgATPase activity is >50,000 D from the N-terminus (50) and not attacked by CAF even under conditions supporting extensive degradation of the myosin heavy chain (17). Furthermore, under the conditions of this study, cleavage of the myosin heavy chain by CAF did not disrupt the quaternary structure of the myosin molecule, nor denature the hydrolytic or the actin-binding sites, but did decrease the affinity of actin for myosin (17).

For the above discussed reasons, we hypothesize that CAF is involved in the production of a species of myosin with “nicked” heavy chains. The ratio of nicked to native myosin may define the diseased state and may be regulated by reactions governing the rate of phosphorylation of myosin (10) or the rate of turnover or properties of CAF inhibitor (54). Future research will decide between these two interesting possibilities.

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