Removal of Z-Lines and α-Actinin from Isolated Myofibrils by a Calcium-activated Neutral Protease*  

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M. Kumudavalli Reddy, Joseph D. Etlinger, Murray Rabinowitz, Donald A. Fischman, and Radovan Zak  

From the Departments of Medicine, Biophysics, Biology and Biochemistry, The University of Chicago, and the Franklin McLean Memorial Research Institute (operated by the University of Chicago for the United States Atomic Energy Commission), Chicago, Illinois 60637  

A calcium-activated factor (CaAF) has been isolated and partially purified from the post-myofibrillar supernatant fraction of rabbit skeletal muscle. The 200-fold purified CaAF hydrolyzed denatured casein, [3H]acetyl hemoglobin, and N-ethyl[3H]maleimide-labeled α-actinin. The proteolytic activity has a pH optimum at 6.9 and is dependent on the presence of Ca2+ (optimum concentration, 10 mM). Digestion of isolated myofibrils with CaAF results in removal of Z-lines and in a parallel loss of a 90,000-dalton protein that has a mobility identical with that of α-actinin as determined by polyacrylamide gel electrophoresis. A protein with the properties of α-actinin (identical electrophoretic mobility, and ability to accelerate the Mg2+-activated ATPase of reconstituted actomyosin) was isolated from the supernatant of CaAF-treated myofibrils. The release of α-actinin from myofibrils by the calcium-activated neutral protease occurs in the absence of detectable change in the electrophoretic profiles of the other myofibrillar proteins, or in the ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) sensitivity of Mg2+-activated ATPase.  

In contrast to the specific removal of Z-lines and of α-actinin by CaAF, trypsin treatment of myofibrils results in extensive degradation of myosin heavy chains and of the inhibitory component of troponin (TN-I), and in loss of EGTA sensitivity of myofibrillar ATPase. The degradation of TN-I and loss of EGTA sensitivity occur before the Z-line disappearance.
isolation of Z-line removing factor. Also, two calcium-requiring proteolytic activities in muscle have been described, but their relation to CaAF is not known. Amino acids and trihydroxocarboxylic acid-soluble peptides were released from isolated myofibrils at neutral pH by a lyophilized 105,000 × g supernatant of rat muscle homogenate in a Ca²⁺-dependent reaction (10). This preparation showed no proteolytic activity with several endopeptidase substrates (such as denatured hemoglobin, casein, and γ-globulin) or with isolated myosin and actin. Limited Ca²⁺-dependent proteolytic cleavage of paramyosin has been demonstrated also in molluscan muscle (11). Finally, several calcium-independent, neutral proteases have been described in muscle (12-13). In this communication, we describe the partial purification and present an analysis of some of the enzymatic properties of the CaAF isolated from skeletal muscle. In particular, the reaction products of the action of CaAF on myofibrils are identified and compared with those obtained by trypsin digestion. Whereas trypsin digests many myofibrillar proteins, CaAF action results in the specific removal of the Z-line protein, α-actinin.

MATERIALS AND METHODS

Reagents—β-Glycerophosphate, EGTA, and dithiothreitol were purchased from Sigma Chemical Co., N-ethyl-H₂-[³H]maleimide (specific activity 0.75 Ci/mM) from New England Nuclear Corp., and [³H]acetic anhydride (specific activity >2000 Ci/mM) from Amer sham/Searle Corp. Hemoglobin was obtained from Sigma Chemical Co. and casein (Hammersten grade) from Nutritional Biochemicals Corp. The DEAE-cellulose used was DE52 from Whatman.

Preparation of Myofibrils and α-Actinin—Myofibrils were prepared from rabbit skeletal muscle according to the procedure of Zak et al. (14) and were stored in 50% glycerol containing 0.25 mM thioglycolic acid at -20°C. Isolated myofibrils were the source of α-actinin, which was extracted and purified as described by Arakawa et al. (15) and stored in 50% glycerol at -20°C.

Preparation of Radioactive Proteins—[³H]Acetylated hemoglobin was prepared according to the method of Hille et al. (16). The product contained 0.18 nmol of [³H]-acetyl groups per 17,000 mg of hemoglobin. Coupling of α-actinin with N-ethyl-[³H]maleimide was performed in 0.1 M Tris-maleate buffer, pH 7.0, at 0°C. Approximately 1 μCi of N-ethyl-[³H]maleimide was added per 10 mg of protein. After the reaction had been allowed to proceed for 10 min, the sample was diluted 20-fold and α-actinin was precipitated twice by ammonium sulfate fractionation. The final precipitate was dissolved in 0.02 M glycophosphate buffer, pH 7.0; it was isoelectrically precipitated by adjustment of the pH to 5.5 with 1 N HCl, redissolved, and dialyzed against the same buffer, and stored in 50% glycerol at -20°C. In two preparations, the specific radioactivity of the conjugate corresponded to 0.07 and 0.23 mmol of N-ethyl-[³H]maleimide per 90,000 mg of α-actinin.

Proteolytic Activity—The proteolytic activity was assayed as follows: with the two labeled substrates, the reaction mixture contained 0.05 M Tris-maleate buffer, pH 6.0, 5 × 10⁻³ M CaCl₂, N-ethyl-[³H]maleimide α-actinin or denatured [³H]maleimide α-actinin and 1 mM NaN₃, and 1 ml of cold 10% trichloroacetic acid. After standing in ice for 10 min, the samples were centrifuged and the radioactivity in the supernatant was determined. The same reaction mixture was used with unlabeled casein as substrate (5 mg/ml), but the incubation time was used 120 min. Aromatic acids released in the trichloroacetic acid supernatant were measured from the absorbance at 280 nm and were expressed as tyrosine equivalents using the molar absorbance of 1163. Control samples containing 5 mM EGTA were run with each reaction, and the values were subtracted from those obtained in the presence of calcium.

Z-Line Removing Activity—The ability of enzyme fractions to remove Z-lines from isolated myofibrils was monitored semiquantitatively by phase microscopy. Enzyme preparations containing 3 mg to 25 μg of protein were incubated at 25°C with 1 to 2 mg of purified myofibrils suspended in Tris-maleate buffer containing 0.1 M KCl and 5 mM CaCl₂. Control samples containing 5 mM EGTA were incubated in the same way. The time required for the complete removal of Z-lines was recorded. The assay has a variability of about 20%.

Enzyme Purification—Calcium-activated factor was extracted and purified from muscle by the following procedure: rabbit skeletal muscle was homogenized in 4 volumes of 4 mM EGTA, pH 7.0, for 1 min at 10,000 × g for 15 min at 4°C. If centrifugation was carried out at 0°C, the pH of the supernatant was adjusted to 6.1 with 1 N acetic acid, and after 15 min the resulting precipitate was removed by centrifugation. The supernatant was further acidified to pH 4.7 to 4.9 with acetic acid and left in ice for 10 min. The precipitate was collected by centrifugation at 10,000 × g for 15 min and incubated in 0.2 M glycophosphate buffer, pH 8.0, containing 4 mM EGTA (70 μl of buffer per kg of muscle). After pH had been adjusted to 7.0 with 1 N KOH, the volume of the extract was adjusted to 200 ml/kg of muscle with cold distilled water and clarified by centrifugation at 20,000 × g for 2 hours. The fraction of the supernatant that precipitates between 20 and 40% ammonium sulfate saturation was adsorbed to a DE52 column (2.5 × 46 cm) equilibrated with 0.02 M glycophosphate buffer, pH 7.5, 1 mM thiglycolic acid, and 2 mM EGTA. The fractions that had Ca²⁺-activated proteolytic and Z-line removing activities were pooled, concentrated by ultrafiltration, and stored frozen. This fraction is referred to as partially purified CaAF.

The activity was also present in supernatant fractions of rat heart and skeletal muscle. Purification of these extracts was similar to that for rabbit skeletal muscle.

CaAF or Trypsin Digestion of Myofibrils—Myofibrils suspended in 0.1 M KCl, 0.01 M Tris-maleate buffer, pH 7.0, and 1 mM NaN₃ were treated either with trypsin or with CaAF at room temperature. All incubations were carried out in 400 ml of 5 mM CaCl₂. For controls, myofibrils were incubated with CaAF but with 1 mM EGTA instead of CaCl₂, without any additions. When trypsin was used, the myofibrillar medium was supplemented with 2 mM MgCl₂ and 2 mM EGTA. The ratio of the volume of the solution in which myofibrils were suspended to the volume of myofibrillar pellet after a 10-min centrifugation at 800 × g was 10:1. The trypsin concentration was 15 μg/ml, and the CaAF concentration was 1-400 μg/ml. After digestion, a 4-fold excess of soybean trypsin inhibitor (Armour) was added and the solution was immersed in ice. The myofibrils were then washed four times by centrifugation (800 × g, 10 min) and resuspended. Finally, the myofibrils were suspended in water and prepared for Na dodecyl-SO₄ gel electrophoresis as described by Ellinger and Fischman (17).

Na dodecyl-SO₄ gel electrophoresis of Myofibrillar Proteins—For Na dodecyl-SO₄ gel electrophoresis, 5% acrylamide gels containing 1:37 (w/w) cross-linker were used with Tris-glycine buffer, pH 8.8 (17). Gels were stained with Coomassie blue in 25% isopropl alcohol, 10% acetic acid for 18 hours and destained in an isopropyl alcohol-acetic acid solution. Densitometric tracings were obtained at 540 nm using a Gilford spectrophotometer with linear transport attachment, model 2410.

Analytic Methods—Inorganic phosphate was measured by the method of Lowry et al. (18). Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer using an Instagel (Packard) counting solution. A correction for quenching was made by the use of an external standard. The radioactivity of ¹²⁵I was measured with an efficiency of about 38%.

RESULTS

Purification of CaAF—Calcium-activated neutral protease was purified approximately 200-fold, as described under "Materials and Methods." The protease activity (Table I) was...
TABLE I

Purification of calcium-activated protease

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume</th>
<th>Protein</th>
<th>Z-line removing activity</th>
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<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
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<tr>
<td>Homogenate</td>
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<td>2.7</td>
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<tr>
<td>Supernatant</td>
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<td>2.9</td>
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<tr>
<td>Crude extract</td>
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<td>16</td>
</tr>
<tr>
<td>20 to 40% (NH₄)₂SO₄ fraction</td>
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<td>16</td>
<td>47</td>
</tr>
<tr>
<td>DE52 fraction</td>
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<td>8</td>
<td>60</td>
</tr>
<tr>
<td>Sephadex G-200 fraction</td>
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<th>Enzyme specific activity (min⁻¹ x mg⁻¹) with different substrates</th>
</tr>
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<tbody>
<tr>
<td>a-Actinin</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
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<td>0.1</td>
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<td>10</td>
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*The incubation mixture contained 1.0 × 10⁴ cpm, corresponding to 0.30 mg of N-ethyl[^H]maleimide-a-actinin.

+ The incubation mixture contained 1.3 × 10⁴ cpm, corresponding to 0.37 mg of[^H]acetyl hemoglobin.

+ The incubation mixture contained 5 mg of denatured casein.

+ The units refer to the incubation time needed for the complete removal of Z-lines.

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Fig. 1. DEAE-cellulose chromatography of the calcium-activated protease (Step 5). CaAF (262 mg of protein) was loaded on a column (2.5 x 46 cm) of DE52 cellulose (Whatman) equilibrated with 0.02 M glycerophosphate buffer, pH 7.5, containing 2 mM EGTA, 1 mM thioglycolic acid, and 1 mM NaN₃. Protein was eluted with a 0 to 0.8 M KCl linear gradient at a flow rate of 36 ml/hour. Fractions (8 ml) were assayed for calcium-activated splitting of [SH]acetyl hemoglobin. The proteolytic activity is given as counts per min x 10⁻⁶. The reaction mixture contained 1.3 × 10⁴ cpm (0.37 mg of hemoglobin) per assay. Complete Z-line removal, +; Z-lines intact, −; O—O, absorbance at 280 nm; —, proteolytic activity.

Fig. 2. Hydrolysis of N-ethyl[^H]maleimide-labeled a-actinin (4 × 10⁴ cpm), 0.16 mg of CaAF (DE52 fraction), 0.05 M Tris-maleate buffer, and 5 mM CaCl₂ or 5 mM EGTA, as indicated. A, time course; pH 6.9; TCA, trichloroacetic acid; B, effect of Ca²⁺ concentration; incubation time, 30 min; C, effect of pH; incubation time, 30 min.

The time course of hydrolysis of N-ethyl[^H]maleimide-a-actinin by CaAF is shown in Fig. 2A. Similar results were obtained with [^H]acetyl hemoglobin and denatured casein. It is apparent in Fig. 2C that the pH optimum for CaAF is at 6.9; maximal activation by CaCl₂ occurs at 10 mM concentration (Fig. 2B).

Digestion of Isolated Myofibrils by CaAF and by Trypsin: Analysis by Na dodecyl-SO₄ Gel Electrophoresis—A comparison of Na dodecyl-SO₄ gel electrophoretic profiles of myofibrillar proteins was made after CaAF and trypsin digestion of isolated myofibrils. The duration of enzyme treatment was selected so that Z-line removal was either partial or complete. The Z-line removal was monitored by phase contrast microscopy of small samples withdrawn from the mixtures at various incubation times. Typical results with a crude (Fraction 3) and partially purified (Fraction 5) CaAF preparation are shown.

The changes in myofibrillar proteins obtained after Z-line removal with CaAF crude extract (purification step 3) are shown in Fig. 3. Na dodecyl-SO₄ gels stained with Coomassie blue and densitometric tracings of the gels before (Fig. 3C) and after (Fig. 3A) CaAF digestion are shown. Band 2 corresponds to a-actinin; it has a molecular weight of 90,000, calculated from its electrophoretic mobility after calibration with proteins of known molecular weight; and N-ethyl[^H]maleimide-a-actinin intermixed with myofibrils migrates with the same
mobility. Band 3 corresponds to troponin component TN-I ($M_r = 24,000$; it has a mobility identical with the middle band of purified troponin). CaAF digestion results in the removal of most of the $\alpha$-actinin (Fig. 3A, band 2). Approximately one-half of the TN-I band is also removed (Fig. 3A, band 3), whereas an additional low molecular weight protein (approximate $M_r = 20,000$, presumably a degradation product of TN-I, appears. No degradation products of myosin heavy chain (band 1) appear.

Treatment of myofibrils with trypsin gives an entirely different pattern of change (Fig. 3B). The most prominent features of trypsin digestion are almost complete removal of TN-I (Fig. 3B, band 3) and the appearance of bands in the region of 100,000 to 155,000 (Fig. 3B, band 1). It is assumed that these bands are myosin degradation products, because digestion of myosin results in the appearance of bands with similar mobilities. Changes produced by trypsin are calcium independent.

To distinguish more clearly between the actions of trypsin and CaAF, we compared the Na dodecyl-SO$_4$ gel electrophoretic profiles following trypsin digestion of myofibrils which resulted in varying degrees of Z-line removal. That TN-I is more susceptible to trypsin digestion than $\alpha$-actinin can be seen from a comparison of the results in Figs. 3 and 4. Two-minute treatment of myofibrils with trypsin (Fig. 4, Gel C) leads to an apparently complete loss of TN-I, whereas the amount of $\alpha$-actinin is relatively unchanged and the Z-line remains intact. Prominent myosin degradation bands appear after prolonged treatment with trypsin (Fig. 4, Gels E and F). This contrasts with the effect of CaAF (Fig. 3), which results in the removal of Z-lines and most of the $\alpha$-actinin, at a time when TN-I removal is incomplete.

In a second group of experiments, the effect of CaAF purified on DE52 cellulose (purification step 5, Table I) was compared with that of trypsin (Fig. 5). Identical results were obtained with CaAF purified through Step 6. A densitometric tracing of

![Fig. 3. Densitometric tracing of myofibrillar proteins by Na dodecyl-SO$_4$ gel electrophoresis after digestion of myofibrils by CaAF and trypsin. Analyses were carried out when Z-line removal was judged to be complete by phase contrast microscopy. The ratio of CaAF (Step 3, Table I) to myofibrils was 1:50. The ratio of trypsin to myofibrillar protein was 1:4000. The amount of protein applied to the 5% Na dodecyl-SO$_4$ polyacrylamide gels was 100 $\mu$g. A, Z-line removed by CaAF; 1-hour treatment; B, Z-line removed by trypsin; 35-min treatment; C, control myofibrils, untreated. The arrows indicate the positions of the following proteins: 1, myosin degradation product; 2, $\alpha$-actinin; 3, TN-I.](http://www.jbc.org/)

![Fig. 4. Na dodecyl-SO$_4$ gel electrophoretic profiles of myofibrils treated with trypsin to varying degrees of Z-line removal. The experiment shown is typical of five experiments. Approximately 100 $\mu$g of protein were applied to each gel; the trypsin to myofibril ratio was 1:4000; the trypsin inhibitor to trypsin ratio was 4:1. The numbers on the left indicate the positions of the following proteins: 1, myosin degradation product; 2, $\alpha$-actinin; 3, TN-I (24,000). A, control myofibrils; B, trypsin plus trypsin inhibitor; 35-min incubation; C, no Z-line removal; 2-min trypsin treatment; D, partial Z-line removal; 8- to 10-min trypsin treatment; E, complete Z-line removal; 35-min trypsin treatment; F, complete Z-line removal with 10 times the amount of trypsin in E.](http://www.jbc.org/)
The Na dodecyl-\(\text{SO}_4\) gel electrophoresis of control myofibrils incubated without enzyme is shown in Fig. 5, and bands for which areas were measured planimetrically after different types of digestion are indicated by hatch marks. Again, trypsin digestion results in the appearance of myosin degradation products that increase in amount when longer periods of digestion are used. In contrast, CaAF produces no myosin degradation products. Both CaAF and trypsin treatment resulted in a decrease of \(\alpha\)-actinin, but removal by CaAF was more complete and was calcium-dependent. Troponin component TN-I, known to be particularly susceptible to proteolysis, was digested by trypsin, but only minimally, if at all, by the purified CaAF.

Thus, trypsin had multiple effects on myofibrils, digesting both myosin heavy chain and TN-I, but affecting \(\alpha\)-actinin to a lesser extent. In contrast, CaAF treatment resulted in almost complete removal of \(\alpha\)-actinin, and in relatively little removal of TN-I, and had no detectable effect on myosin heavy chain.

**Analysis by Assay of EGTA-sensitive ATPase**—The degradation of TN-I by CaAF and trypsin was also assessed in an independent way, by the measurement of the EGTA sensitivity of the Mg\(^{2+}\)-activated myofibrillar ATPase. EGTA sensitivity is defined as the inhibition of Mg\(^{2+}\)-ATPase activity observed after addition of EGTA. It is well known that trypsin treatment of myofibrils results in the loss of EGTA sensitivity due to the degradation of TN-I. The addition of 5 mM EGTA to untreated myofibrillar preparations resulted in 80% inhibition of Mg\(^{2+}\)-ATPase (Fig. 6). Treatment of myofibrils with CaAF under conditions that completely remove Z-lines (Fig. 6, No. 2) gave results identical with those for untreated myofibrils, indicating no loss of EGTA sensitivity. In contrast, treatment of myofibrils with trypsin under conditions where Z-line removal could not be detected by phase microscopy (No. 4) or where partial Z-line removal was observed (No. 1) resulted in complete loss of EGTA sensitivity.

**Release of \(\alpha\)-Actinin from Myofibrils**—Isolated skeletal muscle myofibrils were treated with CaAF for 60 min in the presence of calcium or EGTA. Undigested myofibrils were then removed by centrifugation, the supernatant filtered through Millipore filters, concentrated by vacuum dialysis or by lyophilization, and analyzed either by electrophoresis (Fig. 7) or enzymatically for the presence of \(\alpha\)-actinin (Fig. 8).

Only one major band appeared in the supernatant after CaAF treatment of myofibrils in the presence of calcium (Fig. 7). The mobility of this band was identical with that of purified \(\alpha\)-actinin. In five separate determinations, we recovered in the supernatant 17 to 21% of the \(\alpha\)-actinin removed from myofibrils by digestion with CaAF. These estimates were obtained from planimetric measurements of the \(\alpha\)-actinin band on stained Na dodecyl-\(\text{SO}_4\) gels of untreated and CaAF-digested myofibrils, and of concentrates of the supernatant. There is no release of \(\alpha\)-actinin when digestion is carried out in the presence of EGTA.

We also tried to identify the protein released in the supernatant by enzymatic assay, based on the observation by Seraydarian et al. (19) that the activity of Mg\(^{2+}\)ATPase of reconstituted actomyosin is stimulated by the addition of \(\alpha\)-actinin in the presence of 0.02 M KCl (Fig. 8, No. 4). On the other hand, \(\alpha\)-actinin acts as inhibitor if the KCl concentration is increased to 0.2 M (Fig. 8, No. 3). Concentrated supernatant from myofibrils treated with CaAF and calcium affects its ATPase activity in the same manner as purified \(\alpha\)-actinin (Fig. 8, last panel). The supernatant from myofibrils treated with CaAF plus EGTA, however, shows no effect on ATPase activity (Fig. 8, third panel). It may be concluded that CaAF digestion of myofibrils results in the selective release of a protein having the properties of \(\alpha\)-actinin.

**DISCUSSION**

We have purified CaAF more than 200-fold (relative to the 10,000 \(\times\) g supernatant of muscle homogenate) and have established that, at neutral pH, it contains protease ac-
FIG. 7. Densitometric tracing of Na dodecyl-SO₄ gel electrophoreograms of proteins released by CaAF into post-myofibrillar supernatant. The results are typical of four experiments. Myofibrils were treated with CaAF (purification step 5 or 6) to complete Z-line removal. After 10,000 × g centrifugation for 10 min and filtration of the supernatant through a Millipore filter, the soluble proteins were concentrated and approximately 50 µg were loaded on 5% polyacrylamide gels. The vertical dotted line indicates the position of purified α-actinin on 5% Na dodecyl-SO₄ gels. A, supernatant from CaAF-treated myofibrils. The myofibrillar protein ratio was 1:250; B, supernatant from EGTA plus CaAF-treated myofibrils.

activity which is dependent on the presence of Ca²⁺. The calcium-activated "protease" activity toward three different protein substrates correlated with Z-line removal activity in different fractions during the purification process.

We have further examined the specificity of the effect of CaAF on Z-line removal by analyzing the profiles of myofibrillar proteins by means of polyacrylamide gel electrophoresis. It is well established that individual myofibrillar proteins differ in their susceptibility to endopeptidase attack. Thus the inhibitory component of troponin (22) and C-protein (24) are very sensitive to mild tryptic digestion. Because actin (25) and α-actinin (8), on the other hand, are relatively resistant, procedures involving trypsin treatment are often used in their preparation. The protease sensitivity of myosin varies in different types of muscle (26), but generally the digestion periods required to produce submolecular fragments are longer (27) than those made with TN-I or C-protein. Because trypsin is known to remove Z-lines (8), and because considerable information is available about the effect of trypsin digestion of myofibrils, we compared the action of CaAF on the electrophoretic profiles of myofibrillar proteins with that of trypsin. It became apparent that trypsin and CaAF produce different patterns of changes.

Short treatment with trypsin initially leads to considerable digestion of the inhibitory component of troponin, with the concomitant appearance of a degradation product with an electrophoretic mobility corresponding to a molecular weight of 20,000. This change occurs before any alteration in the Z-line is detected by phase contrast microscopy. When treatment is extended, α-actinin gradually disappears from the gel profiles. In addition, new bands with the mobility of peptides having molecular weights between 100,000 and 155,000 become visible. Comparison with electrophoretic analyses of purified myosin treated with trypsin identified these bands as myosin fragments.

In contrast to trypsin, CaAF treatment, even for prolonged periods of time and at concentrations 10 times higher than those needed for Z-line and α-actinin removal, does not produce degradation products of myosin. The troponin inhibitory component, however, was digested by CaAF, although it is more resistant than α-actinin. In more highly purified preparations of CaAF, only a minor decrease in the TN-I band was noted, and enzymatic analysis of Mg²⁺-activated ATPase did not indicate any loss of calcium-dependent regulatory activity. The EGTA sensitivity was found to be unaltered. In less pure preparations, however, a clear decrease in the area of the TN-I was recorded, and the presence of TN-I degradation product was noticed.

The disappearance of α-actinin from the electrophoretic
electrophoretic mobility of α-actinin was detected. This effect of CaAF is similar to that of trypsin, which also releases α-actinin from the myofibrils without degrading it entirely (9). The fact that no subfragments of α-actinin produced by CaAF could be demonstrated after CaAF digestion if compatible with the view that α-actinin proteolysis may be an all or none process; once a molecule is attacked by protease, its digestion proceeds to small peptides. A similar digestion pattern has been reported for bovine serum albumin (28). It is not yet determined what proportion of the α-actinin is digested before, and how much is digested after its release from the myofibril.

Removal of the α-actinin by CaAF may be secondary to its partial digestion in situ on the myofibril that might unstabilize Z-line structure and lead to release of the α-actinin. An alternative explanation is that another Z-line protein, highly sensitive to endopeptidase digestion, is involved in anchoring α-actinin to the network of thin filaments. The protein composition of the Z-line is not clearly established. A variety of procedures, which apparently have differing modes of action, can remove the electron-dense material of the Z-line. Thus, in addition to the CaAF action the removal of Z-lines can be accomplished by brief treatment with trypsin (8), pancreatic lipase (29), 3 M urea (31), deoxycholate (17), bis(dinitrophenyllysine) (32), and by extraction of glycerinated myofibrils with 0.6 M ATP (33). Electrophoretic analysis of deoxycholate extracts of myofibrils (21) demonstrated that two proteins, with molecular weights of 95,000 and 85,000 can be resolved. Substantial amounts of these two proteins still remain in the fibrils after deoxycholate treatment, however, despite the fact that the dense material seen with the phase contrast microscope is completely removed within 15 s. Thus, there is a strong possibility that other, unidentified components are being released before these Z-line proteins. It is also possible that endopeptidase treatment cleaves a single specific bond holding α-actinin to a non-protein component of the Z-line. The physiological role of CaAF is still unknown at this time. For maximal activity, CaAF and all other calcium-activated proteases studied so far require concentrations of ionized calcium about 100 times higher than that assumed to be present in activated muscle. Possibly significant activity occurs in vivo at lower calcium concentrations. It is of interest that the myocardial cell damage which is associated with Z-line abnormalities has been found to be accompanied by a considerable increase of intracellular calcium (see, e.g. Fleckenstein et al. (34).

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