A Ca^{2+}-Activated Protease Possibly Involved in Myofibrillar Protein Turnover. Partial Characterization of the Purified Enzyme†

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ABSTRACT: The purified Ca^{2+}-activated protease (CAF) isolated from porcine skeletal muscle and capable of removing Z-disks from intact myofibrils is optimally active on either myofibril or casein substrates at pH 7.5 and in the presence of 1 mM Ca^{2+} and at least 2 mM 2-mercaptoethanol. No CAF activity is detected when 1 mM Mg^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} are added singly. When added with 1 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and Fe^{2+} inhibit, whereas Mg^{2+}, Mn^{2+}, and Ba^{2+} have no effect on CAF activity. CAF is irreversibly inhibited by iodoacetate but is unaffected by soybean trypsin inhibitor. $s^0_{20,w}$ = 5.90 S, and sedimentation equilibrium molecular weight = 112 000 for purified CAF. Because purified CAF migrates as two polypeptide chains with molecular weights of 80 000 and 30 000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the CAF molecule must consist of one each of these two polypeptide chains. Approximate molecular dimensions of 38 × 220 Å can be calculated for CAF from calibrated gel permeation column data or from $s^0_{20,w}$ and the molecular weight. Amino acid composition and physical properties of purified CAF distinguish it from the known catheptic enzymes and from other proteases found in blood or in granulocytes. Purified CAF removes Z-disks and the 400-Å periodicity associated with troponin in the I band and partly degrades M lines but causes no other ultrastructurally detectable effects when incubated with myofibrils. These results agree with the earlier finding that purified CAF degrades troponin, tropomyosin, and C-protein but has no effect on myosin, actin, or α-actinin, and suggest that CAF may have a physiological role in disassembly of intact myofibrils during metabolic turnover of myofibrillar proteins.

The preceding paper described the purification from skeletal muscle tissue of a Ca^{2+} activated proteolyticenzyme (CAF)[1] that very rapidly removes Z-disks from myofibrils in vitro. Studies on the effect of purified CAF on purified myofibrillar proteins have shown that CAF degrades troponin, tropomyosin, and C-protein but does not hydrolyze actin, myosin, or α-actinin (Dayton et al., 1975a). Assembly of the myofibrillar proteins into a myofibril has little effect on susceptibility of the individual proteins to degradation by CAF (Dayton et al., 1975a). Because CAF is the first muscle protease that has been shown to degrade intact myofibrils and purified individual myofibrillar proteins, it is possible that this enzyme may be involved in intracellular degradation of myofibrillar proteins during their metabolic turnover. The availability of highly purified CAF (Dayton et al., 1976b) made it possible to study some enzymic and physical properties of CAF in the anticipation that these properties may provide some clues on the possible role of CAF in myofibrillar protein turnover. This paper describes some catalytic and physical properties of the CAF molecule and the effect of purified CAF on the ultrastructure of myofibrils.

Experimental Procedure

Protein Preparations. Purified CAF was prepared from porcine skeletal muscle according to the procedure described in the preceding paper (Dayton et al., 1976b). In some experiments that did not require extremely homogeneous protein preparations, CAF purified through the second DEAE-cellulose chromatography was used because preparative electrophoresis showed that the 60 000-dalton component that contaminated such preparations had no enzymic activity (Dayton et al., 1976b). Use of CAF purified only through the second DEAE-cellulose chromatography reduced the large preparative task associated with making purified CAF because the last chromatographic purification on Sephadex G-150 columns was accompanied by substantial losses of CAF (Dayton et al., 1976b). Tests showed that use of CAF, purified through the second DEAE-cellulose chromatography, had no effect on assays determining the effects of Ca^{2+} concentration, pH, temperature, or 2-mercaptoethanol concentration on CAF activity. Myofibrils and casein used as substrates for CAF were made as described in the preceding paper (Dayton et al., 1976b). Unless otherwise indicated, all preparations were done at 0 to 3 °C with precooled solutions made by using double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers. Protein concentrations were determined by using the biuret method (Gornall et al., 1949) as modified by Robson et al. (1968) or the Folin–Lowry procedure (Lowry et al., 1951).

Ca^{2+} Dependence of CAF Activity. Two separate studies, one using casein as a substrate and the other using myofibrils as a substrate, were done to determine the effect of Ca^{2+}

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[1] Abbreviations used are: CAF, Ca^{2+}-activated factor; EGTA, 1,2-bis(2-dicarboxymethylaminoethoxy)ethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

concentration on CAF activity. Procedures used for both casein and myofibril assays were identical with those described in the preceding paper (Dayton et al., 1976b) except that Ca^{2+} concentration in the assay mixture was varied. CAF activity was determined as described previously for the casein and quantitative myofibril assays (Dayton et al., 1976b). Actual Ca^{2+} concentration of the supernatant from each assay tube was determined by using a Perkin-Elmer atomic absorption spectrophotometer to guard against the possibility of massive Ca^{2+} binding by the substrates.

pH Dependence of CAF Activity. The pH dependence of the proteolytic activity of CAF was determined by using both myofibrils and casein as substrates; pH dependence of CAF activity was identical regardless of which substrate was used, and only the results obtained with myofibrils will be reported here. CAF activity was assayed at pH values between 3.0 and 9.5 in assay mixtures containing 100 mM KCl, 100 mM Tris-acetate adjusted to the desired pH, 10 mM 2-mercaptoethanol, 5 mM $CaCl_2$, 0.1 mM EDTA, 2.5 mg of myofibrils/ml, and 5 μg of purified CAF/ml. All assays were incubated at 25 °C for 30 min. To ensure that pH of the assay mixtures remained constant, pH of each mixture was measured both immediately before and after the 30-min reaction period. Controls were done at all pH values and were handled identically with experimental tubes except that 5 mM $CaCl_2$ was replaced by 10 mM EDTA. The reaction was stopped by addition of enough 100 mM EDTA to make the final EDTA concentration in each assay tube 10 mM. Insoluble material was sedimented by centrifuging at 100 000g_{max} for 30 min. CAF activity at a given pH was expressed as the difference between absorbance at 278 nm of supernatants from assay tubes containing CAF and Ca^{2+} and supernatants from control tubes containing CAF and EDTA.

Temperature Dependence of CAF Activity. Results of studies on the temperature dependence of CAF activity were again identical when either myofibrils or casein were used as substrates. Assay procedures and conditions were identical with those previously described (Dayton et al., 1976b), except that different incubation temperatures were used. CAF activity was determined after various times of incubation at 0, 25, and 37.5 °C.

Effect of 2-Mercaptoethanol Concentration on CAF Activity. Effect of 2-mercaptoethanol concentration on CAF activity was determined by using either myofibrils or casein as a substrate; results were identical regardless of substrate. Assay procedures and conditions were identical with those previously described (Dayton et al., 1976b), except that amount of 2-mercaptoethanol in the assay mixture was varied between 0 and 15 mM 2-mercaptoethanol.

Analytical Ultracentrifugation. Studies using analytical ultracentrifugation were done with a Spinco Model E analytical ultracentrifuge equipped with a RTIC unit and an electronic speed control. Sedimentation velocity runs were conducted at 20 °C using double-sector Kel-F centerpieces and quartz lenses. High-speed meniscus-depletion sedimentation equilibrium runs (Yphantis, 1964) were done by using a Yphantis six-channel centerpiece with sapphire lenses. A partial specific volume of 0.716 $cm^3\ g^{-1}$ was estimated for purified CAF from its amino acid composition (Cohn and Edsall, 1943). All sedimentation equilibrium experiments used only highly purified CAF from Sephadex G-150 chromatography (Dayton et al., 1976b) and were done in 100 mM KCl, 20 mM Tris-acetate, pH 7.5, 10 mM 2-mercaptoethanol, 5 mM EDTA at 18 to 20 °C. Sedimentation velocity runs were done using both highly purified CAF from Sephadex G-150 chromatography and CAF from the second DEAE-cellulose chromatography (Dayton et al., 1976b). These runs indicated that the presence of small amounts of the 60 000-dalton material that contaminates CAF, purified only through the second DEAE-cellulose column, had little effect on the sedimentation coefficient of CAF. Plates were measured with a Nikon 6C profile projector.

Determination of the Stokes' Radius of the CAF Molecule. Stokes' radius of the CAF molecule was determined by using a 1.6 × 80.0 cm Sephadex G-150 column that was calibrated by determining elution volumes from the column of aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A, whose Stokes' radii are known (Ackers, 1964). Ten milligrams of each of these standard proteins were loaded in 1–2 ml, and the column was eluted at 5.0 ml/h and at 2 °C with 20 mM Tris-acetate, pH 7.5, 1 mM NaN_3, 1 mM EDTA. Elution volume from this calibrated column of 10 mg of purified CAF, also loaded in 1–2 ml, was then determined and compared with elution volumes of the standard proteins to determine the Stokes' radius of CAF. An independent estimate of the Stokes' radius of CAF was derived by using the sedimentation coefficient and the molecular weight obtained from sedimentation equilibrium to calculate a frictional coefficient for purified CAF and then using this frictional coefficient to calculate the Stokes' radius. The diffusion coefficient, $D^0_{20,w}$, of purified CAF was also calculated from the sedimentation coefficient and the sedimentation-equilibrium molecular weight.

Amino Acid Analysis. Amino acid analysis of purified CAF was done by oxidizing 0.5–1.0 mg of purified CAF with performic acid (Hirs, 1967). The oxidized sample was then placed in 2.0 ml of constant boiling HCl (5.9 N), and the tube was sealed under vacuum and placed in a mineral oil bath at 108 °C for 24 h. The protein hydrolysate was cooled, evaporated to dryness on a Buchler flash evaporator, redissolved in 2.0 ml of water, and evaporated to dryness two additional times. Amino acid analysis was done by using a Beckman automatic amino acid analyzer. Only highly purified CAF from Sephadex G-150 columns was used for amino acid analysis.

Effect of CAF on Myofibril Ultrastructure. Initial investigations of the effects of CAF on myofibril ultrastructure were done using myofibril preparations. Subsequent experiments, however, were done using glycerinated whole fiber bundles because morphological findings on these bundles were technically more satisfactory than those on unoriented myofibrils. Rabbit psoas muscle, glycerinated according to the procedure of Huxley (1963), was teased into long, thin bundles (250–300 μm in diameter) in a solution of 120 mM KCl, 60 mM Tris-acetate, pH 7.0, 1.2 mM NaN_3. The strips were further chopped into approximately 500-μm lengths using a razor blade, and the small pieces were used in experiments that tested the effects of CAF on myofibril structure.

The effect of CAF on myofibril structure was determined by comparing fiber bundles incubated in four different solutions made as follows: (1) myofibril, no Ca^{2+} control—100 mM KCl, 50 mM Tris-acetate, pH 7.0, 10 mM EGTA, 5 mM 2-mercaptoethanol, 1 mM NaN_3; (2) myofibril, Ca^{2+} control—100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM Ca^{2+}, 5 mM 2-mercaptoethanol, 1 mM NaN_3; (3) CAF, no Ca^{2+} control—100 mM KCl, 50 mM Tris-acetate, pH 7.0, 10 mM EGTA, 5 mM 2-mercaptoethanol, 50 μg of purified CAF/ml; and (4) CAF, Ca^{2+} assay—100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM Ca^{2+}, 5 mM 2-mercaptoethanol, 1 mM NaN_3, and 5, 10, or 50 μg of purified CAF/ml. The final incubation volume was 2.0 ml, and each tube contained approximately 30 glycerinated fiber pieces. Experiments using

three different CAF concentrations (5, 10, and 50 μg/ml) were incubated for 2 h in a shaking water bath at 25 °C. The incubating liquid was then withdrawn by Pasteur pipet, and the pieces were processed for electron microscopy. In addition, treatment at 50 μg of CAF/ml was allowed to proceed for 20 h with a separate set of controls.

Fixation for electron microscopy was done either with Karnovsky's glutaraldehyde/paraformaldehyde mixture or with 2.5% glutaraldehyde followed by 1% OsO_4. The pieces were accurately embedded for longitudinal or cross section in Epon/Araldite after graded acetone dehydration. Sections were stained with 2% uranyl acetate in methanol, poststained with lead citrate, and examined in an RCA EMU-4 electron microscope.

Results

Effect of Ca^{2+} on CAF Activity. Optimum Ca^{2+} concentration for CAF-catalyzed hydrolysis of myofibrils is 1.0 mM (Figure 1), and CAF degradation of myofibrils occurs very slowly below 0.1 mM Ca^{2+}. Ca^{2+} concentrations of 10 mM inhibit CAF hydrolysis of myofibrils, but the reason for this inhibition is unclear. Ca^{2+}-requirement studies were also done using casein as a substrate to determine whether Ca^{2+} stimulation of CAF's proteolytic activity was due to Ca^{2+} binding directly to the CAF molecule and activating its proteolytic abilities, or to Ca^{2+} binding to substrate molecules to change their conformation and make them more susceptible to CAF. If Ca^{2+} stimulates CAF activity by affecting the substrate, it would seem very unlikely that this effect would occur at the same Ca^{2+} concentration for two dissimilar substrates such as casein and myofibrils. If, on the other hand, Ca^{2+} stimulates CAF activity by affecting the CAF molecule itself, the plot of CAF activity vs. Ca^{2+} concentration should have the same shape regardless of the substrate used. Optimum Ca^{2+} concentration for CAF degradation of casein was also 1 mM (results not shown here), and shape of the plot of CAF activity vs. Ca^{2+} concentration was identical for both myofibril and casein substrates. This result suggests that the effect of Ca^{2+} is directly on the CAF molecule and not on the protein substrate.

pH Optimum of CAF. The pH optimum for CAF activity was near pH 7.5. Significant CAF activity existed between pH 6.5 and 8.0, but CAF activity decreased rapidly below pH 6.5 or above pH 8.0. The effect of pH on CAF activity is completely different from the effect of pH on lysosomal proteases, and it seems very unlikely, therefore, that CAF originates from lysosomes. A nonlysosomal location for CAF has been confirmed by careful differential centrifugation studies, which show that CAF is not sedimented with the lysosomal fraction from whole muscle homogenates. Because pH optimum of CAF activity is in the pH range that exists in cytoplasm of normally functioning muscle cells, CAF, unlike all known cathepsins, would not be inhibited by the pH existing in healthy, living cells.

Effect of 2-Mercaptoethanol and Iodoacetate on CAF Activity. In addition to Ca^{2+}, CAF requires a reducing agent to function at maximum efficiency. Purified CAF dialyzed against 1 mM $KHCO_3$, 5 mM EDTA loses 95% of its proteolytic activity after 24 h. This loss of activity is reversible, however, and full activity can be returned by adding a reducing agent such as 2-mercaptoethanol to the enzyme. CAF requires approximately 2 mM 2-mercaptoethanol for optimum activity and is not inhibited by additional amounts of 2-mercaptoethanol up to 15 mM.

Activation of the proteolytic activity of CAF by 2-mercaptoethanol suggests that cysteine side chains are involved either at the active site of the enzyme or in maintaining proper conformation of the molecule. Incubation of 9.0×10^{-6} M purified CAF with 10 mM iodoacetate at pH 7.0 and 0 °C for 10 min before assaying for activity in the casein assay completely inhibits proteolysis of casein by CAF, even though 2-mercaptoethanol concentration in the casein assay is 100 times greater than the concentration of iodoacetate added to the assay. Thus, iodoacetate irreversibly inhibits CAF under conditions where reaction of iodoacetate with proteins is limited primarily to cysteine side chains. Several well-characterized proteases such as papain are also rapidly and irreversibly inhibited by iodoacetate. Detailed studies have shown that the active site of papain contains a cysteine side chain that is involved in peptide bond hydrolysis. Because we did not determine the stoichiometric amount of iodoacetate bound to the CAF molecule under the conditions used to inactivate the enzyme, it is not known whether iodoacetate inhibits CAF by blocking one cysteine side chain at the active site or whether iodoacetate reacts rapidly with several cysteine side chains on the surface of the CAF molecule to alter conformation of the molecule and thereby inhibit its proteolytic activity.

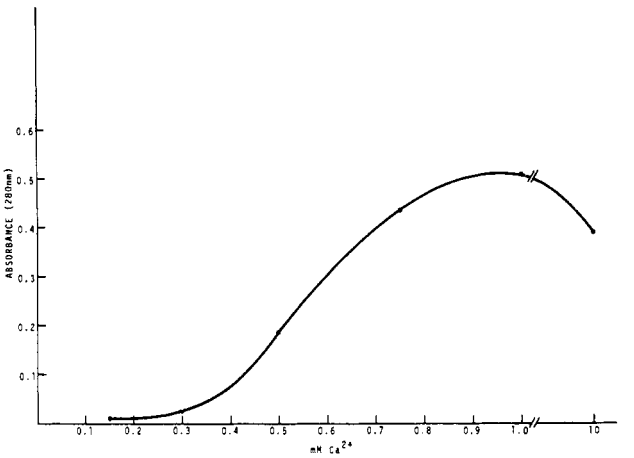

FIGURE 1: Effect of Ca^{2+} concentration on rate of release of soluble peptides from porcine skeletal myofibrils by CAF. Assay conditions: 100 mM KCl, 100 mM Tris-acetate, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, Ca^{2+} as indicated, 2.5 mg of myofibrillar protein/ml, 10 μg of purified CAF/ml, 30 min, 25.0 °C, 2.0 ml final volume. Reaction was stopped by adding 0.1 mM EDTA to a final concentration of 20 mM, and undegraded myofibrils were sedimented at 100 000g_{max} for 30 min. Material soluble in the 100 mM KCl, 100 mM Tris-acetate, pH 7.5, solution was determined by measuring absorbance of the supernatant at 278 nm. Control assays contained all ingredients except CAF.

Effect of Temperature on CAF Activity. Increasing incubation temperature in the range of 0–37.5 °C increases initial rate of hydrolysis of casein by CAF (Figure 2). CAF activity is quite labile in the presence of Ca^{2+} at 37.5 °C, however, and begins to decrease after 5 min of incubation. This decrease in activity is not due to exhaustion of the casein substrate by rapid hydrolysis at 37.5 °C because optical density of peptides released from casein after 5 min at 37.5 °C is only 0.11 (Figure 2) and because addition of more casein substrate after 5 min at 37.5 °C does not result in any additional release of soluble peptides. Incubation of CAF without substrate for 5 min at 37 °C in the presence of 1 mM Ca^{2+} followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the incubated CAF showed that CAF very rapidly autolyzes at 37 °C in the presence of Ca^{2+} (results not shown here); no peptide as large as 12 000 daltons could be detected after 5 min of incubation of CAF at 37 °C in 1 mM Ca^{2+}. Consequently, the rapid de-

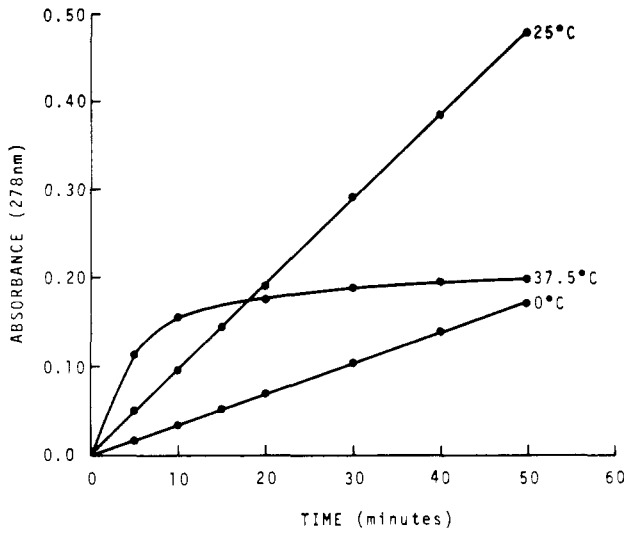

FIGURE 2: Effect of assay temperature on rate of casein hydrolysis by CAF. Assay conditions: 100 mM KCl, 100 mM Tris-acetate, pH 7.5, 10 mM 2-mercaptoethanol, 5 mM $CaCl_2$, 0.1 mM EDTA, 5.0 mg of casein/ml, 5.0 μg of purified CAF/ml, time and temperature as indicated, 2.0 ml final volume. Reaction was stopped by adding 5.0% trichloroacetic acid to a final concentration of 2.5%, and undegraded casein was sedimented at $2000g_{max}$ for 20 min. Material soluble in 2.5% trichloroacetic acid was determined by measuring absorbance of the supernatant at 278 nm. Control assays contained all the ingredients at the same incubation time and temperatures as the sample tube except CAF was omitted.

crease in ability of CAF to degrade casein at 37.5 °C (Figure 2) is probably due to autolysis of the CAF molecule. CAF seems considerably more resistant to autolysis at 0 or 25 °C (Figure 2), and release of peptide material soluble in 2.5% trichloroacetic acid continues linearly for more than 50 min at either 0 or 25 °C. Some autolysis of CAF occurs even at 25 °C, however, and if incubations are prolonged for 12 h, more soluble peptide material is released from casein at 0 than at 25 °C.

Cation and Substrate Specificity of CAF. Because Ca^{2+} was essential for maximum activity of CAF in all three assays of CAF activity used in this study, the effects of some other divalent cations on CAF activity were studied. Mn^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} did not activate CAF when added singly in the absence of Ca^{2+}. In the presence of 1 mM Ca^{2+}, the simultaneous presence of 1 mM Mg^{2+}, Mn^{2+}, or Ba^{2+} had little or no effect on CAF activity (Table I). Co^{2+}, Cu^{2+}, Ni^{2+}, and Fe^{2+}, on the other hand, inhibited Ca^{2+}-activated CAF activity to various degrees when present at 1 mM concentrations (Table I). Susceptibility of CAF to inhibition by these divalent cations may explain why EDTA must be present to maintain CAF activity. If CAF-containing solutions are held without EDTA for more than 12 h, CAF activity is irreversibly lost. This observation was at first interpreted to indicate that EDTA was necessary to chelate trace amounts of Ca^{2+} that contaminate distilled water or some reagents and thereby prevent the rapid autolysis of the CAF molecule that was just discussed. Figure 1 shows, however, that CAF requires at least 0.1 mM Ca^{2+} for appreciable activity. This Ca^{2+} concentration far exceeds that normally found in distilled water or reagents. The effects of Co^{2+}, Cu^{2+}, Ni^{2+}, or Fe^{2+} on CAF activity therefore suggest that inactivation of CAF in the absence of EDTA may be due to reaction with contaminating amounts of one of these divalent cations.

The effect of a known protease inhibitor, soybean trypsin inhibitor, on CAF activity and the ability of CAF to hydrolyze several synthetic substrates were also assayed. Soybean trypsin inhibitor added to CAF assays at a weight ratio of 1:1 soybean trypsin inhibitor:purified CAF (a molar ratio of approximately 5:1 soybean trypsin inhibitor:CAF because of the high molecular weight of CAF; see subsequent discussion) had no effect on ability of CAF to hydrolyze casein. CAF did not catalyze hydrolysis of 2-benzoyl-L-arginine ethyl ester, a substrate for trypsin and papain, of *p*-toluenesulfonyl-L-arginine methyl ester, a substrate for trypsin and thrombin, and of *N*-benzoyl-L-tyrosine ethyl ester, a substrate for chymotrypsin. Other synthetic substrates were not tested, but CAF clearly has a specificity different from papain, another sulfhydryl enzyme, and from trypsin, chymotrypsin, and thrombin.

Amino Acid Composition of CAF. The amino acid composition of purified CAF (Table II) shows that CAF contains relatively small amounts of lysine, arginine, and histidine in relation to the amount of aspartic acid and glutamic acid that

Table I: Effect of Different Divalent Cations on Ca^{2+}-Activated Hydrolysis of Casein by CAF.[a]

Divalent cation	% Act. with 1 mM Ca^{2+} Alone
Mg^{2+}	100
Mn^{2+}	100
Ba^{2+}	95
Co^{2+}	3.2
Cu^{2+}	22.6
Ni^{2+}	7.7
Fe^{2+}	45

[a] Assay conditions: 100 mM KCl, 100 mM Tris-acetate, pH 7.5, 10 mM 2-mercaptoethanol, 1 mM Ca^{2+}, 0.1 mM EDTA, 1 mM of the divalent cation indicated, 5.0 mg of casein/ml, 5.0 μg purified CAF/ml, 25 °C. Reaction was started by addition of CAF, and incubation was for 30 min. Reaction was stopped by adding 5% trichloroacetic acid to a final concentration of 2.5% and undegraded casein was sedimented at 3000 rpm for 20 min. Material soluble in 2.5% trichloroacetic acid was determined by measuring absorbance of the supernatant at 278 nm.

Table II: Amino Acid Composition of Purified CAF.

Amino Acid	CAF[a]	CAF[b]
Lys	60.7 ± 5.0	64.2
His	19.0 ± 1.0	20.1
Arg	42.0 ± 1.5	44.4
Asp	94.7 ± 1.4	100.2
Thr	52.8 ± 0.2	55.9
Ser	101.3 ± 4.8	107.2
Glu	131.4 ± 1.9	139.1
Pro	41.2 ± 1.0	43.6
Gly	133.5 ± 4.5	141.3
Ala	75.9 ± 2.0	80.3
Val	35.8 ± 0.7	37.9
Met	29.1 ± 3.8	30.8
Ile	42.6 ± 2.1	45.1
Leu	75.7 ± 1.7	80.1
Tyr	12.5 ± 3.3	13.2
Phe	38.4 ± 0.9	40.6
Cysteic acid	13.4 ± 0.4	14.2

[a] Figures are residues of amino acid per 1000 total residues and are means plus or minus standard errors for three separate analyses done on different preparations. Only highly purified (from Sephadex G-150 columns, Dayton et al., 1976b) CAF was used for these analyses. [b] Amino acid composition of CAF expressed as number of residues per 112 000-dalton molecule.

it contains. This combination imparts a relatively high net negative charge to the molecule and accounts for the relatively late elution of CAF from DEAE-cellulose (230 mM KCl) (Dayton et al., 1976b), and the relatively rapid migration of CAF (for a molecule of its size) in polyacrylamide gel electrophoresis run in pH 7.5 Tris-HCl buffer (Dayton et al., 1976b). In comparison with some other myofibrillar and sarcoplasmic proteins, CAF contains relatively large amounts of serine and glycine and relatively small amounts of valine, isoleucine, and tyrosine (Table II). The amino acid composition of CAF is clearly different from that of several other proteases such as cathepsin D (Keilová, 1971), prothrombin (Walz et al., 1974), human clotting factor VIII (Legaz et al., 1973), clotting factor X (Jackson, 1972), and two recently isolated neutral proteases from human granulocytes (Ohlsson and Olsson, 1973).

Physical Properties of CAF. Purified CAF appears nearly homogeneous in the analytical ultracentrifuge and sediments as a single boundary with a very slight trailing edge (Figure 3). The sedimentation coefficient of purified CAF is slightly concentration dependent and a least-squares fit of 17 different $s_{20,w}$ values of CAF, determined at different protein concentrations between 1.0 and 4.0 mg/ml, has the equation: $1/s_{20,w} = 0.169 + 0.00309C$; 0.00309 is significantly different from zero at the 0.1% probability level. $s^0_{20,w}$ for purified CAF is 5.90 S with a standard error of 0.06 S. The solvent for all sedimentation velocity runs was 100 mM KCl, 20 mM Tris-acetate, pH 7.5, 5 mM EDTA, 10 mM 2-mercaptoethanol. Concentration *vs.* r^2 plots from meniscus-depletion sedimentation equilibrium (Yphantis, 1964) runs with highly purified CAF were linear (not shown here). Extrapolation of the apparent molecular weights obtained from twelve separate meniscus-depletion sedimentation equilibrium runs, done at different protein concentrations between 0.20 and 1.00 mg/ml to zero protein concentration, was done by using a linear least-squares procedure. Equation of the straight line was $1/M_{app} = 8.88 \times 10^{-6} + 2.60 \times 10^{-6}$(concentration); 2.60×10^{-6} was significantly different from zero at the 1% level of probability, indicating that the molecular weight of CAF was concentration dependent. The molecular weight calculated for purified CAF at zero protein concentration was 112 000 ± 3400 (plus or minus standard error). Sodium dodecyl sulfate–polyacrylamide gels of purified CAF showed that CAF contains two different subunits of 80 000 and 30 000 daltons (Dayton et al., 1976b). Densitometric scans of these sodium dodecyl sulfate–polyacrylamide gels suggested that the 80 000 and 30 000 dalton subunits were present in equimolar ratios (Dayton et al., 1976b). A molecular weight of 112 000 for purified CAF indicates that each CAF molecule contains one 80 000-dalton subunit and one 30 000-dalton subunit.

A linear relationship was found between logarithm of the Stokes radii and the elution volumes of different proteins from a Sephadex G-150 column. CAF elutes from this column just slightly after aldolase, and by using the linear relationship between the Stokes radius and elution volume established for this column by the other proteins of known radii, it can be calculated that the CAF molecule has a Stokes' radius of 43 Å. It is also possible to estimate the frictional coefficient of CAF from the sedimentation coefficient and the molecular weight (Ackers, 1970), and this frictional coefficient can then be used to calculate a second, independent estimate of Stokes' radius of the CAF molecule. The Stokes radius calculated by this second procedure is 46 Å and is in good agreement with the 43 Å obtained directly from the calibrated gel permeation column. A $D^0_{20,w} = 4.54 \times 10^{-7}$ cm^2 sec^{-1} was calculated for purified CAF from $s^0_{20,w}$ and the sedimentation-equilibrium molecular weight.

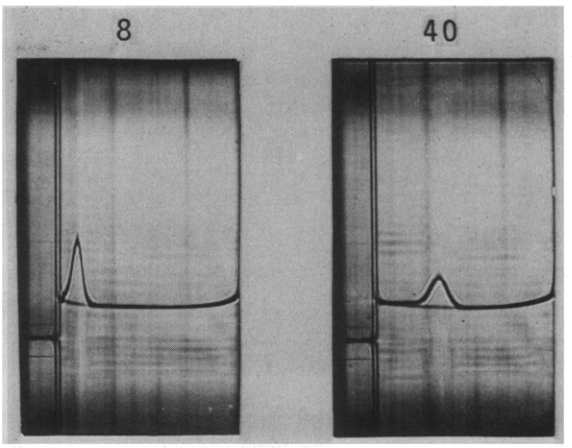

FIGURE 3: Sedimentation pattern of Sephadex G-150-purified CAF. Sedimentation was done in 100 mM KCl, 20 mM Tris-acetate, pH 7.5, 5 mM EDTA, 10 mM 2-mercaptoethanol, 20.0 °C at 4.00 mg of CAF protein/ml and 60 000 rpm for the times indicated. Phase plate angle = 70°.

By making some assumptions concerning hydration and overall shape of the CAF molecule, the Stokes radius can be used to calculate approximate dimensions for the CAF molecule (Tanford, 1961). Assuming 20% hydration and a prolate ellipsoidal shape, dimensions of the CAF molecule calculated from the gel permeation data are 40 × 200 Å. Making the same assumptions, dimensions of 37 × 240 Å are calculated for the CAF molecule from the sedimentation coefficient and molecular weight.

Effects of Purified CAF on Myofibrils. Our earlier studies used sodium dodecyl sulfate–polyacrylamide gel electrophoresis to measure proteolytic breakdown and showed that purified CAF degrades C-protein, troponin-T, and troponin-I and releases α-actinin from myofibrils (Dayton et al., 1975a). Busch et al. (1972) found that P_{0-40} crude CAF preparations very quickly removed Z-disks from myofibrils without causing any other evident ultrastructural changes. Because P_{0-40} crude CAF preparations may contain proteases other than CAF or possess substances that might limit the activity of CAF, it was necessary to examine the ultrastructural effects of purified CAF on myofibrils to determine whether CAF caused ultrastructural deletions in addition to Z-disk removal. Careful examination of the ultrastructural effects of CAF on myofibrils may also provide important evidence about whether CAF is involved in metabolic turnover of myofibrillar proteins.

Control myofibrils incubated under the conditions described in Experimental Procedures were well preserved, and no difference could be seen, either between controls incubated for 2 and 20 h or, after either incubation period, among the three controls themselves. Figure 4a shows a sarcomere from a fiber piece incubated for 20 h in the presence of CAF but no Ca^{2+} (control 3), and Figure 4b shows a sarcomere from a fiber piece incubated for 20 h in the presence of Ca^{2+} but no CAF (control 2). The sarcomeres in these controls show the ultrastructure typical of resting mammalian myofibrils, with well-defined fibrillar Z-disks bisecting the I bands and a well-defined H zone situated at the center of the A band. The M line runs along the center of the H zone. Many I bands showed the 400-Å periodicity that has been attributed to troponin (Endo et al., 1966); this periodicity is most clearly seen in the inset to Figure 4a. Ultrastructure of control Z-disks in cross section

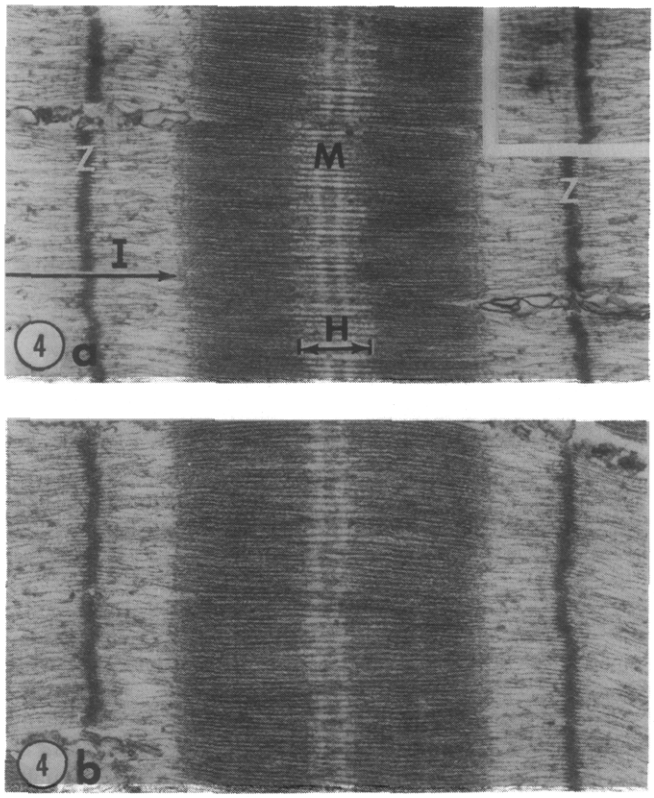

FIGURE 4: Electron micrographs of longitudinal sections of control myofibrils. (a) Longitudinal section through sarcomere from glycerinated rabbit psoas muscle that was incubated for 20 h at 25 °C in the presence of 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM 2-mercaptoethanol, 10 mM EGTA, 1 mM NaN_3, 50 μg of CAF/ml. A Z-disk, M line, I band, and H zone are labeled. A 400-Å periodicity can be seen in occasional areas of the I band (×26 600). This periodicity is seen more clearly by viewing the inset obliquely (inset ×26 000). (b) Longitudinal section through sarcomeres from glycerinated rabbit psoas muscle incubated for 20 h at 25 °C under the same conditions as Figure 4a except that the solution contained no CAF and 5 mM Ca^{2+} was substituted for 10 mM EGTA. Ultrastructure of the myofibril after this treatment is identical with that seen in Figure 4a (×27 300).

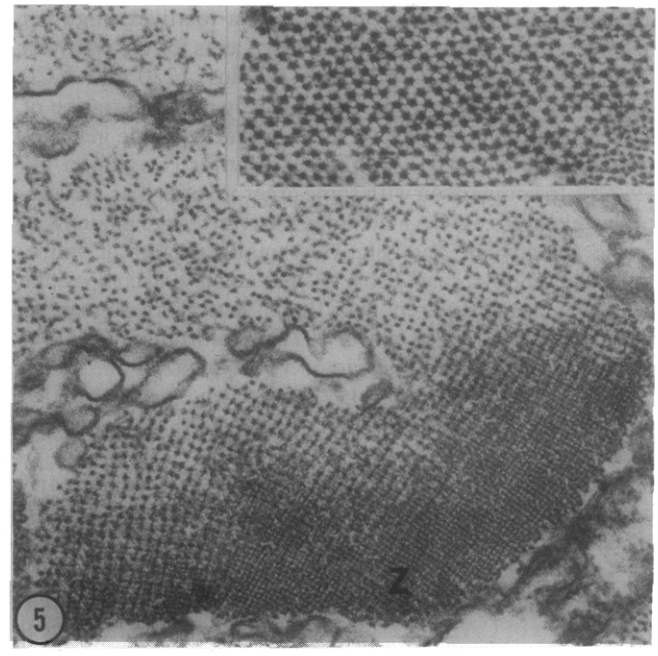

FIGURE 5: This electron micrograph of a cross section through control myofibrils shows a slightly oblique cross section through the Z-disk (labeled Z) and adjacent I-band region of glycerinated rabbit psoas muscle that was incubated for 20 h at 25 °C in the presence of 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM 2-mercaptoethanol, 10 mM EGTA, 1 mM NaN_3, 50 μg of CAF/ml (×67 200). The inset shows a cross section through the M line of the same material. Although the majority of cross-sectioned thick filaments possess their complete set of six radiating M bridges, some do not; this probably reflects slight disruption of the M line during the glycerination process (inset ×67 200).

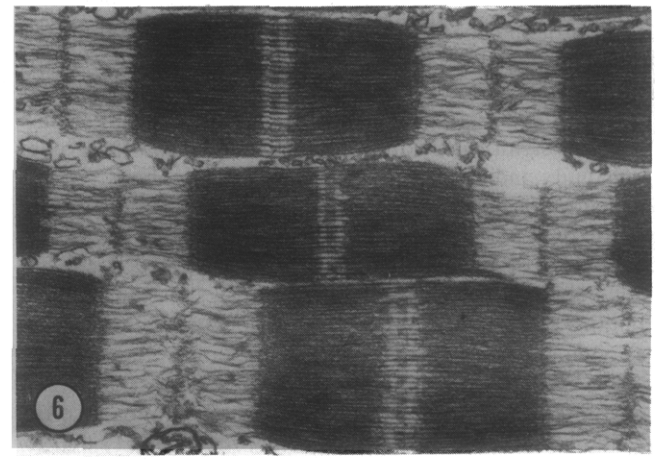

FIGURE 6: Electron micrograph of a longitudinal section of glycerinated rabbit psoas muscle that was mildly treated with CAF. Fibrils were incubated for 2 h at 25 °C in the presence of 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM 2-mercaptoethanol, 5 mM Ca^{2+}, 1 mM NaN_3, 10 μg of CAF/ml. This treatment has greatly reduced Z-disk density and has abolished the 400 Å I-band periodicity but otherwise has not disturbed the normal ultrastructure of the sarcomere (×25 300).

(Figure 5) is similar to the square lattice pattern reported by Landon (1970) and MacDonald and Engel (1971). Thin filaments line up in a square array with a spacing of approximately 180 Å just before their insertion into the Z-disk. Spacing of the Z-disk lattice is approximately 90 Å. Axes of the Z-disk lattice coincide with the axes of adjacent I filaments. A cross section through a control M line is shown in the inset to Figure 5. Thick filaments are present in hexagonal array, and most thick filaments possess their full complement of six radiating M bridges.

Fiber pieces examined after either of the two mildest CAF treatments (i.e., 2 h at either 5 or 10 μg of CAF/ml) showed two well-defined changes (Figure 6): (1) Z-disk density is markedly reduced, suggesting the loss of much Z-disk material, and in cross sections Z-disk remnants could be identified only by small remaining patches of density because most of the Z-disk lattice had been lost (Figure 7); and (2) the 400 Å I-band periodicity is abolished. Apart from these two effects, no other change was observed in either longitudinal or cross sections of myofibrils treated with 5 or 10 μg of CAF/ml for 2 h.

Incubation with 50 μg of CAF/ml for 2 h resulted in complete removal of about 80% of the Z-disks and also slightly affected the M line, but no other ultrastructural changes were seen. No Z-disks remained after 20 h of incubation with CAF (Figure 8), and the M line appeared patchy. Thin filaments, bereft of supporting Z lines, course irregularly through the I band. It is evident, however, that, apart from having their 400 Å periodicity removed (which ocurred at an early stage during the incubation), thin filaments have not been affected to any noticeable extent. Figure 8 was deliberately chosen from an experiment carried out with fiber pieces that had been stretched during glycerination and whose I bands, therefore, were longer than usual. Where the nonoverlapped thin filaments in these long I bands remain in the plane of the section (arrows), they are seen to course out to the site formerly oc-

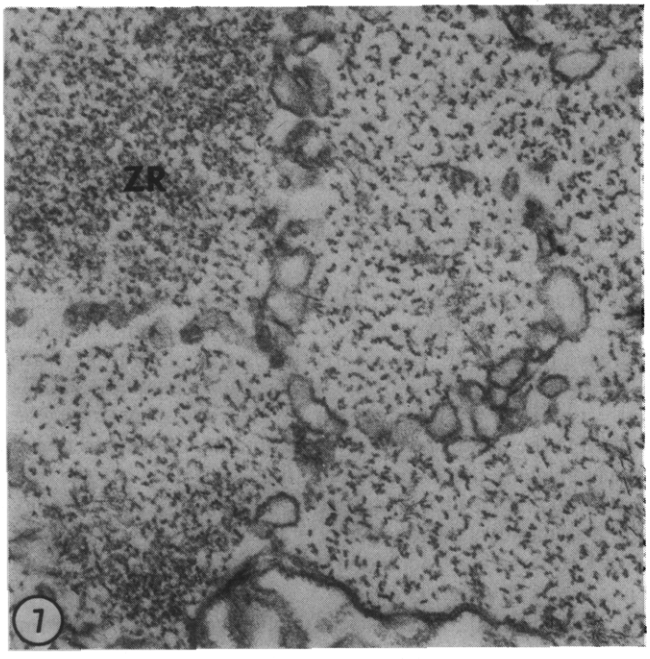

FIGURE 7: A small increase in density is the only criterion that distinguishes Z-disks (labeled ZR) in cross-sectioned I bands of glycerinated rabbit psoas muscle that has been incubated for 2 h at 25 °C in the presence of 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM 2-mercaptoethanol, 5 mM Ca^{2+}, 1 mM NaN_3, 10 μg of CAF/ml (×67 200).

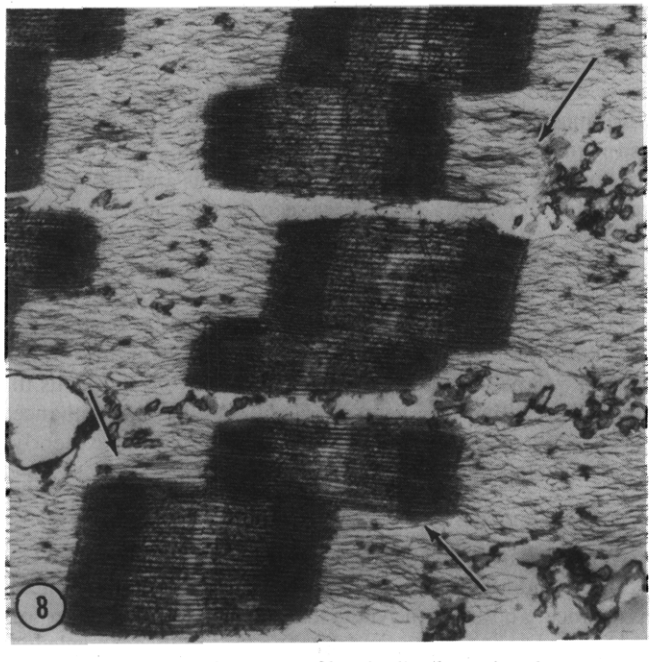

FIGURE 8: Electron micrograph of longitudinally sectioned sarcomeres from glycerinated rabbit psoas muscle that was subjected to 20 h of CAF treatment. Fibrils were incubated for 20 h at 25 °C in the presence of 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM 2-mercaptoethanol, 5 mM Ca^{2+}, 1 mM NaN_3, 50 μg of CAF/ml. M lines are incomplete and are clearly affected by this treatment. Long, wavy, thin filaments fill the wide I bands and are seen, where they remain in the plane of the section, to stretch right out to the site of the former Z-disk (arrows). Areas of densely staining material are seen in the I bands (×22 000).

cupied by the Z-disk. These electron microscope observations are consistent with assays of the effects of purified CAF on individual, purified myofibrillar proteins (Dayton et al., 1975a). These assays showed that purified CAF had no effect on purified actin, but degraded the troponin-T and troponin-I

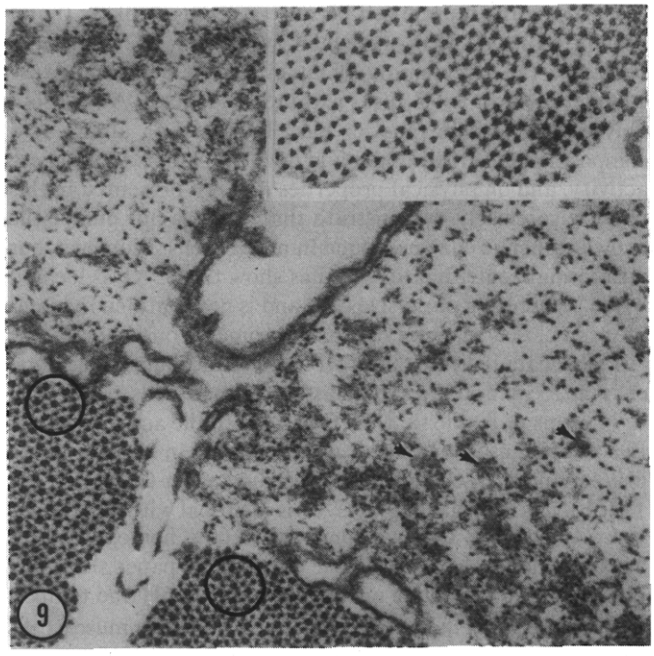

FIGURE 9: Electron micrograph of CAF-treated, cross-sectioned I bands, adjacent out-of-register A bands, and M-line region (inset) from glycerinated rabbit psoas muscle that was incubated for 20 h at 25 °C in the presence of 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 5 mM 2-mercaptoethanol, 5 mM Ca^{2+}, 1 mM NaN_3, 50 μg of CAF/ml. The relatively denser areas seen in the I bands (arrows) are presumably cross sections through the dense areas described in Figure 8. The normal hexagonal array of thick and thin filaments (circle) is seen in the cross-sectioned A-band (×62 500). Some M bridges can still be seen (insert) in cross-sectioned M lines, but most M bridges are missing (inset ×67 200).

subunits of troponin. Figure 8 also shows a few spots of dense material scattered irregularly throughout the I bands. These dense blobs probably represent α-actinin-containing material that, after its release from the Z-disk, is capable of rebinding to the thin filaments (Stromer et al., 1969). In cross-sectioned I bands, these dense spots appear as relatively darker areas (Figure 9). Figure 9 also shows a portion of cross-sectioned A band from an out-of-register adjacent myofibril. Each thick filament in this cross-sectioned A-band is surrounded by its normal complement of six thin filaments. The inset of Figure 9 shows that, after 20 h of CAF treatment, the M-bridge array of the M line is incomplete, although the M bridges are not completely removed.

Discussion

Data in the preceding paper (Dayton et al., 1976b) showed that skeletal muscle contains only very small quantities of CAF (a maximum of 3.4 μg/g of muscle fresh weight as estimated from the increase in specific activity during purification). Consequently, the possibility that CAF actually originates from blood or connective tissue present in small amounts in minced muscle tissue must be considered, especially because blood is known to contain numerous Ca^{2+}-activated proteolytic enzymes involved in coagulation. The molecular weight, amino acid composition, and other properties of CAF described in this paper, however, clearly distinguish it from all blood or connective tissue proteolytic enzymes whose properties are known. Moreover, several attempts to isolate CAF activity from blood by the procedure described in the preceding paper (Dayton et al., 1976b) revealed no CAF activity in blood. The observation by Busch et al. (1972) that extracellular Ca^{2+} stimulates Z-disk removal from myofibrils in 9 h also suggests a location for

CAF inside the muscle cell because it seems unlikely that a protein molecule with molecular weight of 112 000 could penetrate the sarcolemma of intact muscle cells and cause complete removal of Z-disks within 9 h. Hence, CAF is probably endogenous to muscle cells. The data on amino acid composition, on effect of pH and divalent cations on catalytic activity, and on physical properties of CAF presented in this paper also clearly demonstrate that CAF is not one of the known catheptic enzymes found in muscle cells. Moreover, our differential centrifugation studies show that CAF is located in the sarcoplasm of muscle cells and is not confined to membrane-enclosed particles such as lysosomes. Consequently, the available data indicate that CAF is an intracellular proteolytic enzyme located in the sarcoplasm of muscle cells.

It seems probable that CAF was one of the active enzymes in a crude muscle enzyme fraction isolated in 1969 by Kohn (1969), and as discussed in the preceding paper (Dayton et al., 1976b), it is very likely that CAF is identical with the kinase-activating factor that was purified to 60% homogeneity by Huston and Krebs (1968). The data in this paper show that CAF is optimally active under conditions of pH and temperature that exist in the sarcoplasm of normal, living muscle cells in vivo, and because CAF seems to be located in the sarcoplasm, it is imperative that CAF activity be closely regulated in some way to prevent continuous and indiscriminate degradation of myofibrils in living cells. Ca^{2+} is an obligatory requirement for CAF activity in vitro, and it seems likely that Ca^{2+} is involved in controlling CAF activity in vivo. Intracellular free Ca^{2+} concentrations in healthy mammalian skeletal muscle cells are generally 10^{-5} M or less (Jöbsis and O'Connor, 1966; Ridgway and Ashley, 1967; Weber, 1966). This Ca^{2+} concentration is about an order of magnitude less than we have found necessary for appreciable CAF hydrolysis of myofibrils in vitro. It is possible that the free Ca^{2+} requirement of CAF is reduced by some as yet unknown mechanism to a level where the existing intracellular free Ca^{2+} levels are high enough to activate the enzyme. That CAF contains nonidentical subunits of 80 000 and 30 000 daltons proffers the possibility that one of these subunits is catalytic and the other is regulatory. Alternatively, local concentrations of Ca^{2+} in muscle cells may reach levels high enough to activate CAF.

Although the physiological role of CAF is not yet known, that CAF is the first proteolytic enzyme isolated from muscle cells that is capable of degrading intact myofibrils suggests that CAF may be involved in metabolic turnover of myofibrils. We have recently proposed a possible role for CAF in disassembly of myofibrils to monomeric proteins that can then be degraded to amino acids by other muscle proteases (Dayton et al., 1975a). This role is consistent with our electron microscope findings that CAF very quickly removes Z-disks from myofibrils and also partly degrades M lines because at least part of the physiological function of Z-disks and M lines ostensibly is to maintain the three-dimensional architecture of myofibrils. Moreover, our assays of the effects of purified CAF on purified myofibrillar proteins show that CAF degrades tropomyosin and C-protein (Dayton et al., 1975a), and it seems likely that tropomyosin and C-protein contribute to stability of thin and thick filaments, respectively. A physiological role for CAF in disassembly of myofibrils also recognizes the unusual inability of CAF to degrade purified actin and myosin (Dayton et al., 1975a), even though myosin is susceptible to a wide range of proteolytic enzymes. Hence, CAF has a unique specificity for degradation of those structures or proteins that ostensibly serve to keep myosin and actin assembled in the form of myofibrils. A role for CAF in metabolic turnover of myofibrils is supported by our preliminary results showing that CAF activity is elevated in atrophying denervated muscle (Jergenson, Robson, and Stromer, unpublished results) and in muscle from dystrophic chickens (Reville and Zeece, unpublished results). Furthermore, Kohn (1969) found that activity of his soluble proteolytic fraction increased significantly in atrophying rat skeletal muscle, and, as indicated earlier, it is very likely that Kohn's soluble proteolytic fraction contained CAF. Several studies (Issacson and Sandow, 1968; Varley and Dhalla, 1973; Vihert and Pozdyunina, 1969; Young et al., 1959) have shown that intracellular free Ca^{2+} levels increase during pathological or necrotic states in muscle cells, and it is possible that this increase in free Ca^{2+} concentration is sufficient to activate CAF, which may then initiate myofibrillar degeneration. Finally, ability of CAF to very rapidly degrade Z-disks may be related to the findings of a very large number of ultrastructural studies showing that marked alterations in Z-disks are the first structural change observed in myofibrils during rapid atrophy that accompanies a wide variety of muscle pathologies. These Z-disk alterations include hypertrophy (MacDonald and Engel, 1969; Meltzer et al., 1973; Resnick et al., 1968; Santa, 1969; Shafiq et al., 1969), streaming (Engel and Stonnington, 1974; Gauthier and Dunn, 1973; Ghatak et al., 1973,; Goebel et al., 1973; Stonnington and Engel, 1973; Yarom et al., 1974), and disintegration and disappearance (Afifi et al., 1972; Johnson, 1969; Martinez et al., 1973; Pellegrino and Franzini, 1963; Price et al., 1962; Tomanek and Lund, 1973) and occur in conditions as diverse as denervation atrophy, muscular dystrophies, alcoholic myopathy, rhabdomyolysis, the stiff-man syndrome, Marfan's syndrome, and the prune-belly syndrome. Based on these ultrastructural observations during rapid muscle atrophy, the Z-disk is the structural area of the myofibril most affected during atrophy. Disintegration of Z-disks as the initial step in degradation of myofibrils would account for both the masses of disorganized filaments and the gradual reduction in myofibril diameter seen during muscle atrophy (Dayton et al., 1975a; Pellegrino and Franzini, 1963).

Average dimensions of the CAF molecule given by two independent measures used in this study were 38 × 220 Å. An axial ratio of 5.8 is larger than that of most other proteolytic enzymes, and it is possible that CAF serves a structural function as well as initiating metabolic turnover of myofibrils in vivo. Localization at or near the Z-disk would greatly enhance the effectiveness of the small amounts of CAF that evidently exist in skeletal muscle.

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