Ca\(^{2+}\)-dependent Proteolytic Activity in Crab Claw Muscle

EFFECTS OF INHIBITORS AND SPECIFICITY FOR MYOFIBRILLAR PROTEINS

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The claw closer muscle of the Bermuda land crab, Gecarcinus lateralis, undergoes a sequential atrophy and restoration during each molting cycle. We describe here the role of Ca\(^{2+}\)-dependent proteinases in the turnover of myofibrillar protein in normal aneudysial (intermolt) claw muscle. Crab Ca\(^{2+}\)-dependent proteinase degrades the myofibrillar proteins actin, myosin heavy and light chains, paramyosin, tropomyosin, and tropenin-2 and -1. Ca\(^{2+}\)-dependent proteinase activity in whole homogenates and 90,000 \( \times \) g supernatant fractions from muscle homogenates has been characterized with respect to Ca\(^{2+}\) requirement, substrate specificity, and effects of proteinase inhibitors. The enzyme is inhibited by antipain, leupeptin, E-64, and iodoacetamide; it is insensitive to pepstatin A. The Ca\(^{2+}\)-dependent proteinase is a sarcoplasmic cysteine proteinase that shows maximal activation at 1 mM Ca\(^{2+}\) at neutral pH. Since approximately 28\% of the activity remains at 1.5 \( \mu \)M Ca\(^{2+}\), the enzyme is partially active at physiological Ca\(^{2+}\) concentrations. The specificity of crab Ca\(^{2+}\)-dependent proteinase was examined with native myosin with normal ATPase activity as well as with radioiodinated myosin and radioiodinated hemolymph proteins. Hydrolysis of 125I-myosin occurs in two phases, both Ca\(^{2+}\)-dependent: 1) heavy chain (\( M_r \approx 200,000 \)) is cleaved into four large fragments (\( M_r \approx 160,000, 110,000, 73,000, 60,000 \)) and numerous smaller fragments; light chain (\( M_r \approx 18,000 \)) is cleaved to a 15,000-Da fragment; 2) the fragments produced in the first phase are hydrolyzed to acid-soluble material. Although radioiodinated native hemolymph proteins are not susceptible to the Ca\(^{2+}\)-dependent proteinase, those denatured by carboxymethylation are degraded. These data suggest that crab Ca\(^{2+}\)-dependent proteinase is involved in turnover of myofibrillar protein in normal muscle and muscle undergoing proecdysial atrophy.

The role of lysosomal and nonlysosomal pathways in the turnover of myofibrillar proteins has been explored in a number of systems during both steady state and pathological conditions (see Refs. 1–3 for reviews). A Ca\(^{2+}\)-dependent proteinase (4) active at neutral pH has been implicated in the nonlysosomal hydrolysis of myofibrillar proteins. In vertebrate muscle maintained in vitro, increased intracellular levels of Ca\(^{2+}\) stimulate protein turnover (5, 6) and are associated with dissolution of myofilaments (7) and release of \( \alpha \)-actinin from Z lines (8). Incubation of muscle with Ca\(^{2+}\) ionophore increases the release of filaments from myofibrils (9). Increased levels of Ca\(^{2+}\)-dependent proteinase activity have been observed in dystrophic muscle (10) and in muscle caused to atrophy by vitamin E deficiency (11) or hyperthyroidism (12).

The claw closer muscle of the Bermuda land crab Gecarcinus lateralis experiences a cyclical atrophy and restoration during each intermolt period (13). Muscle protein decreases 40\% during proecdysis, resulting in a 4-fold reduction in myofibril cross-sectional area (14). The muscle is restored following ecdysis (13). We have examined the role of Ca\(^{2+}\)-dependent proteinases in this molt-related atrophy (see Ref. 15 for review). The calcium content of atrophic muscle homogenates is twice that of homogenates from normal muscle (16). The activity of the crab Ca\(^{2+}\)-dependent proteinase, which degrades several myofibrillar proteins including actin and myosin, is more than 2-fold greater in proecdysial muscle (16).

Vertebrate Ca\(^{2+}\)-dependent proteinases, like other cysteine proteinases, are inhibited by sulphydryl reagents and substrate analogs leupeptin, antipain, and E-64 (17, 18). Although most Ca\(^{2+}\)-dependent proteinases purified from vertebrate muscle require between 0.1 and 4 mM Ca\(^{2+}\) for half-maximal activation (designated Ca\(^{2+}\)-dependent proteinase II; see Refs. 18–20 for reviews), some forms of the proteinase (Ca\(^{2+}\)-dependent proteinase I) require only micromolar levels of Ca\(^{2+}\) (21–23). It appears that these two forms are unrelated (24, 25). A third form, obtained by autolysis of Ca\(^{2+}\)-dependent proteinase II, also requires micromolar Ca\(^{2+}\) (26, 27). Thus, there may be two forms of Ca\(^{2+}\)-dependent proteinase that are fully active at physiological Ca\(^{2+}\) concentrations within the muscle fiber.

A review on mammalian Ca\(^{2+}\)-dependent proteinases mentions the existence of Ca\(^{2+}\)-dependent proteinase activity in muscle from the horseshoe crab (18); otherwise, the occurrence of Ca\(^{2+}\)-dependent proteinases in vertebrate muscle is largely unexplored. The present study has characterized Ca\(^{2+}\)-dependent proteinase activity in crab claw muscle with respect to substrate specificity, effective Ca\(^{2+}\) concentration, and effects of proteinase inhibitors. These results show similarities between crustacean and vertebrate Ca\(^{2+}\)-dependent proteinases thereby establishing this crustacean system as a simple and convenient model for the role of Ca\(^{2+}\)-dependent proteolysis in myofibrillar protein turnover and its manifestation in the structure of the sarcomere.

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2 The abbreviations used are: E-64, N-[N-(3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine; EGTA, ethylene glycol bis(\( \beta \)-aminoethyl ether)N,N',N''-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MeSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HC, heavy chain; LC, light chain.
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EXPERIMENTAL PROCEDURES

Materials

Animals—G. lateralis, obtained from the Bermuda Biological Station, were maintained either individually or collectively in containers with damp sand, and fed weekly on a diet of lettuce, carrots, and dry cat food. The molt cycle stage of animals was determined according to Skinner (28). Only anecdysial (intermolt) male crabs were used.

Chemicals—E-64 was a gift from Dr. David Johnson, East Tennessee State University. Hepes, EDTA, leucine, casein (low proteolytic activity), leupeptin, antipain, and pepstatin A were purchased from Sigma; fluorescamine from either Sigma or Roche; acrylamide from either Eastman, Sigma, or Bio-Rad; N,N’-methylenebisacrylamide and Chelex 100 from Bio-Rad; ultrapure SDS from British Drug House; and chloramine-T from Mallinckrodt Chemical Works.

Methods

SDS-Polyacrylamide Gel Electrophoresis—Proteins were dissolved in SDS sample buffer (16). Protein was measured by fluorescence emission at 338 nm (excitation λ = 286 nm); bovine serum albumin served as standard (16).

SDS-PAGE was done using a discontinuous gel system (16). Separating gels (7.5% and 10%) were made from a 30% acrylamide and 1% N,N’-methylenebisacrylamide stock. Bio-Rad low molecular weight standards and purified crab muscle myosin (16) were used as protein standards.

Carboxymethylation of Crab Hemolymph Proteins—Hemolymph proteins were denatured by carboxymethylation (28). A 2.5-mL solution of hemolymph proteins (10 mg/mL) in 10 mM sodium bicarbonate, pH 8.0, was brought to 10 mM in 2-mercaptoethanol, and 2.5 mL of a solution containing 8 M guanidine hydrochloride, 100 mM sodium phosphate, pH 8.0, and 3 mM EDTA were added. After 15 min at room temperature, 150 μL of 1 M iodoacetic acid, pH 8.0, was added and the solution stirred in the dark for 20 min. After addition of 35 μL of 2-mercaptoethanol, the solution was dialyzed against 2 liters of Buffer A containing 0.1 mM EGTA to remove free amino acids. They were then dialyzed against Buffer A, adjusted to pH 7.0, 10 g of Chelex 100 replaced EGTA. Reaction mixtures (22 μL) contained dialyzed 90,000 g supernatant fractions (2.8–3.8 mg of protein/mL) and radiolabeled proteins. The concentrations of CaCl\textsubscript{2} or EGTA buffers contained 100 μM CaCl\textsubscript{2} and 100 μM EGTA. The concentration of CaCl\textsubscript{2} was varied to obtain free Ca\textsuperscript{2+} concentrations from 0.01 μM to 10 mM, calculated from the equation of Bremel and Weber (32) using K = 1.9 × 10\textsuperscript{-11} M at pH 7. After incubation for 1 h at 37 °C, protein was precipitated in cold 6% trichloroacetic acid and soluble radioactivity in supernatant fractions was measured as above.

Effects of Proteinase Inhibitors—In one series of experiments, the effects of inhibitors on hydrolysis of unlabelled total proteins were examined. Reaction mixtures (250 μL) containing 200 μL of supernatant fractions (5.6–14.8 mg of protein/mL), 5 mM CaCl\textsubscript{2}, and either 100 μM leupeptin, 100 μM antipain, 100 μM pepstatin A in 1% Me\textsubscript{2}SO, 1% Triton X-100, 100 μM E-64, 5 mM iodoacetamide, or 10 mM EDTA were incubated for 12 h at 37 °C. Trichloroacetic acid-soluble material was determined with fluorescamine using leucine as standard as described (16).

In a second series, the effects of inhibitors on hydrolysis of 131I-casein and 131I-myosin were examined. Reaction mixtures (1 mL) contained 90,000 g supernatant fraction (2.3–3.2 mg of protein/mL), 5 mM CaCl\textsubscript{2}, radiolabeled protein (2 × 10\textsuperscript{5} cpm), and either 100 μM leupeptin, 100 μM E-64, or 10 mM EDTA, and incubated at 37 °C. Since hydrolysis of 131I-myosin was linear between 1 and 4 h (see Fig. 4), the effects of inhibitors on Ca\textsuperscript{2+}-dependent proteinase activity were monitored during this time interval. Hydrolysis of 131I-casein was linear during the first hour of incubation. The specific radioactivities in these experiments were 4 × 10\textsuperscript{5} cpm/μg of myosin and 1.6 × 10\textsuperscript{6} cpm/μg of casein.

Reconstitution Experiments—Low speed pellets (16,000 × g), which were washed four times in 100 mL of Buffer A and resuspended in 2 volumes of Buffer A, and 90,000 g supernatant fractions were used in reconstitution experiments. Four sets of reaction mixtures were used: pellet alone (50 μL of resuspended pellet containing between 1.1 and 2.1 mg of protein and 200 μL of Buffer A), supernatant fraction alone (200 μL containing between 1.0 and 1.7 mg of protein and 50 μL of Buffer A), and pellet (50 μL) and supernatant fraction (200 μL) combined. To each mixture 50 μL of 131I-casein was added. After incubation at 37 °C, protein was precipitated in cold 6% trichloroacetic acid, and acid-soluble primary amines were determined by fluorescamine. For SDS-PAGE, mixtures were dissolved in 2 volumes of SDS sample buffer.

To determine the proteolytic patterns at low Ca\textsuperscript{2+} concentrations, pellets were washed with Chelex 100-treated Buffer A and supernatant fractions were dialyzed against 4 2-liter changes of Buffer A, adjusted to pH 7.0, as described above, except that 5 g of Chelex 100 replaced EDTA. Compositions of reaction mixtures were the same as above with 5 mM Ca\textsuperscript{2+}; CaCl\textsubscript{2}/EGTA buffer stock was added to obtain free Ca\textsuperscript{2+} concentrations of 10 and 100 μM. After incubation for 96 h at 37 °C, mixtures were dissolved in 2 volumes of SDS sample buffer for SDS-PAGE.

RESULTS AND DISCUSSION

Washed pellets from low speed spins contained the myofibrillar proteins characteristic of arthropod muscle (15, 33): myosin heavy chain (Mr = 200,000), paramyosin (Mr = 105,000), troponin-T (Mr = 47,000), actin (Mr = 43,000), tropomyosin (Mr = 40,000), troponin-I (Mr = 29,000), and myosin light chain (Mr = 18,000) (Fig. 1A, lane d). We have not identified troponin-C in claw muscle homogenates. Other arthropod troponin-Cs have molecular weights of 17,000–18,000 Da (33) and would be indistinguishable from myosin.
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FIG. 1. Reconstitution experiment: myofibrillar proteins (A) and soluble proteins in 90,000 $\times$ g supernatant fraction (B) from muscle homogenate separated on 10% SDS-PAGE. A, myofibrillar proteins were incubated with (lanes a–c) or without (lanes d and e) soluble proteins for 0 h (lanes a and d) or 12 h (lanes b, c, and e) at 37 °C. Reaction mixtures contained either 5 mM $Ca^{2+}$ (lanes a, b, d, and e) or 10 mM EGTA (lane c). Positions of molecular weight standards are indicated at left: A, actin; HP, hemolymph proteins; MHC, myosin heavy chain; MLC, myosin light chain; P, paramyosin; TM, tropomyosin; TNT, troponin-I; TNT, troponin-T. B, soluble proteins were incubated with 5 mM $Ca^{2+}$ for 0 h (lane a) and 12 h (lane b). Both actin (A) and a large soluble protein (arrow) were susceptible to $Ca^{2+}$-dependent proteinase activity. Supernatant fraction also contained a protein (asterisk) slightly larger than tropomyosin.

LC on one dimensional SDS-polyacrylamide gels. Washing pellets in low salt buffer followed by low speed centrifugation extracted some myosin and enriched pellets in proteins associated with thin filaments. As a result, the large amount of actin often obscured troponin-T (Fig. 1A). In addition to muscle proteins, the 90,000 $\times$ g supernatant fraction contained a collection of six proteins ($M_r = 68,000–90,000$) from the hemolymph, in all likelihood the six subunits of crustacean hemocyanin (34). These proteins are seen in the hemolymph of land crabs electrophoresed under similar conditions (16).

In the presence of 5 mM $Ca^{2+}$ there was no detectable release of trichloroacetic acid-soluble material from myofibrillar proteins incubated alone (Fig. 2). SDS-PAGE confirmed that there was no apparent degradation of myofibrillar proteins in these mixtures (Fig. 1A, lane e). Supernatant fractions incubated alone showed only a small amount of proteolysis (Fig. 2), which was associated with hydrolysis of residual amounts of actin and a soluble protein larger than myosin HC (Fig. 1B, lane b, arrow) but not other soluble proteins. However, large amounts of acid-soluble primary amines were released when myofibrillar protein and supernatant fractions were incubated together (Fig. 2). SDS-PAGE demonstrated that myosin HC, paramyosin, actin, tropomyosin-1 and -I, and tropomyosin were hydrolyzed (Fig. 1A, lane b). As myosin HC was degraded, two new bands appeared at 160,000 and 110,000 (Fig. 1A, lane b, arrowheads). Although the degradation of myosin LC was not detected in reconstitution experiments, hydrolysis of $^{125}$I-myosin (see below) as well as previous studies (16) showed that LC is susceptible to $Ca^{2+}$-dependent proteolysis. All six hemolymph proteins remained undegraded, showing the specificity of the $Ca^{2+}$-dependent proteinase for myofibrillar proteins. EGTA appeared to inhibit proteolysis completely (Fig. 1A, lane e).

At lower $Ca^{2+}$ concentrations the proteolytic pattern was essentially the same as that at 5 mM, although the lower proteolytic activity necessitated prolonged incubations of 96 h. Myosin HC was degraded as fragments appeared at 160,000 and 110,000 (Fig. 3, lanes b and c). Actin, tropomyosin-T, and tropomyosin were also hydrolyzed while degradation of paramyosin, troponin-I, and myosin LC (not shown) was not evident under these conditions. However, given that these three proteins showed little degradation at 5 mM $Ca^{2+}$ (Fig. 1A, lane b), it is not surprising that hydrolysis could not be detected at low $Ca^{2+}$-dependent proteinase activities. Paramyosin would not be as susceptible to hydrolysis as myosin since paramyosin constitutes the core of the thick filament.

Degradation of myofibrillar proteins was completely inhibited by EGTA (Fig. 3, lane a); hemolymph proteins remained unhydrolyzed in low $Ca^{2+}$ (Fig. 3, lanes b and c). Although the concentration of $Ca^{2+}$ in these fibers is not known, the low $Ca^{2+}$ concentrations selected were probably within the normal physiological range, particularly since troponin and sarcoplasmic reticulum would bind $Ca^{2+}$, which would reduce the amount of free $Ca^{2+}$ available for the $Ca^{2+}$-dependent proteinase. Thus these data show that contractile proteins are degraded at physiological levels of $Ca^{2+}$ and that substrate specificity is similar over a 500-fold range in $Ca^{2+}$ concentration.

These reconstitution experiments demonstrated that the $Ca^{2+}$-dependent proteinase is localized in the sarcoplasm and is not closely associated with either microsomes or myofibrils, since activity remained in 90,000 $\times$ g supernatants of muscle homogenates. Crustacean muscle contains very small amounts of connective tissue, vascular elements, and nerves; satellite cells are absent (14). Both hemolymph and epidermis are significant contaminants of muscle. However, epidermis contains less than 5% of the $Ca^{2+}$-dependent proteinase activity that found in the same amount of claw muscle while no $Ca^{2+}$-dependent proteinase activity was detected in homogenized hemolymph (data not shown). Since hemolymph contains hemocytes, which are also found in connective tissue (35), it is unlikely that these phagocytic cells contribute to $Ca^{2+}$-dependent proteolysis in muscle homogenates. Thus it appears that most of the $Ca^{2+}$-dependent proteinase activity originates in claw muscle fibers.
Physiological levels of Ca\(^{2+}\) in the sarcoplasm and is involved in troponin-T.

Crab Ca\(^{2+}\)-dependent proteinase (Ref. 16, see below), mini-supernatants showed a similar response to Ca\(^{2+}\), except that maximum Ca\(^{2+}\)-dependent proteinase activity remained at 1.5 mM rather than 5 mM Ca\(^{2+}\) (36).

The difference between the two preparations probably resulted from the absence in 90,000 × g supernatants of sarcoplasmic reticulum and troponin, both of which would sequester Ca\(^{2+}\).

Group-specific inhibitors have been used to characterize various vertebrate proteolytic enzymes in vivo and in vitro. Pepstatin inhibits aspartic proteinases such as cathepsin D (37); leupeptin, antipain, and E-64 inhibit cysteine proteinases, including vertebrate muscle Ca\(^{2+}\)-dependent proteinases (18, 21–23, 26). Sulphydryl reagents such as iodoacetamide, and N-ethylmaleimide also inhibit vertebrate Ca\(^{2+}\)-dependent proteinases (17, 18, 21), demonstrating the requirement of a thiol group for catalysis.

Inhibitors of cysteine proteinases also reduce crab muscle Ca\(^{2+}\)-dependent proteinase activity. Table I shows the effects of several inhibitors on Ca\(^{2+}\)-dependent proteinase activity in muscle homogenates. Although both 100 μM antipain and leupeptin inhibited proteolysis by about 50% neither was as effective as EGTA, which inhibited more than 90%. Neither

Proteolytic activity in 90,000 × g supernatant fractions of muscle homogenates increased with increasing concentrations of Ca\(^{2+}\) from 0.10 μM to 1 mM (Fig. 4); further increases to 5 and 10 mM Ca\(^{2+}\) depressed proteolysis. This broad response to Ca\(^{2+}\) suggests there is more than one form of crab Ca\(^{2+}\)-dependent proteinase and that each form differs in Ca\(^{2+}\) sensitivity. Supporting data are the presence of two peaks of Ca\(^{2+}\)-dependent proteinase activity following aminohexyl-Sepharose chromatography (data not shown). Since substantial Ca\(^{2+}\)-dependent proteinase activity remained at 1.5 μM Ca\(^{2+}\) (28% of the rate at 1 mM Ca\(^{2+}\)), it appears that a significant portion of Ca\(^{2+}\)-dependent proteinase functions at physiological levels of Ca\(^{2+}\) in the sarcoplasmic reticulum and is involved in myofibrillar protein turnover in vivo. The short incubation time used (1 h), combined with the remarkable stability of crab Ca\(^{2+}\)-dependent proteinase (Ref. 16, see below), minimized possible autolysis to a form with greater Ca\(^{2+}\) sensitivity.

Ca\(^{2+}\)-dependent proteinase activity in whole muscle homogenates showed a similar response to Ca\(^{2+}\), except that maximum activity occurred at 5 mM rather than 1 mM Ca\(^{2+}\) (36). The difference between the two preparations probably re-
pepsstatin A (100 μM), which was dissolved in 1% MeSO, nor 1% MeSO alone had any effect on activity. Iodoacetamide (5 mM) inhibited Ca²⁺-dependent proteinase activity by 54%. E-64 (100 μM) was as effective an inhibitor as excess EGTA; as little as 10 μM yielded the same amount of inhibition as higher concentrations (data not shown). The greater effectiveness of E-64 as compared to other substrate analogs, such as leupeptin and antipain, possibly resulted from its ability to bind irreversibly to the Ca²⁺-dependent proteinase (38). The effects of inhibitors on hydrolysis of radiiodinated myosin and casein in 90,000 × g supernatant fractions were qualitatively similar to those observed on proteolysis in muscle homogenates in that both E-64 and EGTA inhibited hydrolysis to a greater extent than leupeptin (Table II). E-64 was again as effective as excess EGTA in inhibiting proteolysis. These similarities between the degradation of radiiodinated substrates and unlabeled myofilaments in the presence of specific inhibitors show that the more sensitive assay conditions employing [¹²⁵I]-casein to characterize the catalytic properties of crab Ca²⁺-dependent proteinase are valid.

The specificity of crab Ca²⁺-dependent proteinase was examined with radiiodinated substrates. [¹²⁵I]-myosin was degraded in the presence of 5 mM Ca²⁺, resulting in the release of acid-soluble radioactivity; 10 mM EGTA completely inhibited hydrolysis (Fig. 5). Hydrolysis of [¹²⁵I]-myosin occurred in two phases; there was a slow release of label during the first 10–15 min, followed by a more rapid hydrolysis. Preincubation for 20 min at 37 °C before addition of Ca²⁺ did not change this pattern. Under identical conditions, radiiodinated native hemolymph proteins were not hydrolyzed (Fig. 5).

Autoradiograms of SDS-polyacrylamide gels showed that when Ca²⁺ was present in the reaction mixture, both [¹²⁵I]-myosin HC and LC were degraded into several major fragments and many minor fragments (Fig. 6, A and B). The molecular weights of the major fragments obtained from the hydrolysis of [¹²⁵I]-myosin HC were 160,000, 110,000, 73,000, and 60,000 Da (Fig. 6A, lanes b and c). The 160,000 and 110,000 fragments correspond to fragments observed in 10% gels (Fig. 6A, lane b). These same fragments had been sized previously at 140,000 and 105,000 Da (16); electrophoresis on 7.5% gels (Fig. 6A) provided a more accurate estimate of molecular weights above 100,000. The two most prominent bands (M₁ = 73,000 and 60,000) were not observed previously (16) since their molecular weights and mobilities in acrylamide gels are similar to those of hemolymph proteins (Fig. 1B).

In addition to the major fragments, numerous minor fragments, ranging in molecular weight from 25,000 to 100,000, appeared. [¹²⁵I]-Myosin LC was degraded to a 15,000 molecular weight fragment (Fig. 6B, lane b). After prolonged exposure (22 h) to the proteinase, the major fragments produced during the early phase of digestion were hydrolyzed without the appearance of additional fragments (Fig. 6B, lane c). The autoradiograms showed that the short lag period before release of acid-soluble radioactivity resulted from the generation of large fragments before their subsequent hydrolysis to acid-soluble material (Fig. 5).

It was possible that the fragments generated from Ca²⁺-
dependent proteolysis in the 90,000 × g supernatant fractions were subsequently degraded by other proteolytic enzymes active at neutral pH. To determine whether the Ca\textsuperscript{2+}-dependent proteinase was responsible for the complete hydrolysis of the \textsuperscript{125}I-myosin, reaction mixtures were incubated in the presence of 5 mM Ca\textsuperscript{2+} for 2 h during which most of the myosin was degraded. EGTA was then added to one mixture to a final concentration of 10 mM. The release of acid-soluble radioactivity was measured and compared to the release from a mixture in which 10 mM EGTA was present throughout the incubation period. Both hydrolytic phases were Ca\textsuperscript{2+}-dependent since EGTA inhibited hydrolysis whether or not myosin was first cleaved into fragments (Fig. 7). Furthermore, the hydrolytic rates were similar in both after the addition of EGTA, 0.2 ng/h in mixtures with EGTA throughout the incubation and 0.3 ng/h in mixtures in which EGTA was added at 2 h. Proteolysis in mixtures containing Ca\textsuperscript{2+} was 10-fold greater (3.0 ng/h). Ca\textsuperscript{2+}-independent proteolysis usually amounted to less than 10% of the total proteolytic activity when Ca\textsuperscript{2+} is present (Tables I and II). Since there was little degradation of myofibrillar proteins in reactions lacking Ca\textsuperscript{2+} (Figs. 1, 2, and 9), we conclude that other neutral proteinases play a relatively minor role in turnover of contractile proteins in claw muscle and that crab Ca\textsuperscript{2+}-dependent protease can completely degrade both myosin HC and LC to acid-soluble material.

Previous studies (16), as well as results presented here (Figs. 1 and 3), showed that hemolymph proteins were not degraded by crab Ca\textsuperscript{2+}-dependent proteinase. Experiments with radiiodinated native hemolymph proteins confirmed our earlier observations; these proteins were not hydrolyzed by crab muscle Ca\textsuperscript{2+}-dependent proteinase (Fig. 8, lane c). However, radiiodinated hemolymph proteins denatured by carboxymethylation were susceptible to Ca\textsuperscript{2+}-dependent proteolysis (lane b), which was inhibited by EGTA (lane e).

Since hemolymph proteins, which are not normally hydrolyzed by crab Ca\textsuperscript{2+}-dependent proteinase, became susceptible to proteolysis when denatured, it seemed possible that degradation of \textsuperscript{125}I-myosin resulted from denaturation with chloramine-T. In fact, \textsuperscript{125}I-myosin had no ATPase activity (data not shown). However, it is clear from reconstitution experiments (Figs. 1 and 3) and earlier studies (16) that HC and LC in native myosin in myofibrillar fractions and whole muscle homogenates are degraded by Ca\textsuperscript{2+}-dependent proteinase. To ascertain that native myosin was hydrolyzed, purified crab muscle myosin was used as substrate. This myosin preparation had a K\textsuperscript{+-EDTA} ATPase activity of 1.3 μmol of ATP·mg\textsuperscript{-1}·min\textsuperscript{-1}, which is comparable to Limulus muscle myosin (39). A Ca\textsuperscript{2+}-dependent proteinase, partially purified by organomercurial-Sepharose chromatography, contained two major proteins (M, 95,000 and 40,000). Incubation of that enzyme preparation with purified myosin cleaved HC to numerous fragments only in the presence of Ca\textsuperscript{2+} (Fig. 9). Thus, native myosin, either purified or as an integral part of a myofilament, is susceptible to Ca\textsuperscript{2+}-dependent proteolysis in vitro.

Although crustacean claw muscle Ca\textsuperscript{2+}-dependent proteinase resembles the cysteine proteinases purified from vertebrate muscle, there are some important differences. In the presence of Ca\textsuperscript{2+}, vertebrate Ca\textsuperscript{2+}-dependent proteinase undergoes extensive autolysis. The Ca\textsuperscript{2+}-dependent proteinase in chicken skeletal muscle is completely inactivated in 4 h at 25 °C (40); rabbit skeletal muscle Ca\textsuperscript{2+}-dependent proteinase has a half-life of 8.1 min at 37 °C (41). In contrast, the crustacean Ca\textsuperscript{2+}-dependent proteinase remains active even after incubation at 37 °C for 48 h (16). The enzymes from vertebrates differ from crab Ca\textsuperscript{2+}-dependent proteinase with respect to substrate specificity. Vertebrate muscle Ca\textsuperscript{2+}-dependent proteinase degrades myosin HC, troponin, tropomyosin, and C protein (19). The crab Ca\textsuperscript{2+}-dependent proteinase hydrolyzes actin and myosin LC as well as myosin HC, troponin, tropomyosin, and paramyosin (data described here and in Ref. 16). C protein is absent from arthropod muscle (33).

The differences in substrate specificity may result from different catalytic properties of the proteinases or different susceptibilities of crustacean and vertebrate myofibrillar proteins. A Ca\textsuperscript{2+}-dependent proteinase purified from Ehrlich ascites tumor cells that degrades the intermediate filament proteins vimentin and desmin but not myofibrils, actin, tubulin, azocasein, bovine serum albumin, ovalbumin, fibrinogen, neurofilaments, or histones is an example of the former (42). It appears that Ca\textsuperscript{2+}-dependent proteinases, which occur in various tissues in addition to muscle (19, 20), have specialized functions tailored to a particular cell type.

The many ultrastructural and biochemical similarities between crab claw muscle atrophy and atrophies induced by...
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