Identification of Two Protease Inhibitors from Bovine Cardiac Muscle*

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Lloyd Waxman and Edwin G. Krebs
From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

SUMMARY

Low salt extracts from homogenates of bovine cardiac muscle contain two protease inhibitors, one specific for the calcium-activated protease from this tissue and the other for trypsin and chymotrypsin, but no other serine proteases, including plasmin, thrombin, and subtilisin. The former, which can be separated from the protease by chromatography on DEAE-cellulose, is a protein with a molecular weight of 270,000. Its action is not based on the sequestering of calcium, and it is present in large excess over the amount of calcium-activated protease in this tissue. The trypsin inhibitor, which has a molecular weight of 70,000, is estimated to be present at approximately 300 µg/g, wet weight, of tissue. The identification of inhibitors such as these in the cytoplasm may explain why nonlysosomal proteolytic activity has been thought to be insignificant in the overall turnover of intracellular protein and suggests that a re-evaluation of this possibility is necessary.

A calcium-activated protease from rabbit skeletal muscle was first characterized by Krebs and co-workers (1, 2) and later purified to homogeneity by Dayton et al. (3, 4). This enzyme is a heterodimer of molecular weight 110,000 that requires millimolar calcium for full activity, and it has been shown to degrade only certain components of the contractile apparatus, including tropomyosin, troponins-T and -I, but not troponin-C, actin, or myosin (4, 5); incubation of the enzyme in the presence of myofibrils produces degradation of the Z band and at a slower rate, the M line (4, 5).

In attempting to purify the enzyme from cardiac muscle by procedures similar to those used successfully with skeletal muscle, no calcium-stimulated proteolytic activity toward casein could be detected in crude extracts, and yet, after chromatography on DEAE-cellulose, this activity was easily measured. When the column fractions were then assayed for inhibitory activity, a region was found which, when added back to fractions containing proteolytic activity, did indeed inhibit the calcium-stimulated hydrolysis of casein. A preliminary description of this substance and its resolution from a second protein having inhibitory activity toward trypsin is presented in this communication. The identification of these inhibitors opens new horizons into the possible broad distribution of nonlysosomal proteases and their potential function in initiating cellular proteolysis.

MATERIALS AND METHODS

The assays of proteases and protease inhibitors were conducted as follows. Calcium-activated protease activity was assayed using casein as substrate. The reaction mixture contained the enzyme sample, 5 mg/ml of casein, 50 mM Tris-Cl (pH 7.4), and 3 mM 2-mercaptoethanol in a total final volume of 500 µl. The reaction was initiated by the addition of CaCl2 to a final concentration of 5 mM and allowed to proceed for 20 min at 25°C. The reaction was terminated by the addition of 150 µl of ice cold 10% trichloroacetic acid, the mixture was spun for 10 min at top speed in a clinical centrifuge, and the absorbance of the supernatant was read at 280 nm. Control reactions contained 1 mM EDTA instead of CaCl2. One unit of activity is defined as a change in 0.1 absorbance unit at 280 nm/20 min.

Assay for the inhibitor of the calcium-activated protease was performed by a similar procedure with the exception that the inhibitor samples were incubated with 5 units of partially purified protease (purified to the stage of gel filtration, see Fig. 2) for 15 min at 25°C prior to the addition of casein and calcium. Absorbance changes derived from the digestion of proteins other than casein occurring during either the preincubation or assay were determined from assays performed in the absence of casein; calculations of inhibitor activity were corrected for this value. One unit of inhibitor is defined as that amount of inhibitor which will inhibit one unit of calcium-activated protease by 50% under the conditions of the standard assay. The assay for trypsin inhibitor was performed by the identical procedure with that presented above except that 4 µg of three times crystallized trypsin were used in each assay; trypsin activity was based on casein hydrolysis. The activity and inhibition units were the same as defined for the calcium-activated protease.

The activities of trypsin toward tosylarginine methyl ester and chymotrypsin toward benzoyl tyrosine ethyl ester were carried out as described in the Worthington Enzyme Manual (6). The effect of the trypsin inhibitor on the esterase activity of these enzymes was determined by preincubating varying amounts of inhibitor with 5 µg of protease for 15 min at 25°C in a total volume of 200 µl and at the end of which time, 100 µl was removed for the assay.

RESULTS

Skeletal muscle contains an active calcium-stimulated protease that is readily detected from assays of unfractionated muscle extracts (2, 3, 5). If cardiac muscle was extracted under similar conditions to those used for skeletal muscle, no calcium-sensitive protease, based on casein hydrolysis, could be detected. The chromatography on DEAE-cellulose of a low ionic strength extract of bovine cardiac muscle, however, revealed one peak of calcium-activated protease activity which eluted at 0.25 M NaCl (Fig. 1), in agreement with the elution position reported for the skeletal muscle enzyme by Dayton et al. (3) and Reddy et al. (5). When the active fractions were pooled, concentrated by addition of ammonium sulfate to 60% saturation, and chromatographed on Sephadex G-150, the activity eluted in a single peak that corresponded to a molecular weight of 110,000 (Fig. 2A), also in agreement with that found for the skeletal muscle enzyme (2, 3). The protease exhibited a requirement for calcium of nearly 1 mM; this value was observed with both casein (7) and reduced and carboxymethylated, maleylated lysozyme (8, 9) as the substrates. The enzyme was not activated by magnesium at any
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level tested or by a combination of magnesium with less than 1 mM calcium. Moreover, lanthanum, which has the same ionic radius as calcium (10), activated the protease only 20% as well as calcium at 1 mM. The pH optimum of the cardiac calcium-activated enzyme measured with either casein or lysozyme as substrate, was between pH 7.0 and 8.0. This suggests that the origin of the enzyme is probably nonskeletal. Recently, Revelle et al. (11) have shown this explicitly by cell fractionation studies with skeletal muscle.

Because the protease could not be detected in the crude extract, but only after ion exchange chromatography, the fractions from this latter step were tested for their ability to inhibit the calcium-activated protease. As indicated in Fig. 1, a peak of inhibitory activity was detected which eluted at a lower ionic strength than the protease (75 mM NaCl). These fractions from the ion exchange chromatography also inhibited trypsin-catalyzed proteolysis (Fig. 1). The fractions were pooled, concentrated by precipitation upon addition of ammonium sulfate to 60% saturation and chromatographed on Sepharose 6B (Fig. 2B). This procedure separated the inhibitor of the calcium-activated protease from the trypsin inhibitor; the apparent molecular weights of the two inhibitors were 260,000 to 270,000 and 70,000, respectively, and both had been 30- to 50-fold purified over the crude extract at this stage. Both inhibitors were shown to be proteins by their sensitivity to proteolytic, but not other hydrolytic enzymes (Table I) and the fact that their activities are diminished at elevated temperatures (>50°C).

Both inhibitors completely block the activity of their respective proteases (Fig. 3), and no inhibitory activity which had the same chromatographic properties as the two proteins described here could be detected in erythrocyte lysates or plasma. The trypsin inhibitor also blocks the caseinolytic activity of chymotrypsin but has no effect on other serine proteases, and from Lineweaver-Burk plots (12), it is shown to be a competitive inhibitor of the esterase activities of these two enzymes (Fig. 4). The calcium-activated protease inhibitor is highly specific and has no effect on any other protease which has been tested, including trypsin, chymotrypsin, subtilisin, papain, thermolysin, plasmin, thrombin, ficin, or bromelain. Its mechanism of inhibition is yet not known because of the difficulties in applying the usual graphical methods of analysis to tightly binding inhibitors (13, 14).

A titration similar to that presented in Fig. 3A was performed in order to calculate the relative amounts of inhibitor and protease in heart muscle. The activities were separated on DEAE cellulose and the respective peaks were pooled and dialyzed to remove low molecular weight ultraviolet-absorbing substances which do not precipitate with trichloroacetic acid. It was then possible to titrate protease activity with increasing amounts of inhibitor as described above. From these data, it was shown that there is approximately 10 times as much inhibitor present as there is protease in the low salt extract. From a similar type of experiment, it was estimated that cardiac muscle contains approximately 240 units of trypsin inhibitor/g, wet weight, of tissue. If it is assumed that, as for many proteolytic inhibitors (15), a simple stoichiometry exists between the proteolytic enzyme and its inhibitor, then it can be calculated that cardiac muscle contains approximately 300 μg of trypsin inhibitor/g, wet weight, of tissue.

**DISCUSSION**

A calcium-dependent factor that was capable of activating phosphorylase b kinase was first described by Meyer et al. in 1964 (1). Drummond and Duncan (16) observed a similar factor in the isoelectric precipitate (pH 5.1) of cardiac muscle extract on DEAE-cellulose. One hundred grams of fresh bovine heart, trimmed of fat and connective tissue, was ground in a meat grinder. A 10-g portion was then homogenized for 1 min at high speed in a Waring Blender in a buffer consisting of neutralized 4 mM EDTA, 50 mM NaCl, 3 mM 2-mercaptoethanol, and 5 mM TrisCl, ml/g of pH 7.4 (2.5 tissue), and the homogenate was centrifuged for 40 min at 27,000 x g in the SS-34 rotor of a Sorvall centrifuge. The supernatant was decanted and the pH was adjusted to 7.4 with 1 M NaOH and the conductivity lowered to 1 mho by the addition of ice cold deionized water. All operations were carried out at 4°C. The extract was then applied to a column of DEAE-cellulose (1.5 x 25 cm) equilibrated in 0.1 mM EDTA, 10 mM NaCl, 3 mM 2-mercaptoethanol, and 5 mM Tris-Cl, pH 7.4, and the column was washed with 10 volumes of this buffer following application of the sample. A linear gradient was run, 150 ml on each side, from 10 to 500 mM NaCl; each fraction contained 2.5 ml. Aliquots (150 ml) were taken to assay for calcium-activated protease activity, and 50 μl for inhibitory activity, as described under "Materials and Methods."
extracts. The supernatant obtained from this procedure contained an inhibitory substance which could prevent the activation of phosphorylase b kinase. At this time, however, it was not clear that the calcium-dependent factor was a protease because it inactivated itself very rapidly and appeared not to be working as an enzyme, but possibly stoichiometrically (1, 16). Subsequently, Huston and Krebs (2) identified the skeletal muscle factor as a protease and purified it to near homogeneity. Dayton et al. (3) then purified the enzyme to homogeneity from porcine skeletal muscle. At the present time, there is evidence for the existence of a similar protease in brain (17, X3), platelets (19), and calf uterus (20), which suggests that this factor may be found in many tissues.

Our interest in the control of protein degradation and the possible function of nonlysosomal proteases in this process has led to a re-examination of the properties of the calcium-activated protease. From the data presented here, the heart enzyme, which is presumably identical with the calcium-dependent phosphorylase kinase activation factor of Drummond and Duncan (16), is similar in size (110,000 daltons), calcium-dependent phosphorylase kinase activation factor of Drummond and Duncan (16), is similar in size (110,000 daltons), calcium

TABLE I
Properties of the cardiac muscle protease inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca²⁺-activated protease inhibitor</th>
<th>Trypsin inhibitor</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>40</td>
<td>63</td>
</tr>
<tr>
<td>RNase</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>DNase</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
<td>5 min, 50°C</td>
<td>48</td>
<td>68</td>
</tr>
<tr>
<td>10 min, 100°C</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>Papain</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pepsin</td>
<td>6</td>
<td></td>
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Fig. 2. Gel filtration profiles of the calcium-activated protease, its inhibitor, and the trypsin inhibitor. A, the fractions which contained calcium-activated protease activity after DEAE-cellulose chromatography were pooled and concentrated by precipitation with solid ammonium sulfate to 60%, and the redissolved pellet was applied to a column of Sepharose 6B (2.5 x 95 cm). The elution buffer consisted of 0.1 mM EDTA, 50 mM NaCl, 3 mM 2-mercaptoethanol, and 5 mM Tris-Cl, pH 7.4; 2.5-ml fractions were collected. Inset, molecular weight calibration curve for this column. A denotes the position of the calcium-activated protease; B, that of the trypsin inhibitor. The standards and their molecular weights were: 1, catalase (228,000); 2, aldolase (160,000); 3, lactate dehydrogenase (148,000); 4, glyceraldehyde-3-phosphate dehydrogenase (144,000); 5, malate dehydrogenase (70,000); 6, bovine serum albumin (66,000); 7, ovalbumin (45,000); 8, cAMP-dependent protein kinase catalytic subunit (40,000); 9, myoglobin (17,000). B, the fractions which contained both protease inhibitors were pooled after DEAE-cellulose chromatography and treated with solid ammonium sulfate to 60%. The redissolved pellet was applied to a column of Sepharose 6B (2.5 x 90 cm) as described above. Inset, molecular weight calibration curve for this column. Only the molecular weight of the calcium-activated protease inhibitor was determined on this column (denoted by the arrow). The standards used and their molecular weights were: 1, β-galactosidase (540,000); 2, pyruvate kinase (240,000); 3, catalase (228,000); 4, aldolase (190,000); 5, lactate dehydrogenase (148,000). The fractions which contained trypsin inhibitory activity were rechromatographed on Sephadex G-150 (shown in the inset to A) in order to obtain the molecular weight.

Fig. 3 (left). Titration of proteolytic activity with partially purified inhibitors. A, increasing amounts of calcium-activated protease inhibitor purified through the gel filtration step were added to 2.5 units of calcium-activated protease, and the mixtures were assayed as described under "Materials and Methods." B, increasing amounts of trypsin inhibitor which had been purified through the DE52 step were added to tubes containing 4 μg of trypsin (●) or 4 μg of chymotrypsin (○) and assayed as described under "Materials and Methods." Fig. 4 (right). Lineweaver-Burk plot for the inhibition of chymotrypsin by the partially purified trypsin inhibitor. Reciprocal plot for the inhibition of benzoyl tyrosine ethyl ester hydrolysis by chymotrypsin (2.4 μg) with increasing amounts of the trypsin-specific inhibitor which had been purified through gel filtration. The assay was conducted as described under "Materials and Methods."
requirement (1 mM), and pH optimum (7.4) to the purified skeletal muscle calcium-activated protease (3, 4). The inhibitor of the calcium-dependent protease described in this study is also presumably a component of the “inhibitory factor” of Drummond and Duncan (16); the latter contains, in addition, the trypsin inhibitor also identified in this present study.

The inhibitor is unusually large, having a molecular weight of 270,000 and from the evidence presented here, it interacts directly with the protease rather than by chelating calcium since no level of calcium up to 25 mM can reverse the inhibition. Although the inhibitor seems to be present in large excess over the protease, it is not at all clear what governs their interaction or how DEAE-cellulose promotes their dissociation. It is quite possible that a large fraction of the protease is still bound to the myofibrillar fraction, although re-extraction with 0.1 NaCl or 0.1% Triton X-100 does not alter the relative amounts of protease and inhibitor.

Although there have been several reports of the existence of nonlysosomal proteases and peptidases in muscle (21-26), most discussions of the site and control of intracellular protein degradation have implicated the lysosome because of its high concentration of protein hydrolases and the apparent absence of significant proteolytic activity in other cell fractions. It is quite possible, however, that a number of cellular proteases have gone undetected because of the presence of inhibitory substances such as those described here. This suggestion is attractive because it would help to explain the marked heterogeneity in turnover rates of different cell proteins (27), an aspect of protein degradation which cannot be satisfactorily reconciled with the nondiscriminating feature of lysosomal digestion.

On the other hand, the cytoplasmic proteases which are associated with the inhibitors that have been reported here may actually be involved in processing one or more of the cell’s proteins before it can assume its proper function after synthesis. The role of the inhibitors in controlling this kind of process is equally intriguing and both possibilities are currently under investigation.

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REFERENCES
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L Waxman and E G Krebs


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