Limited Autolysis Reduces the Ca\(^{2+}\) Requirement of a Smooth Muscle Ca\(^{2+}\)-activated Protease*

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Chicken gizzard smooth muscle contains large amounts of Ca\(^{2+}\)-activated protease activity. Approximately 15 mg of purified enzyme can be obtained from 1 kg of fresh muscle. The enzyme consists of two subunits (Mr = 80,000 and 30,000) present in a 1:1 molar ratio. In the presence of CaCl\(_2\), the 80,000/30,000-dalton heterodimer (form I) is rapidly converted by limited autolysis to a 76,000/18,000-dalton species (form II). Both the 80,000- and 30,000-dalton subunits are degraded simultaneously. Moreover, the Ca\(^{2+}\) dependence for autolysis (K\(_{a50}\) = 300 \(\mu\)M) is identical for both subunits. Neither the time course nor the Ca\(^{2+}\) dependence of the autolytic conversion reaction is altered by 10- and 20-fold molar excesses of substrate. Limited autolysis markedly reduces the Ca\(^{2+}\) requirement for substrate degradation. Using N-[ethyl-\(^{2-}\)H]maleimide-labeled 27,000-dalton cardiac myosin light chains as substrate, the Ca\(^{2+}\) requirement of form I was found to be quite high (K\(_{a50}\) = 150 \(\mu\)M). Under similar conditions, the Ca\(^{2+}\) requirement of form II was 30-fold lower (K\(_{a50}\) = 5 \(\mu\)M). Limited autolysis did not alter the specific activity of the enzyme. Our results demonstrate that smooth muscle contains an abundant amount of Ca\(^{2+}\)-activated protease. Moreover, autolysis of this enzyme may play an important regulatory role by converting the native form to a species that is fully active at physiological levels of intracellular calcium ion.

Ca\(^{2+}\)-requiring proteases have been isolated from several tissue sources (1-8). Most of these enzymes appear to have a neutral pH optimum and are potently inhibited by reagents that react with sulfhydryl groups (1-8). Until recently, there was much skepticism about any important role for these enzymes in normal cell function because of the excessively high concentrations of Ca\(^{2+}\) required for activation, generally in the millimolar range. However, several recent reports have described forms which are fully active at micromolar concentrations of Ca\(^{2+}\) (9-12). The relationship between the high and low Ca\(^{2+}\)-requiring enzymes has not been established with certainty. At least one group has purified these two forms from skeletal muscle and shown that while both are heterodimers consisting of 80,000- and 30,000-dalton subunits, they differ only in Ca\(^{2+}\) requirement and elution profile from DEAE-cellulose (12). Most recently, Imahori and colleagues have proposed that a Ca\(^{2+}\)-activated neutral protease isolated from chicken skeletal muscle (Mr = 80,000) can be converted by limited autolysis from a form that requires millimolar levels of Ca\(^{2+}\) to one that is sensitive to Ca\(^{2+}\) in the micromolar range (10, 11).

Recently, we have purified a Ca\(^{2+}\)-activated protease from smooth muscle. Large amounts of this enzyme can be prepared from either chicken gizzard or bovine aortic smooth muscle. Like the protease originally purified from skeletal muscle by Dayton et al. (2), the native form of the smooth muscle enzyme is a heterodimer consisting of 80,000- and 30,000-dalton subunits bound with a molar ratio of 1:1. In this study we report the purification of the Ca\(^{2+}\)-activated protease from chicken gizzard smooth muscle. In addition, we demonstrate that the native enzyme can be converted by limited autolysis from a high to a low Ca\(^{2+}\)-requiring form.

**EXPERIMENTAL PROCEDURES**

**Materials**

All chemical reagents were purchased from Sigma, as were the chromatographic media, DEAE-Sepharose, and Reactive Red-120 agaroose. Sephacryl S300 was obtained from Pharmacia Fine Chemicals.

**Methods**

**Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 7.5% slab gels according to the procedure of Fnorr and Pearson (13) with modifications to improve gel stability (14). Nondenaturing gel electrophoresis was performed in 5% tube gels at 4°C using a Fairbanks buffer system as described previously (15). Electrophoresis grade reagents were obtained from Bio-Rad. Tube gels stained with Coomassie blue were scanned with an ISCO model 1310 gel scanner and slab gels in a Beckman DU-8 spectrophotometer using the gel-scanning accessory and peak integrator.

**Characterization Studies**—Isoelectric focusing was performed in the horizontal mode using the LKB Multiphor as previously described (16). Gels contained 2% Ampholines, pH 3-10 (Pharmacia), and 10% glycerol. Approximately 30 \(\mu\)g of enzyme was applied to the surface of the gel, and focusing was conducted at 4°C with 10 watts, constant power, for 6 h. Sedimentation coefficients were determined by sedimentation of 50 \(\mu\)g of purified enzyme in 5-30% glycerol gradients as previously described (17). Markers included 100 \(\mu\)g each of catalase (113 S), alcohol dehydrogenase (7.4 S), and bovine serum albumin (4.35 S). Determinations of Stokes radius were obtained by gel filtration in a Sephacryl S200 column (0.9 × 60 cm) equilibrated with 20 mM MOPS, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 M NaCl. Markers included 1 mg each of catalase (5.2 nm), aldolase (4.2 nm), bovine serum albumin (3.5 nm), ovalbumin (2.5 nm), and cytochrome c (1.4 nm). The sedimentation coefficient and Stokes radius were used to calculate molecular weight and frictional ratio according to the procedures described by Siegel and Monty (18).

**Protease Assays**—Ca\(^{2+}\)-activated protease activity was determined by using N-[ethyl-\(^{2-}\)H]maleimide-labeled 27,000-dalton bovine

1. The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
myosin light chains as substrate. The light chains were partially purified from cardiac myosin by a modification of the method of Pireas and Perry (19). The 20,000- and 27,000-dalton light chains were separated by covalent chromatography on p-hydroxymercuribenzoate agarose (20). The 27,000-dalton light chains eluted from the column with 0.1 M cysteine were dialyzed free of thiol and labeled stoichiometrically with N-[ethyl-2-3H]maleimide (3 mol of ~SH per mol of light chain) in the presence of 6 M urea. After treatment with 5 mM dithiothreitol and dialysis to remove all reactants, approximately 10 μM light chains were added to a typical incubation mixture consisting of 50 mM MOPS, pH 7.0, 2 mM dithiothreitol, 2 mM EGTA, and various concentrations of CaCl₂ in a total volume of 50 μl. Assays were conducted under linear reaction conditions at 25 °C and were terminated at 30 s by the sequential addition of 10 μl of bovine serum albumin (10 mg/ml) in 50 mM EGTA and then 100 μl of 20% trichloroacetic acid. Following sedimentation at 10,000 × g for 15 min, a 100-μl aliquot of the supernatant was counted in a Beckman Beta liquid scintillation counter. Activity is expressed as nanomoles of N-ethylmaleimide-peptide(s) released per min per mg of protease.

RESULTS

Enzyme Purification—Ca²⁺-activated protease was typically prepared from 1 kg (trimmed weight) of fresh chicken gizzards. Gizzards were trimmed to remove mucosal and fibrous tissue and homogenized in a Waring blender in 3 volumes of 40 mM MOPS, pH 7.2, 2 mM EGTA, 1 mM EDTA, and 1 mM dithiothreitol. The homogenate was sedimented at 10,000 × g for 30 min and then subjected to ammonium sulfate fractionation. The precipitate obtained between 30 to 60% saturation was collected by sedimentation and resuspended in 20 mM MOPS, pH 7.0, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol (Buffer A). This material was dialyzed for 24 h against 15 liters of Buffer A and then applied to a column (5 × 18 cm) of DEAE-Sephacel equilibrated in the same buffer. A single peak of activity was resolved from this step (data not shown), and the active fractions were pooled (about 200 ml), concentrated to 100 ml (Amicon ultrafiltration cell, PM-10 filter), and applied to a column (5 × 100 cm) of Sephacryl S300 equilibrated in Buffer A plus 0.5 M NaCl. Gel filtration also resolved a single peak of protease activity (data not shown). The active fractions from this step were pooled (100 ml) and applied to a column (1.5 × 20 cm) of Reactive Red-120 agarose equilibrated with Buffer A. As shown in Fig. 1, the entire sample was loaded onto the column and then washed with approximately 150 ml of Buffer A plus 0.5 M NaCl. All protease activity was adsorbed to the column. Specific elution was achieved by washing the Reactive Red-120 agarose next with Buffer A alone. A sharp peak of protein with some tailing was eluted, and approximately 80% of the protease activity applied to the column could be recovered. All fractions containing protease activity were pooled (180 ml), concentrated (Amicon ultrafiltration cell, PM-10 filter) to approximately 10 ml, and then dialyzed against Buffer A. This concentrated protease served as the source of enzyme for all subsequent studies described in this report.

Table I summarizes the purification results. Approximately 15 mg of purified enzyme was obtained from this representative preparation. The protease was purified approximately 1429-fold. The true yield of activity could not be determined, since the initial extract had no detectable protease activity. This was due to the presence of an uncharacterized inhibitor(s). Similar difficulties in assaying for Ca²⁺-activated protease activity in crude muscle extracts have been described by other investigators (22).

The purified enzyme is shown in Fig. 2. Two components were present: an 80,000-dalton subunit and a 30,000-dalton subunit. Densitometric scanning of the gel shown in Fig. 2 gave a ratio of peak area of 2:7:1 (80,000 to 30,000) yielding a molecular ratio of approximately 1:1.

Effects of Autolysis on Subunit Composition—The protease was found to undergo autolysis in the presence of Ca²⁺. In preliminary experiments with the enzyme it was found that the rate of autolysis and the kinds of fragments produced were strongly affected both by the Ca²⁺ concentration and the temperature at which experiments were conducted. It became apparent, however, that certain discreet intermediate autolytic products could be identified. As shown in Fig. 3, both the 80,000- and 30,000-dalton subunits of the protease were degraded. Limited proteolysis of the 80,000-dalton subunit yielded a 76,000-dalton polypeptide, while the 30,000-dalton subunit was converted to a peptide with a molecular weight of 18,000. To distinguish between the precursor and its autolytic product, we have referred to them as form I (i.e. the native enzyme consisting of 80,000- and 30,000-dalton subunits) and form II (i.e. the autolytic product consisting of 76,000- and 18,000-dalton subunits). Prolonged incubation of the protease in the presence of CaCl₂ resulted in the generation of other fragments and ultimately in loss of enzyme activity.

![Figure 1](image1.png)

**Figure 1.** Chromatography of chicken gizzard Ca²⁺-activated protease on Reactive Red-120 agarose. Pooled fractions containing protease activity obtained from the Sephacryl S300 column (volume, 150 ml; protein concentration, 0.2 mg/ml) were applied to a column (1.5 × 20 cm) of Reactive Red-120 agarose equilibrated in Buffer A. At the point designated by A, the column was washed with about 150 ml of Buffer A containing 0.5 M NaCl. At point B, elution was initiated with Buffer A containing no NaCl. Protease activity was determined by incubation of 3 μl from each fraction with 10 μM substrate for 30 s at 25 °C as described under "Methods." Protease activity.

| Step | Protein* | Activity† | Purification |
|------|----------|-----------|--------------|
| Extract | 42,600 | 12,400 | 0.14 |
| Ammonium sulfate precipitate | 12,400 | 37 | 1.96 |
| DEAE-Sephacel | 436 | 5.2 | 37 |
| Sephacryl S300 | 84 | 27.5 | 1.96 |
| Reactive Red-120 agarose | 15 | 200 | 1.429 |

*Activity was measured as nanomoles of N-[ethyl-2-3H]maleimide-labeled peptides released from purified 27,000-dalton cardiac myosin light chains under standard assay conditions as described under "Methods."

†For the purified protease K₉₀ = 10.1.
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conversion of form I to form II was determined by Lineweaver-Burk analysis to be approximately 300 \(\mu\)M.

To determine if the presence of substrate could alter either the rate or Ca\(^{2+}\) dependence of the conversion of form I to form II, 0-, 10-, and 20-fold molar excesses of purified 27,000-dalton cardiac myosin light chains were incubated with the protease, and autolysis was monitored as described in Figs. 4 and 5. As summarized in Table II, neither the rate nor Ca\(^{2+}\) dependence of the conversion was altered by the presence of these concentrations of substrate.

Characterization of Forms I and II—Physical data obtained on the two forms of the Ca\(^{2+}\)-activated protease suggest that both are asymmetric in shape (i.e. ellipsoid) and that

Since it was noted that degradation of both the 80,000- and 30,000-dalton subunits occurred very rapidly at 25 \(^\circ\)C, a time course was conducted on ice. Fig. 4 summarizes results of this study. Even at 0 \(^\circ\)C, limited autolysis of the 80,000- and 30,000-dalton subunits was rapid at high concentrations of CaCl\(_2\) (1 mM) and, moreover, degradation of both occurred simultaneously. Identical results were obtained over a protein concentration range of 0.05 to 2.5 mg/ml (approximately 0.5 to 23 \(\mu\)M enzyme). While the rate of autolysis was reduced at lower concentrations of Ca\(^{2+}\) (i.e. 0.5 mM), as shown in Fig. 5 the 80,000- and 30,000-dalton subunits were degraded to the same extent at corresponding concentrations of Ca\(^{2+}\). The concentration of Ca\(^{2+}\) necessary for half-maximal autolytic

![Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified chicken gizzard Ca\(^{2+}\)-activated protease. Approximately 8 \(\mu\)g of purified enzyme was applied to a 7.5% polyacrylamide slab gel as described under "Methods."](image)

![Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified Ca\(^{2+}\)-activated protease from chicken gizzard smooth muscle (I) and the product resulting from limited autolysis (II). Purified enzyme (0.4 mg/ml) was incubated for 60 s at 0 \(^\circ\)C in the presence of a buffer containing 20 mM MOPS, pH 7.0, and 1 mM dithiothreitol. The reaction was begun by the addition of CaCl\(_2\) to a final concentration of 0.5 mM. Aliquots containing 5 \(\mu\)g of protein were applied to the gels as described under "Methods." From left to right: I, enzyme prior to the addition of CaCl\(_2\); middle lane, a sample taken at 20 s; II, a sample obtained at 60 s.](image)

![Fig. 4. Time course of autolysis. Purified enzyme (0.4 mg/ml) was incubated at 0 \(^\circ\)C under conditions similar to those described in Fig. 3 except that the CaCl\(_2\) concentration was 1 mM. Aliquots of the enzyme (5 \(\mu\)g) were obtained at the indicated time points, and autolysis was stopped by adding the aliquot directly to 10 \(\mu\)l of a sodium dodecyl sulfate-containing buffer (13). Following electrophoresis, gels were stained with Coomassie blue, and lanes were scanned for protein bands. The percentage of conversion (80,000 to 76,000 daltons and 30,000 to 18,000 daltons) was calculated from the relative peak ratios obtained by densitometric scans of the gels using the peak integrator of the Beckman DU-8 spectrophotometer. ○, 80,000- to 76,000-dalton conversion; O--O, 30,000- to 18,000-dalton conversion.](image)

![Fig. 5. Calcium dependence of autolysis. Buffer conditions were similar to those described in Fig. 3 except that several different calcium concentrations were used. The enzyme concentration was 0.4 mg/ml, and the temperature was 25 \(^\circ\)C. Assays were terminated at 15 s in the manner described in Fig. 4. The percentage of conversion was determined by densitometric scanning as described in Fig. 4. ○, 80,000- to 76,000-dalton conversion; O--O, 30,000- to 18,000-dalton conversion.](image)
both are simple heterodimers. The sedimentation coefficient of form II was found to be smaller than that of form I (Table III), while the Stokes radii of both were identical. As indicated by the increase in frictional ratio of form II (f/f₀), autolysis enhanced asymmetric shape. The calculated molecular weights for both forms were in good agreement with molecular weights obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Table IV). Moreover, the calculated mo-

**TABLE II**

Effect of substrate on the time course and Ca²⁺-dependence of autolysis

For both the time course and Ca²⁺-dependence studies, the protease concentration was 1 μM, and autolysis was monitored and quantitated by gel electrophoretic methods as described in Figs. 4 and 5. The calcium concentration required for half-maximal autolytic conversion (K₀, Ca²⁺) was determined at 0, 10, and 20 μM substrate concentrations as described in Fig. 5. The time course of autolytic conversion at 0 °C in the presence of 0.5 mM CaCl₂ was followed at 0, 10, and 20 μM substrate concentration and the time required for 50% conversion (t₁/₂) determined from densitometric scans as described in Fig. 4. No difference was noted between the 80,000- and 30,000-dalton subunits, and thus only a single value for K₀, Ca²⁺, and t₁/₂ is given in the table.

| Substrate concentration | 0 | 10 | 20 |
|-------------------------|---|----|----|
| μM                      |   |    |    |
| K₀, Ca²⁺ (M)            | 5.4 | 5.6 |
| t₁/₂ (seconds)          | 20 | 21 | 22 |

The substrate was purified 27,000-dalton cardiac myosin light chains.

K₀, Ca²⁺ = calcium concentration required for half-maximal conversion.

t₁/₂ = time required for 50% conversion.

**TABLE III**

Properties of forms I and II

| Form I                  | Form II                |
|-------------------------|------------------------|
| s₂₀,W (g/ml)            | 6.4                    |
| Stokes radius           | 4.0                    |
| Molecular weight (*)    | 110,000 ± 3,500 94,000 ± 3,200 |
| Calculated molecular weight (S) | 108,000 92,400 |
| f₀/f₀                  | 1.25                   |
| pH optimum              | 6.9                    |
| pI                      | 4.9                    |

* Determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Value presented represents the mean of four separate determinations ± S.E.

**TABLE IV**

Molar ratios of the subunits for forms I and II of the Ca²⁺-activated protease.

Approximately 5 μg of purified enzyme obtained from each separation method was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The areas under each subunit determined by densitometric scanning were expressed as a peak ratio (80,000/30,000 for form I and 76,000/18,000 for form II). Molar ratios were determined by correction of peak ratios for differences in subunit molecular weight.

| Form I                  | Form II                |
|-------------------------|------------------------|
| Peak ratio Molar ratio  | 2.7:1 1:1              |
| Peak ratio Molar ratio  | 4.1 1:1                |

Ion exchange chromatography (DEAE-Sepharose)

Gel filtration (Sephacryl S2000)

Glycerol gradient sedimentation

Nondenaturing gel electrophoresis

Isoelectric focusing

FIG. 6. Nondenaturating polyacrylamide gel electrophoresis of the Ca²⁺-activated protease. Approximately 50 μg of the native protease (form I) and its autolytic product (form II) were applied to 5% polyacrylamide tube gels and subjected to electrophoresis for 6 h at 4 °C as described under “Methods.” Some gels were sliced into 2-mm segments, and each segment was eluted overnight into 200 μl of Buffer A containing 0.15 M NaCl. Each eluate (5-μl aliquot) was assayed for 1 min for protease activity as described under “Methods.” Other gels were stained with Coomassie blue (15) and scanned at 660 nm. ● ● ●, protease activity; ○ ○ ○, Aαβ, A, form I, B, form II; NEM, N-ethylmaleimide.

FIG. 7. Polyacrylamide gels of the two forms of Ca²⁺-activated protease. A, nondenaturating polyacrylamide tube gels stained with Coomassie blue as described in Fig. 6. a, form I; b, form II. B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protease obtained from the nondenaturin gels shown in A. a, form I; b, form II.
Despite its larger size, form I was found to migrate slightly faster than form II, possibly as a result of its more acidic composition (Table III). Fig. 7 shows the nondenaturing gels stained with Coomassie blue (Panel A) and also sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples obtained from the nondenaturing gels (Panel B). The molar ratio of the subunits remained constant regardless of the separation methods to which forms I and II were subjected, including nondenaturing gel electrophoresis, as shown in Table IV. Moreover, the 76,000- and 18,000-dalton subunits of form I were readily cross-linked in the presence of 20 mM dimethylsuberimidate at 25 °C. Both the 76,000- and 18,000-dalton subunits disappeared coinciding with the appearance of a new protein band, $M_s = 94,000$ (Fig. 8). Similar results were obtained with form I, except that the extent of cross-linking was only 15–35%.

The Ca$^{2+}$ requirement for substrate degradation for both forms of the protease was determined as shown in Fig. 9. Form I showed a rather broad activation curve with half-maximal activity measured at 150 μM calcium ion concentration. In contrast, form II was fully active at 10 μM calcium ion, and the estimated calcium ion concentration required for half-maximal activation was 5 μM. When these data were analyzed in accordance with the Hill equation (23), a coefficient of 2.3 was obtained for form I, while a coefficient of 5.75 was determined for form II (Fig. 10). The Hill coefficient for form I is difficult to interpret in view of the likelihood of two events occurring simultaneously: autolysis and substrate degradation. On the other hand, the coefficient of 5.75 for form II suggests the possibility of multiple calcium ion-binding sites exhibiting positive cooperativity.

**DISCUSSION**

The role of Ca$^{2+}$-activated proteases in cell function is uncertain. The protease purified from rabbit skeletal muscle may be important in dissolution of myofibrils by degrading proteins of the Z line (24–27). Of particular concern is how the various enzymes purified to date can be active at physiological intracellular concentrations of Ca$^{2+}$, since most seem to require millimolar levels of Ca$^{2+}$ for full activity (1–8).

Several recent studies have reported the presence of Ca$^{2+}$-activated protease activities which are active at micromolar levels of Ca$^{2+}$ (9–12). In one study two forms with identical subunit composition were purified and found to require either high or low concentrations of Ca$^{2+}$ for activity (12). Most recently, Imahori and colleagues, working with an apparently unique form of the Ca$^{2+}$-activated protease from chicken skeletal muscle, have reported that limited proteolysis converts a high Ca$^{2+}$-requiring enzyme to a low calcium-requiring form (10, 11). Their enzyme, which consists of a single 80,000-dalton polypeptide that requires Ca$^{2+}$ in the millimolar range for activity, was converted to a 76,000-polypeptide that was active at micromolar levels of Ca$^{2+}$ (10, 11).

Our results are similar to those of Imahori and colleagues.
Some important differences are notable. Firstly, the chicken gizzard smooth muscle Ca\(^{2+}\)-activated protease, like that from cardiac muscle (7), skeletal muscle (2), and platelets (8) is a heterodimer. As detailed in this report, the 80,000- and 30,000-dalton subunits maintain in a 1:1 molar ratio through a variety of separation methods. In addition, the molecular weight of the holoenzyme calculated from the Stokes radius and sedimentation coefficient (i.e. 108,000) is consistent with the subunit composition proposed. Because the chicken gizzard protease is a heterodimer and both subunits were autolytically degraded simultaneously and at comparable Ca\(^{2+}\) concentrations, it is not clear whether limited proteolysis of only one of the subunits or both is prerequisite for the change in Ca\(^{2+}\) requirement for substrate degradation. A second major difference is the Ca\(^{2+}\) requirement for substrate degradation. In general, both forms I and II of the chicken gizzard enzyme require lower levels of Ca\(^{2+}\) for activity than do the high and low Ca\(^{2+}\)-requiring forms of the chicken skeletal muscle protease described by Imahori and colleagues (10, 11). For example, while the low Ca\(^{2+}\)-requiring skeletal muscle enzyme is half-maximally active at a calcium ion concentration of 40-50 \(\mu\)M, form II of the gizzard enzyme is fully active at this concentration and half-maximally active at 5 \(\mu\)M. This disparity may result from the intrinsic difference in subunit composition between the chicken skeletal muscle and smooth muscle enzymes. On the other hand, it may also reflect differences in assay methods or in calculation of calcium ion concentration.

Neither the calcium ion dependence nor the time course of autolysis was altered by the presence of 10- and 20-fold molar excess of substrate. Moreover, the same parameters were unaffected over a 50-fold range in protease concentration incubated in the absence of substrate. Recently, Mellgren et al. have shown that autolytic inactivation of the rabbit skeletal muscle Ca\(^{2+}\)-activated protease is unaffected by a 20,000-fold molar excess of substrate (28). Collectively, these results suggest that autolysis of the Ca\(^{2+}\)-activated protease may result from an intramolecular process.

Form II of the smooth muscle Ca\(^{2+}\)-activated protease like the precursor, form I, is a heterodimer. The 75,000 and 18,000 subunits maintain a 1:1 molar ratio through a variety of separation steps (Table IV) and can be cross-linked in the presence of dimethylsuberimidate. The low Ca\(^{2+}\) requirement of this form of the protease suggests that it could be the active species at physiological concentrations of intracellular Ca\(^{2+}\). At present, however, we have not identified form II occurring spontaneously in smooth muscle. In addition, while form II is quite sensitive to low concentrations of Ca\(^{2+}\), the conversion process (i.e. I \(\rightarrow\) II) requires relatively high levels of Ca\(^{2+}\) (\(K_{d,50} = 300 \mu\)M). Thus, if Ca\(^{2+}\)-dependent autolysis is an important step which regulates the amount of micromolar Ca\(^{2+}\)-sensitive protease present in smooth muscle, the autolytic process must occur in a localized region of the cell where transient calcium ion concentrations are high. On the other hand, our findings do not exclude the possibilities that other factors may alter the Ca\(^{2+}\) dependence of autolysis or that other proteases within the cell may catalyze the conversion.

It seems likely that the Ca\(^{2+}\)-activated protease plays an important role in smooth muscle function. A large amount of this enzyme is present in both chicken, gizzard and bovine aorta (29). The yield of protease from 1 kg of chicken gizzard smooth muscle is 3 to 20 times greater than from comparable amounts of cardiac (7) and skeletal (2, 4, 5) muscles. This difference may be due to the variety of purification methods used to obtain the enzyme, differences in the amount or kinds of endogenous inhibitors present, or intrinsic content of the protease in different types of muscle. In any case, the precise role of the Ca\(^{2+}\)-activated protease in smooth muscle function is not yet established. Most recently, we have found that when added to a contracted chemically skinned strip of smooth muscle, form II of the protease causes immediate and permanent loss of tension (30). This implies a potential role for the protease in smooth muscle contractile or cytoskeletal protein degradation.

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