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Use of an Active-Site Inhibitor of Stromelysin to Elucidate the Mechanism of Prostromelysin Activation

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A COOH-terminally truncated recombinant form of prostromelysin-1 (MMP-3; EC 3.4.27.17) was activated by incubation at elevated temperature or by the addition of aminophenylmercuric acetate (APMA). By using an inhibitor of mature stromelysin to trap intermediates, it was found that the two methods of activation occurred by different mechanisms. Heat activation was achieved by a single-step bimolecular cleavage which was dependent on the presence of a small amount of mature enzyme. In contrast, APMA activation occurred by a complex multistep mechanism which consisted of intramolecular cleavages within the NH_2-terminal pro portion of the molecule followed by a bimolecular cleavage at the NH_2-terminus of the mature stromelysin. In spite of the different mechanisms of activation, both methods generate indistinguishable active enzymes. © 1995 Academic Press, Inc.

INTRODUCTION

Stromelysin-1 (MMP-3) is a neutral metalloproteinase which has been postulated to play a pivotal role in the pathologic destruction of cartilage. This is based in part on the fact that stromelysin is induced by the inflammatory mediator interleukin-1 (*1*) and is capable of cleaving many of the components of cartilage and basement membrane including proteoglycans and fibronectin (*2–4*). This enzyme has also been implicated as a natural activator of fibroblast procollagenase (*5*) and progelatinase (*6*) as well as neutrophil procollagenase (*7*). For these reasons, the development of potent specific inhibitors of this enzyme could prove invaluable in the treatment of arthritic conditions. Structural studies, such as X-ray crystallography and NMR spectroscopy (*8*), of the active enzyme will contribute much to the development of these inhibitors but require large amounts of stable material. Since stromelysin is secreted as an inactive zymogen, it is necessary to treat the prostromelysin *in vitro* to generate the active enzyme (*9*). However, full-length stromelysin upon activation is not stable and further cleaves itself to a lower-molecular-weight form (*10*) which appears to have a heterogeneous COOH-terminus (P. M. Cameron, unpublished work). To avoid this problem, a COOH-terminally truncated form of the enzyme has been expressed in *Escherichia coli*. This enzyme has been purified to homogeneity and characterized as having k_{cat} and K_m values for hydrolysis of a peptide substrate similar to the values for full-length stromelysin (*11*).

In order to produce a pure, homogeneous preparation of the active form of

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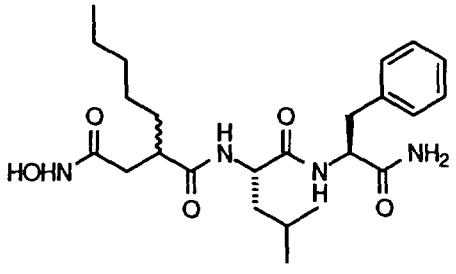
recombinant prostromelysin, different methods of activation were considered. Several activation procedures have been reported, including the addition of proteases such as trypsin, chymotrypsin, neutrophil elastase, plasma kallikrein, plasmin (*12*), and cathepsin G (*13*) as well as the organomercurial aminophenylmercuric acetate (APMA) (*10*). In addition, the use of elevated temperatures has also been reported to lead to activation of stromelysin (*14*). We excluded those methods which involve the addition of proteases due to the complications which could arise if the added protease were not completely removed following activation. In this study we make use of an inhibitor of active stromelysin to compare the mechanism of activation of prostromelysin by means of the addition of APMA or by incubation at elevated temperature.

MATERIALS AND METHODS

Materials

Tricine SDS acrylamide gels and buffers were purchased from Novex (Encinitis, CA). HPLC separations were performed on an ABI Model 140A separation system equipped with a Model 1000S diode array detector using 2.1 × 30-mm RP300 and BU300 columns (Applied Biosystems, Inc., Foster City, CA); PVDF Problot membranes were also purchased from Applied Biosystems. HPLC grade TFA, dimethyl formamide (DMF), and acetonitrile were purchased from Pierce (Rockford, IL). APMA, DMSO, EDTA, MES, Tris-HCl, amido black, 1,10-phenanthroline, and boric acid were from Sigma (St. Louis, MO); Coomassie brilliant blue R-250 and molecular weight standards were from Bio-Rad (Richmond, CA). Sequencing grade trypsin was purchased from Boehringer Mannheim (Indianapolis, IA). Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) was purchased from Wako Chemical (Richmond, VA). Hydroxamate **I** (ICI-U24278) (*15*), an inhibitor of mature stromelysin, was kindly provided by Dr. William Hagmann, Medicinal Chemical Research, Merck Research Laboratories. Amino acid sequence analyses were performed using an automated gas-phase 2020 sequencer from Porton Industries (Tarzana, CA). Peptides were covalently coupled to arylamine supports using a Sequelon AA attachment kit from MilliGen/Biosearch (Burlington, MA).



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Activation of Stromelysin

Escherichia coli-derived truncated prostromelysin was produced and purified as previously described (*11*). Stock solutions of the proenzyme were at 3.4 mg/ml in 20 mM MES buffer, pH 6, containing 5 mM $CaCl_2$ and 0.1 M NaCl. Prior to activation, prostromelysin was diluted to 600 μg/ml (20 μM) in 50 mM Tris-HCl, pH 7.2, containing 1 mM $CaCl_2$ and stored at −20°C until use. Except for the concentration-dependent heat activation study, the prostromelysin was mixed with an equal volume of the same buffer containing 4% DMSO with or without inhibitor immediately prior to the activation procedure. When inhibitor was included, the reaction mixture was incubated at room temperature for 20 min before activation was initiated. Conditions for the partial heat activation of prostromelysin were determined experimentally. It was found that 10 μM prostromelysin in the presence of 2% DMSO was 50–70% activated after 30 min at 53°C. For APMA activation, 2 mM APMA (from a 100× stock solution in DMSO) was added to the reaction mixture and the mixture was incubated at 37°C for the indicated time. Activation was terminated by the addition of 10 mM 1,10-phenanthroline or 20 mM EDTA.

Electroblotting of Proteins from SDS Tricine Gels to PVDF Problot

Tricine gels (10–20% gradient) and buffers were purchased from Novex and used according to the manufacturer's instructions. The proteins were electroblotted to PVDF Problot according to the method of Matsudaira (*16*) using 12.5 mM Tris/96 mM glycine/10% methanol as blotting buffer. Blots were stained with 0.1% amido black in 50% methanol (*17*).

Concentration Dependence of Heat Activation

Two concentrations (20 and 2 μM) of prostromelysin were placed at 53°C for the indicated times. The more concentrated samples were then diluted 10-fold and 1,10-phenanthroline was added to a final concentration of 10 mM. A portion of each sample was used for quantitation of pro and active forms by reverse-phase HPLC (see below).

Quantitation of the Relative Amounts of Prostromelysin and Mature Stromelysin by Reverse-Phase HPLC

Quantitation was performed by chromatographing each sample on a RP300 reverse-phase column using a complex linear gradient of acetonitrile in 0.1% TFA which had been optimized for the separation of pro and active stromelysin. The 210-nm absorbance for each peak was integrated using a Nelson Turbochrom data system and converted to mass units using a known amount of bovine ribonuclease to calculate the number of area units per microgram of protein. After conversion from microgram to molar amounts, the percentage of prostromelysin was calculated as:

$$\frac{\text{moles prostromelysin}}{\text{moles prostromelysin} + \text{moles mature stromelysin}} \times 100.$$

Alkylation of Cysteine with SBD-F

The procedure for alkylation with SBD-F was a modification of that reported by Kirley (*18*). The sample to be alkylated was mixed with an equal volume of 200 mM borate, pH 8.0, containing 4 mM EDTA. SBD-F in DMF was added to yield final concentrations of 2 mM SBD-F and 5% DMF. The alkylation reaction was carried out at 60°C for 30 min. Under these conditions, the alkylation of cysteine is highly specific.

Isolation of Cysteine-Containing Peptides

SBD-F-alkylated peptides were chromatographed on BU300 (for isolation of the pro piece) or RP300 (for isolation of tryptic peptides) reverse-phase HPLC columns (2.1 × 30 mm) at a flow rate of 100 μl/min using a gradient elution where the A buffer was 0.1% TFA in water and the B buffer was 0.1% TFA in acetonitrile. SBD-F reacted with a free sulfhydryl fluoresces and has a characteristic uv absorbance maximum at 383 nm. The column eluate was monitored at 210 and 370 nm (the absorbance maximum of 383 nm was outside the range of the detector used, but detection at 370 nm offered sufficient sensitivity).

Trypsin Digestion

The cysteine-containing pro peptide (2 μg) which had been isolated by reverse-phase HPLC was placed under vacuum for 10 min to remove the TFA and acetonitrile. Trypsin (200 ng) was added in 100 μl of 0.1 M ammonium bicarbonate, pH 7.9, and the reaction was allowed to proceed at 37°C for 18 h. An additional 200 ng of trypsin was added and the incubation was continued for another 4 h. The tryptic peptides were then separated by reverse-phase HPLC on a RP300 column (2.1× 30 mm) equilibrated with 0.1% TFA in water and eluted with a linear gradient from 0 to 50% acetonitrile over 25 min. Absorbance was monitored at 210 and 370 nm as above and the cysteine-containing peptide was collected for sequencing.

Amino Acid Sequence Analysis

Electroblotted bands were excised and placed in the reaction cartridge of the gas-phase sequencer without further treatment. The cysteine-containing tryptic peptide was covalently coupled to a Sequelon AA membrane using water-soluble carbodiimide according to the manufacturer's instructions. The samples were subjected to automated gas-phase Edman degradation on a Porton sequencer using the normal cycle and reagents as supplied by the manufacturer.

RESULTS

Mechanism of Heat Activation of Truncated Prostromelysin

To better understand the mechanism of activation of prostromelysin, we performed heat activation under conditions such that only part of the proenzyme was processed (see *Materials and Methods*). Truncated prostromelysin (10 μM) was

FIG. 1. SDS–PAGE of the products of partial heat activation of stromelysin. The products of partial heat activation were separated on a 10–20% tricine SDS gel and stained with Coomassie blue R250. Lane 1: Molecular weight standards. Lane 2: Heat-activated stromelysin. A duplicate gel was electroblotted to PVDF and the NH_2-terminal sequences of the bands were determined and are displayed to the right of lane 2.

incubated for 30 min at 53°C. Three bands were observed on Coomassie-stained SDS-PAGE gels after this partial heat activation. Figure 1 shows that these bands had the appropriate molecular masses for prostromelysin (29 kDa), mature stromelysin (20 kDa), and the NH_2-terminal pro portion (9 kDa). A duplicate gel was electroblotted to PVDF and each of the bands excised and subjected to NH_2-terminal sequence analysis. The NH_2-terminal sequence found for each band is shown to the right of lane 2 on Fig. 1. It can be seen that the 9-kDa peptide has the same NH_2-terminal sequence as the 29-kDa proenzyme, while the 20-kDa protein has the NH_2-terminal Phe which has been found to be the NH_2-terminus of mature stromelysin generated by APMA activation (*12*). Also, it was noted that no intermediate forms were evident. These results are consistent with heat activation occurring by means of a direct cleavage at or near the NH_2-terminus of the mature enzyme. This would differ significantly from the proposed mechanism of activation by APMA (*12*) which included the generation of an intermediate by an intramolecular cleavage followed by a bimolecular cleavage to the mature enzyme.

In order to further elucidate the mechanism by which heat activation was occurring, 10 μM truncated prostromelysin was activated for 30 min at 53°C in the presence of increasing amounts of hydroxamate **I** (*15*), an inhibitor of mature stromelysin (K_i = 42 nM (*11*)). The reaction was stopped by the addition of 20 mM EDTA and the samples were analyzed for percentage activation using RP-HPLC as described under *Materials and Methods*. Table 1 shows that activation of 10 μM prostromelysin is >90% inhibited by significantly less than an equimolar amount

TABLE 1
Amount of the Hydroxamate Stromelysin Inhibitor Necessary to Inhibit Heat Activation of Truncated Stromelysin

Hydroxamate (μM)	Molar ratio (inhib: prostrom)	% Active	% Inhibition
0.	—	60.0	0
0.016	1:625	57.0	5.0
0.08	1:125	40.8	32.0
0.4	1:25	4.7	92.2
2.0	1:5	1.7	97.2
10.0	1:1	1.9	96.8

Note. Truncated prostromelysin (10 μM) was incubated with the indicated amount of inhibitor for 20 min at room temperature. Partial heat activation was accomplished by incubation for 30 min at 53°C. The reaction was stopped by the addition of 20 mM EDTA. Percentage activation was assessed quantitatively by integration of the peaks generated on reverse-phase HPLC as described under *Materials and Methods.* Percentage inhibition was calculated using the % active when no inhibitor was present as 100%.

of inhibitor (Table 1; 0.4 μM). From these data, it appears that heat activation is mediated by only a small percentage of the molecules present. The most direct explanation of this result is that a low level (<2% by NH_2-terminal sequence analysis (data not shown)) of mature stromelysin present in the preparation is responsible for the cleavage observed. This would mean that heat activation is a bimolecular process, in contrast to the intramolecular process which has been proposed for APMA activation. Additional evidence for the bimolecular nature of this reaction was obtained by comparing the rate of heat activation at two different concentrations (2 and 20 μM). Figure 2 shows that indeed the disappearance of prostromelysin is

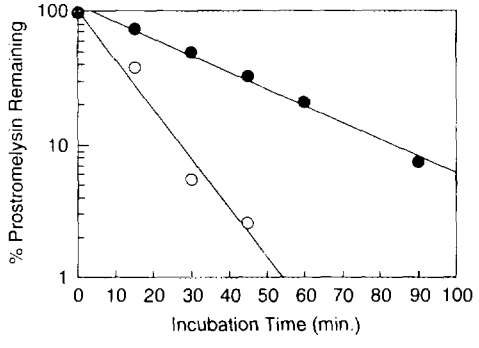

FIG. 2. Concentration dependence of heat activation. Prostromelysin at 2 μM (closed circles) or 20 μM (open circles) was incubated at 53°C for the indicated number of minutes (x axis). The % prostromelysin remaining was calculated at described under Materials and Methods and plotted on a log scale versus time of incubation (y axis).