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Fragmentation of an Endogenous Inhibitor upon Complex Formation with High- and Low-Ca^{2+}-Requiring Forms of Calcium-Activated Neutral Proteases

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ABSTRACT: The interaction of an endogenous inhibitor for the calcium-activated neutral protease (CANP or calpain EC 3.4.22.17) with CANP was examined by SDS–polyacrylamide gel electrophoresis, immunoblot analysis, and gel filtration. Fragmentation of the inhibitor (M_r 110K) by mCANP, a high-Ca^{2+}-requiring form, was shown only in the presence of Ca^{2+} ions of millimolar order, with decreased inhibitor activity recovered from gel extracts in the 110-kDa area. This fragmentation took place even when the inhibitor could completely inhibit the caseinolytic activity of mCANP. The fragmented inhibitor retained considerable inhibitor activity after the CANP–inhibitor complex was dissociated by the addition of EDTA, and 69% of the initial activity was recovered from the mixture reacted with excess mCANP lacking the 110-kDa band. A C-terminal fragment of CANP inhibitor produced in *Escherichia coli* (M_r 40K) was also hydrolyzed by mCANP in the presence of Ca^{2+}. The interaction of both forms of the inhibitor with μCANP, a low-Ca^{2+}-requiring form, led to the same phenomena in the presence of micromolar levels of Ca^{2+}. CANP inhibitor could not completely inhibit the autolysis of mCANP and μCANP, indicating that these were intramolecular events. Gel filtration analysis revealed that the mass of the smallest fragment with inhibitor activity was about 15 000 daltons. These results suggest that CANP inhibitor may act in the manner of a suicide substrate.

The calcium-activated neutral protease (CANP[1] or calpain, EC 3.4.22.17) is an intracellular cysteine protease distributed ubiquitously in various tissues and cells of vertebrates and is presumed to participate in various cellular functions mediated by Ca^{2+} (Suzuki et al., 1984; Pontremoli & Melloni, 1986). CANP has at least two isozymes with different calcium sensitivities: μCANP and mCANP, active at micro- and millimolar Ca^{2+}, respectively. Each CANP is composed of two different subunits, a large catalytic subunit with M_r 80K and

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[1] Abbreviations: CANP, calcium-activated neutral protease; mCANP and μCANP, CANPs which are active in the presence of millimolar and micromolar Ca^{2+}, respectively; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s); BPB, bromophenol blue; sample diluter, 125 mM Tris-HCl, pH 6.8/4% SDS/10% 2-mercaptoethanol/20% glycerol/0.005% BPB.

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a small regulatory subunit with M_r 30K. Inomata et al. (1983) reported that the large subunit of μCANP (79 kDa) is larger than that of mCANP (76 kDa). Comparison of the amino acid sequences of rabbit μCANP and mCANP determined by cDNA cloning revealed that the large subunits of μ- and mCANPs are different, whereas the small subunits are identical (Emori et al., 1986a,b). Activation of both CANPs requires their autolysis in the presence of Ca^{2+}. The molecular weight of the catalytic large subunit in active μCANP is 76 000, while this appreciable decrease in molecular weight is not shown in the case of mCANP activation (Inomata et al., 1985). CANP activity seems to be regulated by Ca^{2+} and an endogenous inhibitor, but little is known about the inhibition mechanism.

CANP inhibitor, termed calpastatin, coexists with CANP in various tissues (Murachi, 1983; Parkes, 1986). It is a heat-stable protein acting specifically on CANP, but various values have been reported for its molecular weight. Previously, we purified CANP inhibitors with different molecular sizes (HMW; 110 kDa, LMW; 26 kDa and 24 kDa on SDS polyacrylamide gel electrophoresis) from rabbit skeletal muscle and demonstrated that 1 mol of HMW and LMW inhibitor subunit inhibits 5 and 1 mol of mCANP, respectively, and that the conversion of the HMW form to the LMW form was caused by proteases in the cytosol (Nakamura et al., 1984, 1985). This suggests the existence of multiple reactive sites for inhibition.

Recently, the amino acid sequences of the CANP inhibitor from rabbit liver and erythrocyte were predicted by analyzing the base sequences of cDNA (Emori et al., 1987; Imajoh et al., 1987a). These results indicate that the erythrocyte and liver inhibitors comprise three and four tandemly repeated structures of about 140 residues, respectively, and that each domain, expressed in *Escherichia coli*, possesses inhibitor activity (Imajoh et al., 1987b; Emori et al., 1988). Similar repetitive domains have been reported for the calpastatin from pig heart (Takano et al., 1986; Maki et al., 1987a).

However the mechanism of inhibition remains unknown. Melloni et al. (1982) reported that, in the presence of Ca^{2+}, CANP and the inhibitor both from human erythrocytes, which is a tetramer composed of 60-kDa subunits, dissociate into 80- and 30-kDa subunits and into 60-kDa subunits, respectively, and that the 60-kDa subunit of the inhibitor binds to the 80-kDa subunit of CANP to form a 140-kDa complex. The reversible interaction between mCANP and its inhibitor was shown by Imajoh et al. (1985), while Shigeta et al. (1984) have shown evidence for the fragmentation of a 70000-Da calpastatin molecule after elution from an immobilized calpain column. To resolve the controversy, we analyzed the reaction between CANP inhibitor and m- and μCANPs.

We report here that both the intact form and the C-terminal fragment of CANP inhibitor are cleaved during complex formation with m- and μCANPs to smaller fragments which still retain CANP inhibitor activity and that autolytic activation of m- and μCANPs occurs simultaneously.

Materials and Methods

Casein (nach Hammersten) was a product of Merck, Darmstadt, West Germany. Phenylmethanesulfonyl fluoride was obtained from Sigma Chemical Co., St. Louis, MO, and leupeptin was from Peptide Institute Inc., Osaka, Japan. DE-52 and DE-53 were products of Whatman, Kent, U.K. Sephadex G-75 and phenyl-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and Ultrogel AcA 34 was from LKB, Bromma, Sweden. Peroxidase-conjugated anti guinea pig immunoglobulin derived from goat serum was obtained from Cappel Laboratories, Malvern, PA. The electrophoresis calibration kits for determination of molecular weights were purchased from Bio-Rad, Richmond, CA, and Pharmacia Fine Chemicals. Freund's complete adjuvant and Affi-Gel–protein A were obtained from Difco Lab., Detroit, MI, and Bio-Rad, respectively. Monoclonal antibodies against CANP (3A11D12, 4C7G2, 1H6F4, 4B9F1) were isolated as described before (Kasai et al., 1986). Immunostain kits for Western blotting were generous gifts from Konishiroku, Tokyo, Japan. All other chemicals were of analytical grade.

Preparation of mCANP and μCANP. mCANP and μCANP were purified from rabbit skeletal muscle according to the method described by Kawashima et al. (1984) and Inomata et al. (1983).

Preparation of CANP Inhibitor. CANP inhibitor was purified from rabbit skeletal muscle by previously reported procedures (Nakamura et al., 1985). CANP inhibitor from rabbit liver was purified as follows. Rabbit liver was removed immediately after slaughter and minced. The minced materials (1.2 kg) were homogenized with 3 volumes of buffer A (20 mM Tris-HCl, 5 mM EDTA, pH 7.5) containing 5 μM leupeptin and 5 mM PMSF in order to prevent undesirable proteolysis of the CANP inhibitor. After centrifugation at 12000*g* for 15 min, the supernatant was filtered through glass wool and solid TCA was immediately added to the filtrate to 15% (w/v) with stirring. The precipitate obtained by centrifugation was suspended in 0.1 M Tris containing 5 μM leupeptin and 5 mM PMSF, and the pH was adjusted to 7.5 with 1 M NaOH. After being stirred overnight, the suspension was readjusted to pH 7.5 and centrifuged, and the supernatant was dialyzed against buffer A containing 5 μM leupeptin and 5 mM PMSF with several changes. The dialyzate was heated at 85 °C for 5 min, cooled to 4 °C, and centrifuged to remove precipitate. The supernatant was dialyzed against water and then lyophilized. The powder was dissolved in buffer A and dialyzed against the same buffer, and the solution was applied to a DE-52 column (3 × 30 cm) equilibrated with buffer A. After being washed with buffer A, the proteins, including CANP inhibitor, were eluted with buffer A containing 0.2 M NaCl. The active fractions were pooled and salted out between 25 and 70% saturated ammonium sulfate. The precipitate collected by centrifugation was dissolved in buffer A containing 25% saturated ammonium sulfate and was applied to a phenyl-Sepharose CL-4B column (1.5 × 13 cm) equilibrated with the same buffer. CANP inhibitor passed directly through the column, while the large amounts of other proteins were adsorbed. The inhibitor was dialyzed against water with several changes and was lyophilyzed. The freeze-dried powder was dissolved in buffer A containing 0.1 M NaCl and subjected to gel filtration on a Ultrogel AcA 34 column (5 × 130 cm) equilibrated with the same buffer. Fractions containing CANP inhibitor were collected and dialyzed against buffer A. The dialyzate was applied to a DE-53 column (0.8 × 16.5 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient of 0–0.5 M NaCl in a total volume of 100 mL of the same buffer. The active fractions, which eluted between 0.05 and 0.15 M, were collected, dialyzed against buffer A, and then were rechromatographed on a DE-53 column (0.6 × 19 cm). Purified CANP inhibitor gave a single band with a M_r of 110000 on SDS–polyacrylamide gel electrophoresis. A C-terminal fragment of rabbit liver CANP inhibitor was produced in *E. coli* according to the procedure described by Imajoh et al. (1987). The purified truncated inhibitor showed a single band with a mass of 40 kDa on

SDS–polyacrylamide gel electrophoresis and contained two internal repeat structures (373 amino acid residues).

Assay of CANP Inhibitor. The inhibitor activity was measured with alkali-denatured casein, which was dissolved in 0.1 M NaOH and adjusted to pH 7.5 with 1 M HCl, as a substrate. Increasing amounts of sample were added to fixed amounts of mCANP or μCANP, and the reaction was started by adding substrate at a final concentration of 0.24% in a mixture containing 0.1 M Tris-HCl (pH 7.5), 28 mM 2-mercaptoethanol, and 6 mM (mCANP assay) or 0.25 mM (μCANP assay) $CaCl_2$. After incubation for 10 min at 30 °C, an equal volume of 10% TCA was added, and the mixture was centrifuged. The absorbance of the supernatant at 280 nm was determined. A control experiment was done identically but with the omission of $CaCl_2$. One unit of CANP activity was defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at 280 nm in 1 h under the standard assay conditions. One unit of inhibitory activity was defined as the amount of inhibitor that inhibited 1 unit of CANP activity completely. In some cases the fluorescence of the supernatant after the reaction (excitation, 275 nm; emission, 303 nm) was monitored with an RF503A (Shimadzu) difference spectrofluorophotometer. Inhibitor activity was estimated from the straight portion of the decreasing slope of a plot of proteolytic activity [fluorescence intensity (%)] vs concentration of samples. The fluorescence intensity of the TCA-soluble peptides was expressed as units calculated from a calibration curve between known amounts of rabbit mCANP and the fluorescence of product measured in the absence of CANP inhibitor. The radioactive assay for CANP inhibitor was performed with ^{14}C-succinylated bovine serum albumin as a substrate by a similar procedure described by Nakamura et al. (1984).

Preparation of Polyclonal Antibody to CANP Inhibitor. The truncated inhibitor (a C-terminal fragment) heated at 100 °C for 3 min in 0.1% SDS was used as the antigen. Polyclonal antibodies to CANP inhibitor were raised in guinea pigs by the intradermal injection of antigens emulsified with Freund's complete adjuvant on days 0, 21, and 49. Antibody specificities of serum from the ear vein were determined by the enzyme-linked immunosorbent assay (ELISA) after Kasai et al. (1986). Blood was collected from the heart, and the immunoglobulin fractions were purified by Affi-Gel–protein A column chromatography.

Electrophoresis and Western Blot Analysis. Gel electrophoresis in the presence of SDS was performed according to the procedure of Laemmli (1970) on 10% or 15% polyacrylamide slab gels. When necessary, the gel was sliced into 2-mm segments without fixation and staining, and gel extracts were assayed for inhibitor activity. In some cases, gels were electroblotted onto nitrocellulose sheets and immunostained with the inhibitor-specific polyclonal antibody by the method of Towbin et al. (1979). Blotted proteins were incubated with polyclonal antibodies against CANP inhibitor followed by incubation with horseradish peroxidase conjugated goat anti guinea pig immunoglobulin and then visualized with a Konishiroku immunostain kit.

Results

Electrophoretic Analysis of the Interaction between mCANP and the Inhibitor. A fixed amount of CANP inhibitor (M_r 110K) from rabbit skeletal muscle was incubated with various amounts of mCANP in the presence or absence of 6 mM Ca^{2+}, and each sample was subjected to SDS–polyacrylamide gel electrophoresis (Figure 1). When excess amounts of CANP inhibitor (activity is in excess) and

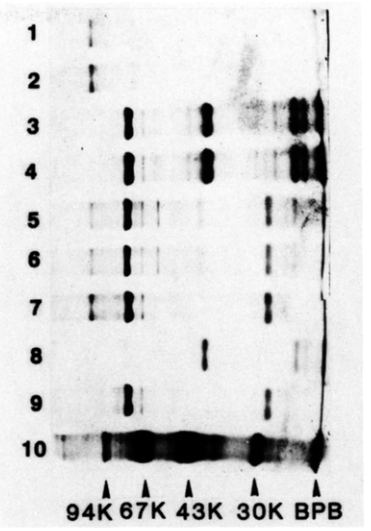

FIGURE 1: SDS–polyacrylamide gel electrophoresis of mixtures of CANP inhibitor and mCANP in various ratios incubated for 15 min: CANP inhibitor (2.7 units) was incubated with various amounts of mCANP at pH 7.5 for 15 min at 30 °C in the presence (lanes 2–6) or absence (lanes 1 and 7) of 6 mM Ca^{2+}. mCANP activity of the reaction mixtures was as follows: 0 (lanes 1 and 2), 5.4 (lanes 3 and 4), and 1.4 units (lanes 5–7). In control experiments, mCANP only (1.4 units) was incubated in the presence (lane 8) or absence (lane 9) of Ca^{2+}. The reaction mixtures were heated to 100 °C with sample diluter for 5 min, except that excess amounts of EDTA were added to lanes 4 and 6 before heating, and were subjected to SDS–polyacrylamide gel electrophoresis. In lane 10, a mixture of protein standards was applied: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

mCANP were mixed in the presence of Ca^{2+} (lanes 5 and 6), the electrophoretic pattern showed a distinct decrease in the 110-kDa band with the faint appearance of a new component with M_r 56K. In immunoblot analysis, a band reacting with antiserum against mCANP, but not with anti 40-kDa inhibitor, was detected at about 55 kDa (data not shown). It appears that the inhibitor cannot completely inhibit autolysis of mCANP. The interaction of CANP inhibitor with a 2-fold excess of mCANP led to the complete disappearance of the 110-kDa band of the inhibitor and the 30-kDa band of mCANP with the appearance of a 40-kDa band (lanes 3 and 4). This band was consistent with an autolysis product of the large subunit of mCANP (lane 8). No covalently bound enzyme–inhibitor complex was detected. These results suggest that the large subunit of mCANP may be bound to the CANP inhibitor molecule in the presence of Ca^{2+} with the degradation of the inhibitor, and that excess CANP not bound to the inhibitor may be autolyzed. To confirm the fragmentation of CANP inhibitor during complex formation with mCANP, mixtures of mCANP and the inhibitor were electrophoresed in the presence of SDS, and gel extracts were assayed for inhibitor activity with alkali-denatured casein as a substrate as described under Materials and Methods. Figure 2 shows that the activity of CANP inhibitor in the 110000-Da region after the reaction with mCANP corresponding to 40% of the inhibitor activity (panel B) decreased and that no activity was recovered in the 110-kDa area while weak activities appeared in the lower molecular weight areas after interaction with equal or excess amounts of mCANP (panels C and D). In control experiments, the mixture of mCANP and CANP inhibitor incubated in the absence of Ca^{2+} gave a similar pattern to that of panel A (data not shown). These gel extracts were also assayed with ^{14}C-succinylated bovine serum albumin as a substrate, and the same result was obtained (data not shown).

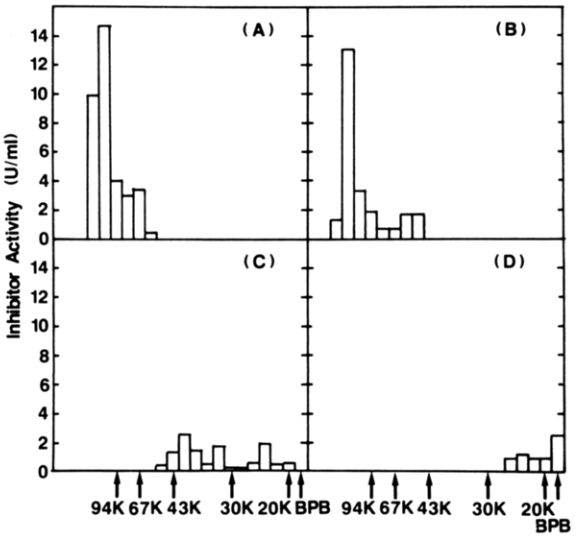

FIGURE 2: Identification of CANP inhibitor activity in fragments after interaction with mCANP. CANP inhibitor (20 units) was incubated in 20 mM Tris-HCl (pH 7.5) for 15 min at 30 °C in the presence of 6 mM Ca^{2+} with various amounts of mCANP: (A) 0, (B) 8, (C) 20, and (D) 41 units. Excess amounts of EDTA were added to the reaction mixtures to dissociate the CANP inhibitor–mCANP complex, and the mixtures were subjected to SDS–polyacrylamide gel electrophoresis. Gel extracts prepared as described under Materials and Methods were assayed for inhibitor activity. Arrows indicate the positions where the following marker proteins were extracted from the gel: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa).

These results provide evidence for the fragmentation of CANP inhibitor during the interaction with mCANP in the presence of Ca^{2+}. It was shown that fragmented inhibitors are qualitatively active, but the activity in gel extracts might not reflect the quantitative activity because of the dispersion of fragments and different efficiencies of extraction from the gel.

Recovery of CANP Inhibitor Activity from the CANP–Inhibitor Complex. We examined directly the total activity of CANP inhibitor and its fragments after interaction with mCANP. Fixed amounts of CANP inhibitor and various amounts of mCANP were mixed in the activity ratios of 1:2, 1:1, and 1:0.3, in 20 mM Tris-HCl buffer, pH 7.5, containing 28 mM mercaptoethanol and 6 mM $CaCl_2$. After 15 min at 30 °C, the mCANP–inhibitor complex was dissociated by adding EDTA to 12 mM and heating the mixture at 100 °C for 5 min in the presence or absence of 2% SDS to assure dissociation of the complex and to inactivate mCANP. The mixtures were diluted 1:50 with 0.1 M Tris-HCl, pH 7.5, to lower the SDS concentration to less than 0.0016%, which did not interfere with the CANP assay. Aliquots of the diluted mixtures were assayed with alkali-denatured casein as a substrate for inhibitor activity. A control experiment was done similarly without $CaCl_2$. Table I shows that considerable amounts of CANP inhibitor activity were recovered from the reaction mixtures of mCANP and the inhibitor after addition of EDTA and heat inactivation of CANP. Surprisingly, CANP inhibitor interacted with mCANP at a ratio 1:2 still retained 69% of initial activity although no intact inhibitor molecule (110 kDa) was detected by SDS–polyacrylamide gel electrophoresis (Figure 1). This indicates that CANP inhibitor bound to mCANP molecules can be dissociated from the enzyme–inhibitor complex by omitting Ca^{2+} and that it can still inhibit freshly added mCANP even as fragments.

Identification of CANP Inhibitor Fragments by Immunoblot Analysis. Attempts were made to identify CANP inhibitor fragments immunologically after the interaction with mCANP. Mixtures containing fixed amounts of the inhibitor and mCANP incubated as described above were electrophoresed and subjected to Western blot analysis using anti 40-kDa inhibitor antibody. Figure 3A illustrates a decrease (lanes 2 and 3) or disappearance (lane 4) of the immunoreactive band with M_r 110K corresponding to an increase in mCANP added in the presence of Ca^{2+}. The interaction of excess inhibitor with mCANP resulted in the formation of faint immunostained fragments with M_r of 70K and 80K. Any other immunoreactive fragments were not detected in the mixtures of inhibitor and equal or excess amounts of mCANP. When very large amounts of sample were subjected to immunoblot analysis or the interaction was performed under mild

Table I: Recovery of Inhibitor Activity after Interaction of CANP Inhibitor (I) with mCANP (E)[a]

	inhibitor activity units (%)[b]	
	–SDS, 100 °C, 5 min	+SDS, 100 °C, 5 min
E + I + Ca^{2+}		
I < E (1:2)[c]	12.0 (69.0)	12.0 (69.0)
I = E (1:1)	13.5 (77.6)	13.5 (77.6)
I > E (1:0.3)	16.8 (96.6)	16.5 (94.8)
I + EDTA	17.4 (100)	17.4 (100)

[a] CANP inhibitor was incubated in 20 mM Tris-HCl (pH 7.5) for 15 min at 30 °C in the presence of 6 mM Ca^{2+} with various amounts of mCANP. Excess amounts of EDTA were added to the mixtures, and the mixtures were heated to 100 °C for 5 min in the presence or absence of SDS. Mixtures were assayed with alkali-denatured casein for residual inhibitor activity. [b] The relative residual activity when only inhibitor incubated in the absence of Ca^{2+} was taken as 100%. [c] Numbers in parentheses are the mCANP to CANP inhibitor activity ratios.

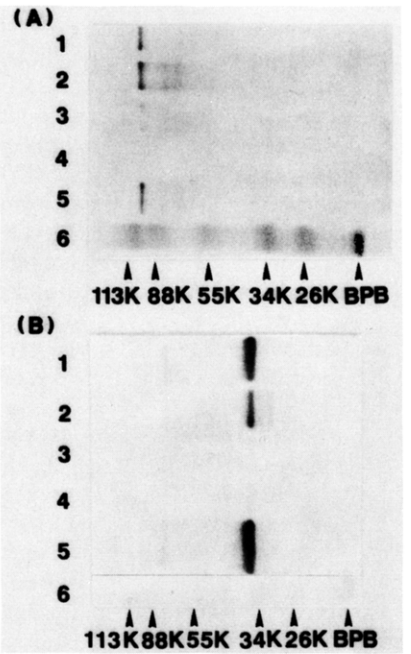

FIGURE 3: Immunoblot analysis of mixtures of CANP inhibitor and mCANP in various ratios incubated for 15 min: (A) Intact forms of CANP inhibitor (M_r 110K; 0.9 unit) were incubated in 20 mM Tris-HCl, pH 7.5, for 15 min at 30 °C in the presence (lanes 2–4) or absence (lanes 1 and 5) of 6 mM Ca^{2+} with various amounts of mCANP: (lanes 1 and 3) 0.9 unit; (lane 2) 0.3 unit; (lane 4) 3.0 units; (lane 5) none. (B) The C-terminal fragments of CANP inhibitor (M_r 40K; 0.9 unit) were incubated in the same manner as in (A). After incubation the reaction was stopped by the addition of excess EDTA, and the mixtures were subjected to SDS–polyacrylamide gel electrophoresis. They were then electroblotted and immunostained with anti-40-kDa inhibitor antibody. In lane 6, the prestained molecular weight standards were applied.

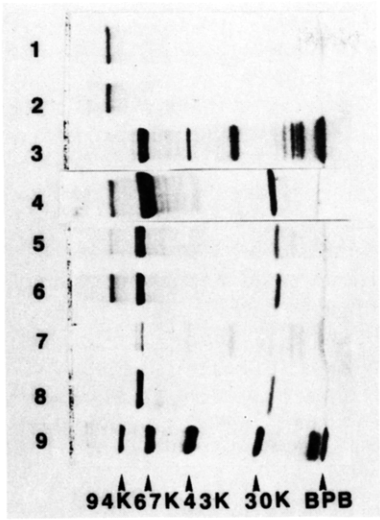

FIGURE 4: SDS–polyacrylamide gel electrophoresis of the mixture of CANP inhibitor and μCANP in various ratios incubated for 15 min. CANP inhibitor (0.5 unit) was incubated in 20 mM Tris-HCl (pH 7.5) for 15 min at 30 °C in the presence (lanes 2, 3, and 5) or absence (lanes 1, 4, and 6) of 250 μM Ca^{2+} with various amounts of μCANP: (lanes 1 and 2) none; (lanes 3 and 4) 1.0 unit; (lanes 5 and 6) 0.25 unit. μCANP (0.25 unit) was also incubated in the presence (lane 7) or absence (lane 8) of 250 μM Ca^{2+} without CANP inhibitor. The reaction mixtures were heated to 100 °C with sample diluter for 5 min and then subjected to SDS–polyacrylamide gel electrophoresis. In lane 9, marker proteins were applied; phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

conditions with lower concentrations of Ca^{2+}, stained bands around 25 kDa were seen, but the intensities of these bands did not correspond to the loss of the 110-kDa band.

We examined the interaction of the C-terminal fragment of the inhibitor (M_r 40K) with mCANP (Figure 3, panel B). The truncated inhibitor was also degraded by mCANP in the presence of Ca^{2+}. Even when mCANP was incubated with excess inhibitor, the 40-kDa band decreased with the appearance of new immunostained bands (30 kDa) as shown in lane 2.

We also examined the interaction of CANP inhibitor from rabbit liver with mCANP from rabbit skeletal muscle in a similar manner. All results were the same as those shown in Figures 1–3 and Table I (data not shown).

Interaction of CANP Inhibitor with μCANP. The reaction of CANP inhibitor with μCANP was similarly examined except that the reaction mixtures were incubated in the presence of 250 μM Ca^{2+}. Figure 4 shows the same electrophoretic pattern of CANP inhibitor as that given by mCANP, that is, a decrease or disappearance of the 110-kDa band. In contrast, the large subunit of μCANP (79 kDa) was converted to a 76-kDa band that differed from that of mCANP even when an excess amount of the inhibitor was present. Though the change appears obscure in Figure 4, the application of smaller amounts led to clear identification of the 76-kDa band (data not shown). In the absence of Ca^{2+} this subunit remained unchanged (lanes 4 and 6). These results suggest that CANP inhibitor cannot completely inhibit autolysis of μCANP and that the resultant active form of μCANP degrades its inhibitor. In immunoblot analysis, changes in the CANP inhibitor similar to those of the inhibitor after interaction with mCANP were shown (Figure 5). A decrease or disappearance of the 110- or 40-kDa immunostained band was detected that corresponded to the amount of μCANP added, and other immunoreactive materials were not seen except for faint bands with M_r of 30K and 24K.

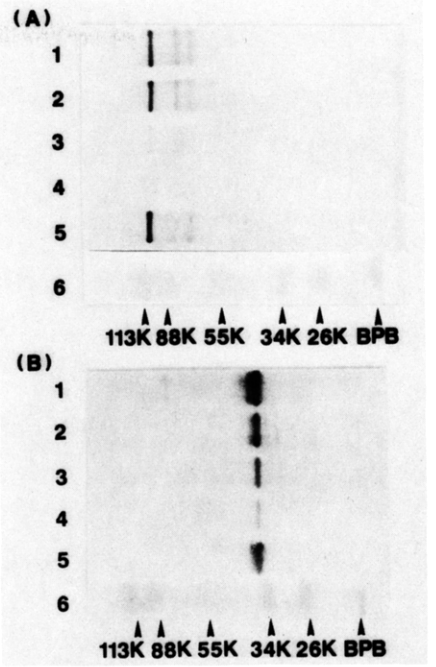

FIGURE 5: Immunoblot analysis of mixtures of CANP inhibitor and μCANP in various ratios incubated for 15 min. (A) Intact forms of CANP inhibitor (M_r 110K; 0.4 unit) were incubated in 20 mM Tris-HCl, pH 7.5, for 15 min at 30 °C in the presence (lanes 2–4) or absence (lanes 1 and 5) of 250 μM Ca^{2+} with various amounts of μCANP: (lanes 1 and 3) 0.4 unit; (lane 2) 0.1 unit; (lane 4) 0.8 unit; (lane 5) none. (B) The C-terminal fragments of CANP inhibitor (M_r 40K; 0.5 unit) were incubated in the same manner as in (A). After incubation, the reaction was stopped by the addition of excess EDTA, and the mixtures were subjected to SDS–polyacrylamide gel electrophoresis. They were electroblotted and immunostained with anti-40-kDa inhibitor antibody. In lane 6, the prestained molecular weight standards were applied.

Gel Filtration Analysis of CANP Inhibitor Fragments Produced by mCANP. To confirm the presence of immunologically negative but active fragments, a mixture of CANP inhibitor and mCANP in an activity ratio of 1:2 was incubated in the presence of 6 mM Ca^{2+} at 30 °C for 15 min. After the addition of EDTA to 12 mM, the mixture was subjected to gel filtration on a Sephadex G-75 column (1:1 × 27.0 cm) in the presence of 6 M urea. CANP inhibitor activity was eluted after the main protein peak, indicating that inhibitor fragments dissociated from the complex retain inhibitor activity (Figure 6A). Aliquots of each fraction were subjected to SDS–polyacrylamide gel electrophoresis, which revealed that the main protein peak contained the large subunit of mCANP, originally bound to the inhibitor, and autolysis products of excess CANP and that the peak with inhibitor activity consisted of proteins with molecular weights of ca. 15 000 (Figure 6B). These peptides were probably the smallest fragments with CANP inhibitor activity.

Discussion

The susceptibility of CANP inhibitor to proteases has been reported (Nakamura et al., 1985; Mellgren & Carr, 1983) and the change of rat liver calpastatin from a high molecular weight form to a 34-kDa form during hydrazine-induced anemia has also been shown (Yamato et al., 1983). It was of great interest to know the physiological significance of proteolytic processing of CANP inhibitor, especially whether the inhibitor could be proteolyzed to smaller forms during the interaction with CANP. Although there have been several reports describing the interaction between CANP and the inhibitor, the conclusions were contradictory.

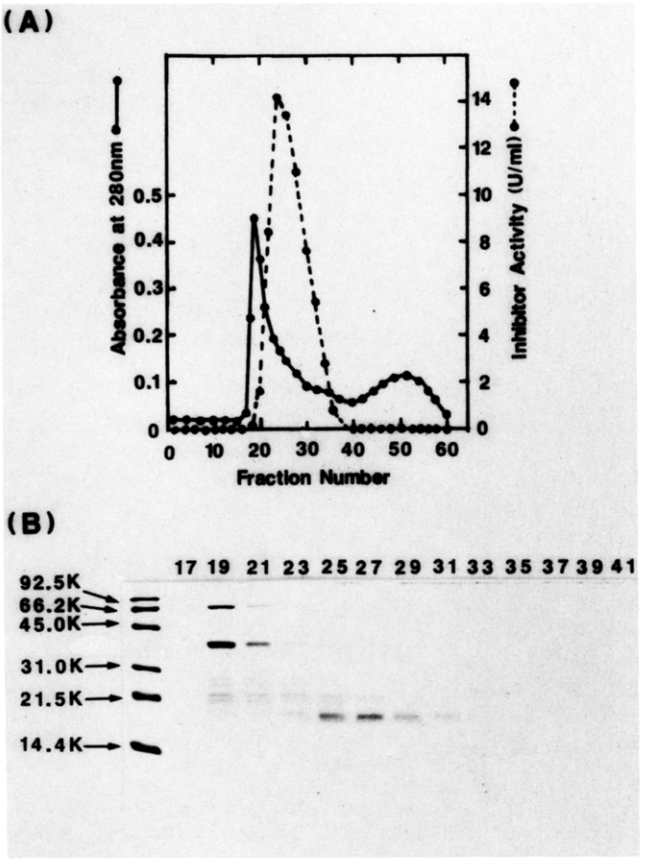

FIGURE 6: Gel filtration of CANP inhibitor fragments degraded by mCANP. (A) CANP inhibitor (43 units) was incubated in 20 mM Tris-HCl, pH 7.5, at 30 °C for 15 min in the presence of 6 mM Ca^{2+} with mCANP (91 units). After incubation, the reaction was stopped by the addition of excess EDTA, and the mixture was lyophilized. The freeze-dried powder was dissolved in 20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 6 M urea and then applied to a 1.1 × 27.0 cm column of Sephadex G-75 equilibrated in the same buffer. Fractions of 0.5 mL were collected and assayed with alkali-denatured casein for inhibitor activity (dotted line). (B) 7.5 μL of each fraction was heated for 5 min at 100 °C with equal amounts of sample diluter and then electrophoresed on a 15% polyacrylamide slab gel in the presence of SDS. The molecular weight standards used were phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

The present study reveals that the fragmentation of both an intact form (110 kDa) and a truncated form (40 kDa) of CANP inhibitor occurs during complex formation with m- and μCANPs, even in the presence of sufficient inhibitor to cause complete enzyme inactivation, and that the resultant fragmented inhibitors retain activity. Shigeta et al. (1984) investigated the interaction of 70 000-Da calpastatin and calpain immobilized to Sepharose and indicated that the fragmentation of calpastatin occurs, but their study lacked quantitative analysis of the interaction of calpastatin with calpain. Mellgren et al. (1986) and Mellgren and Carr (1983) reported that 1 mol of bovine cardiac inhibitor could inhibit up to 4 mol of bovine heart peak II calcium-dependent protease and that the inhibitor could be proteolyzed to low molecular weight forms by the protease even at a molar ratio of protease to inhibitor of 1:1, namely, a 1:4 activity ratio. This suggests that the fragmentation of the inhibitor can take place even when it can completely inhibit the enzyme, in agreement with our data. In contrast, DeMartino and Croall (1984) and Melloni et al. (1984) observed the reversible interaction of CANP inhibitor with CANP by gel filtration on Sephacryl S-300 and by the equilibrium distribution coefficients into Ultrogel AcA 34, respectively. Their methods seem unsuitable for investigating quantitative interactions, and subtle changes in the inhibitor, such as nick formation, may be missed.

Attempts to identify the inhibitor fragments by immunoblot analysis failed, indicating that the epitopes recognized by the anti-40-kDa inhibitor were preferentially degraded under the conditions of the analysis. Mellgren et al. (1986) observed weak immunoreactive bands with M_r 30K and 25K after the interaction of the inhibitor with equimolar calcium-dependent protease, but they could detect no immunostained bands in the mixture incubated with excess enzyme. The region containing the epitopes is probably sensitive to proteolysis by CANP. In contrast, the reactive sites of CANP inhibitor seem to be conserved because fragmented inhibitor with little immunoreactivity retained 69% of the initial inhibitor activity (Table I). Successful use of gel filtration in the presence of 6 M urea suggested that the smallest fragment with CANP inhibitor activity was about 15 000 Da. Shigeta et al. (1984) indicated the fragmentation of 70-kDa calpastatin into various inhibitory peptides of molecular weight ranging from 14K to 70K and all four repetitive domains (each composed of approximately 140 amino acid residues) expressed in *E. coli* have been reported to possess inhibitory activity against calpains I and II (Maki et al., 1987b), in agreement with our data.

Even excess inhibitor could not completely inhibit autolysis of either mCANP and μCANP. This fact provides further evidence that the active forms of m- and μCANPs are generated by not only intermolecular but also intramolecular autolysis (Inomata et al., unpublished results). First, part of m- and μCANPs may be activated by intramolecular autolysis in the presence of Ca^{2+}, and then, the resultant active enzymes may proteolyze the substrate-like sites of CANP inhibitor molecule to smaller forms. Fragmented inhibitor binds to the CANP molecule, so that the modified CANP slows down enormously its catalytic reaction. In other words, CANP inhibitor may act in the manner of a suicide substrate for CANP.

Recent studies suggest the importance of the plasma membrane as a site for the autocatalytic activation of CANP (Imajoh et al., 1986; Pontremoli et al., 1985; Gopalakrishna & Barsky, 1986). Imajoh et al. (1987c) reported that CANP inhibitor inhibits the association of CANP with the membrane as well as the proteolysis of membrane-bound substrate and that the inhibitor fragments generated by limited proteolysis by endoproteinase Arg C do not inhibit the breakdown of membrane-bound substrate but the binding of CANP to membranes. Furthermore, Emori et al. (1988) reported that the four domains of the CANP inhibitor produced in *E. coli* differed from each other in inhibition of m- and μCANPs. Further study will be necessary to clarify whether proteolytic processing of CANP inhibitor occurs in vivo or whether each domain has any functional difference. If CANP inhibitor has multivalent functions, fragmentation of CANP inhibitor would seem to be essential for the elaborate regulation of CANP.

Registry No. Ca, 7440-70-2.

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