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A fragment of an endogenous inhibitor produced in *Escherichia coli* for calcium-activated neutral protease (CANP) retains an inhibitory activity

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A C-terminal fragment of an endogenous rabbit liver inhibitor for calcium-activated neutral protease (CANP) was produced in *Escherichia coli* and its inhibitory activity was examined after purification. The truncated inhibitor (373 amino acid residues), which contains two internal repeat structures, inhibits 2 mol CANP whereas the native liver inhibitor (639 residues), containing four internal repeat structures, inhibits 4 mol CANP. This supports the hypothesis that the repeating unit is the functional unit of inhibition. The results also indicate that post-translational modification of the inhibitor is not essential for inhibition.

Ca^{2+}-activated neutral protease; Calpain; Proteinase inhibitor

1. INTRODUCTION

Calcium-activated neutral protease (CANP, calpain, EC 3.4.22.17), a non-lysosomal cysteine protease, participates in various cellular functions mediated by Ca^{2+} [1-3]. Thus, an understanding of the regulation of its activity is very important for elucidating its biological role. The activity is regulated mainly by Ca^{2+} and an endogenous inhibitor, CANP inhibitor [2,4-6]. Recently, we deduced the complete amino acid sequence of the rabbit inhibitor from the cDNA base sequence as a step in the analysis of the mechanism of inhibition of CANP [7]. The sequence contains four internal repeats of about 140 residues each. Since CANP inhibitors from various sources inhibit 3-8 mol CANP [6], we assumed that the repeating unit is the functional unit of inhibition. To confirm this hypothesis, a cDNA fragment encoding the C-terminal half of the inhibitor was expressed in *E. coli*, and the inhibitory activity of the protein product was examined together with the activity of native liver inhibitor.

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2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were obtained from the sources indicated: casein, Merck; isopropyl-β-D-thiogalactopyranoside (IPTG), Wako Pure Chemicals; phenylmethanesulfonyl fluoride (PMSF), ampicillin and kallikrein, Sigma; TPCK-trypsin, Millipore; α-chymotrypsin, Worthington; proteinase V8 (*Staphylococcus aureus*), Miles Laboratories; papain and bromelain, Boehringer Mannheim; TSK-gel phenyl-5PW, Toyo-Soda.

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Lysylendopeptidase was a gift from Dr T. Masaki, Ibaraki University.

2.2. *Assay of CANP and its inhibitor*

The proteolytic activities of low- and high-Ca^{2+}-requiring CANPs (μCANP and mCANP) were measured at 100 μM and 5 mM $CaCl_2$, respectively, using casein as a substrate [8]. The assay of the inhibitor for CANP was carried out as in [5].

2.3. *Preparation of CANP, CANP inhibitor, cathepsins B and H, and ingensin*

μCANP and mCANP were purified from rabbit skeletal muscle (spect. act. 400 and 180 U/mg, respectively) [9]. An endogenous inhibitor for CANP was purified to homogeneity (fig.1B) from rabbit liver essentially as in [10]. Cathepsins B and H were prepared from rat liver [11]. Ingensin was prepared from rat liver as in [12].

2.4. *Plasmid*

During the course of cDNA cloning of the CANP inhibitor [7], a clone λCJ5S was isolated which contained in λgt10 a 2 kb insert DNA for the 3′-region of the inhibitor mRNA (3.8 kb). λCJ5S was subcloned into the *Eco*RI site of the pUC8 vector. A resulting plasmid pCJ5S was found to express a *lac*-fused protein as judged by the DNA sequence (see fig.3). The produced protein was expected to encode a fused protein of 373 amino acid residues composed of 6 amino acid residues from the *lacZ* gene and polylinker, and the C-terminal half (367 residues) of the rabbit CANP inhibitor.

2.5. *Bacterial culture and induction of truncated CANP inhibitor*

Cells of *E. coli* K-12 HB101 carrying pCJ5S were grown at 37°C to late-log phase in 500 ml of χ-broth [13], and further grown in 1 l M9 medium [13] containing 50 mg ampicillin for 2 h. Then, 1 ml of 1 M IPTG was added (final concentration: 1 mM), and the cells were incubated for 1.5 h with shaking.

2.6. *Other methods*

The DNA sequence was determined by the dideoxy chain termination method [14]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as in [15]. The amino acid composition and protein sequence were analyzed by a Hitachi 835 amino acid analyzer and an Applied Biosystems 470A protein sequencer, respectively. The concentration of protein was determined by amino acid analysis.

3. RESULTS

3.1. *Purification of truncated CANP inhibitor expressed in* E. coli

Cells of *E. coli* K-12 HB101 harboring pCJ5S expressed large amounts of a 40 kDa polypeptide when induced with IPTG. The expressed inhibitor was heat-stable, simplifying its purification. The truncated CANP inhibitor was purified from the cell lysates according to the method established for the 107 kDa inhibitor from human liver [10]. Cultured cells (~2 g) were washed with water, and suspended in 20 ml Tris-HCl, pH 7.5, containing 5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A) supplemented with 10 mM PMSF. Cells were disrupted with a Branson sonifier, model 185. Cell debris was removed at 15000 rpm in a Hitachi RPR-20 rotor at 0°C. To the supernatant fraction, obtained after heating the extract for 10 min in boiling water, was added solid NaCl to a final concentration of 1 M. The sample was applied to a phenyl-Sepharose CL-4B column (3 × 5 cm) pre-equilibrated with buffer A containing 1 M NaCl. The column was eluted with the same buffer, and the flow-through fraction was collected and dialyzed against buffer A. The dialyzed sample was adsorbed to a DE-52 column (1.8 × 30 cm), and eluted with a linear gradient of 0–0.4 M NaCl in buffer A. The inhibitor fraction eluted at 0.1 M NaCl was made 10 mM with respect to HCl, and applied to a TSK-gel phenyl-5PW RP column (4.6 × 75 mm). The column was developed with a linear gradient of 0–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, and the elution was monitored at 210 nm. The purified truncated inhibitor, which was eluted at 40% (v/v) acetonitrile, was dried in a Savant Speed Vac concentrator, dissolved in 5 mM EDTA and 5 mM 2-mercaptoethanol, and stored at −20°C.

The purified truncated inhibitor showed a single band with a molecular mass of 40 kDa on SDS-PAGE (fig.1A). More than 10 mg of the homogeneous protein was obtained from a 1 l culture.

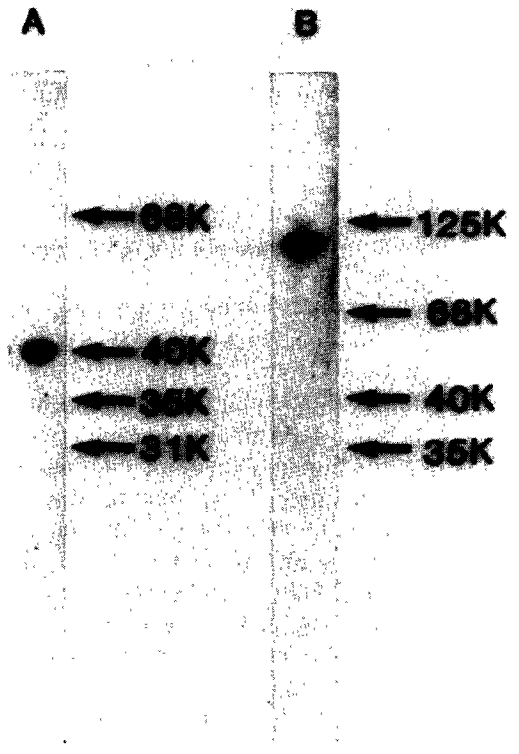

Fig.1. SDS-PAGE of the purified inhibitors. (A) The truncated inhibitor; B, the liver inhibitor. The approximate molecular masses of marker proteins are indicated.

3.2. *N-terminal sequences of the truncated and liver CANP inhibitors*

The amino acid composition of the purified inhibitor agreed with that deduced from the cDNA sequence (table 1). The N-terminal sequence analysis of the truncated inhibitor showed that the protein produced was a polypeptide fused to a hexapeptide derived from the *lacZ* gene and polylinker (table 2) as expected from the DNA sequence. The seventh Gly residue of the truncated inhibitor protein corresponded to Gly-352 in the translation product of the mRNA for the CANP inhibitor as judged from the amino acid sequence (table 2, see fig.3). Thus, the truncated inhibitor contained the C-terminal half (367 residues) of the CANP inhibitor.

The N-terminus of the liver CANP inhibitor was assigned to Glu-80 of the translation product from the protein sequence analysis (table 2). Thus, the liver inhibitor loses the N-terminal 79 residues during the maturation of the primary translation product.

3.3. *Inhibition of CANP by the purified inhibitors*

The purified truncated and liver inhibitors inhibited the caseinolytic activity of mCANP. The activity of CANP decreased linearly with increasing amounts of the inhibitors. From the values of the extrapolated intercepts, 1 mol of the truncated and liver inhibitors inhibited 1.9 and 4.1 mol mCANP, respectively (fig.2). Similar stoichiometric inhibitions were observed for μCANP by both inhibitors (not shown). These inhibitors had no effect on other proteases examined: ingensin, cathepsins B and H, kallikrein, trypsin, α-chymo-

Table 1

Amino acid composition of the truncated inhibitor

Amino acid	Deduced value from cDNA[a]		Experimental value (mol%)
	Residues	mol%	
Asx			9.7
(Asp)	35	9.4	
(Asn)	6	1.6	
Thr	21	5.6	3.8
Ser	40	10.7	10.9
Glx			14.7
(Glu)	42	11.3	
(Gln)	13	3.5	
Pro	42	11.3	11.9
Gly	14	3.8	5.8
Ala	38	10.2	12.6
Cys	2	0.5	–[b]
Val	11	2.9	3.1
Met	4	1.1	1.3
Ile	9	2.4	1.7
Leu	28	7.5	8.1
Tyr	2	0.5	0.5
Phe	3	0.8	1.8
Lys	49	13.1	10.2
His	3	0.8	0.6
Arg	11	2.9	3.4
Total	373	99.9	100.1

[a] Value corresponding to residues 352–718 of the rabbit inhibitor [7] with an extension of the N-terminus by six residues (see fig.3)

[b] Not determined

Table 2

N-terminal sequence of the CANP inhibitors

Cycle number	Phenylthiohydantoin-amino acid released (pmol)	
	Truncated inhibitor	Liver inhibitor
1	Thr (33)	Glu (72)
2	Met (241)	Lys (69)
3	Ile (183)	Thr (8)
4	Thr (25)	Ala (67)
5	Asn (145)	Ser (8)
6	Ser (7)	Arg (14)
7	Gly (78)	Ser (7)
8	Lys (185)	Lys (39)
9	Pro (172)	Glu (39)
10	Leu (101)	Pro (58)
11	Leu (157)	Val (35)
12	Pro (140)	-[a]

[a] Not determined

Proteins used for the Edman degradation were 15 μg (375 pmol) and 10 μg (90 pmol) of the truncated and liver inhibitors, respectively. Recoveries of phenylthiohydantoin-Ser and -Thr were usually 5–10% and 20%, respectively. For sequence, see legend to fig.3

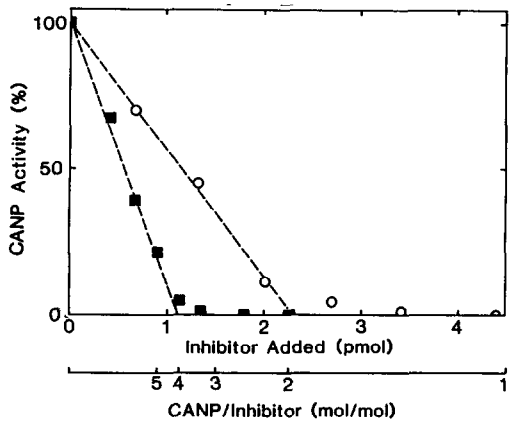

Fig.2. Inhibition of the activity of CANP by the purified inhibitors. The caseinolytic activity of mCANP (0.5 μg; 4.5 pmol) was measured in the presence of various amounts of the truncated inhibitor (○) or the liver inhibitor (■). Molar ratios of mCANP/inhibitor are also indicated. The amounts of the inhibitors were estimated using the molecular masses of 40 and 68 kDa for the truncated and liver inhibitors, respectively (see fig.3).

trypsin, bromelain, papain, lysylendopeptidase and proteinase V8. These results indicate that the truncated inhibitor containing the C-terminal half of the liver inhibitor is active and has the same specificity as the native inhibitor, though the maximum number of CANP molecule inhibited differs on a molar basis.

4. DISCUSSION

Our previous study indicated that the primary translation product of the CANP inhibitor is composed of 718 amino acid residues and contains four repeating structural units, hypothetical functional units, as shown in fig.3 [7]. Takano et al. [16] have also proposed a similar hypothesis on the basis of the partial sequence of the porcine inhibitor. The isolated liver inhibitor (M_r 110000 by SDS-PAGE) lacks the N-terminal 79 residues, but contains the four repeating units. The truncated inhibitor used in this study corresponds to the region consisting residues 352–718 and contains two repeats.

We have now shown that the truncated inhibitor, in which no post-translational modifications should be present, has the same inhibitory activity as the native inhibitor. This indicates that post-translational modification of the inhibitor is not essential for inhibition, although such

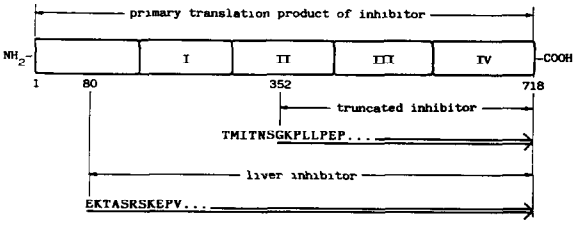

Fig.3. Schematic structure of the CANP inhibitor. Four internal repeats of about 140 amino acids each are indicated in Roman numerals. The N-terminal sequences of the truncated inhibitor and the liver inhibitor are shown by the one-letter abbreviations [7]. The first 6 residues of the truncated inhibitor, TMITNS are derived from the *lacZ* and polylinker; (ATG)ACCATGATT ACGAATTCG. The initiating Met corresponding to ATG could not be detected in the final product. The sequence from Gly-7 was identical to that from Gly-352. The N-terminal sequence of the liver inhibitor was assigned to the sequence from residue 80 in the cDNA sequence of the inhibitor.

modification(s) does occur in the liver inhibitor, as evidenced by the fact that the M_r estimated by SDS-PAGE (110000) is significantly larger than the value (68000) calculated from the amino acid sequence [7].

1 mol of the liver inhibitor, which has four internal repeats, inhibits 4 mol CANP. 1 mol of its C-terminal fragment containing two internal repeats, inhibits 2 mol CANP. The fact that the number of CANP inhibited by these inhibitors corresponds to the number of repeating units in their sequences, strongly supports the hypothesis that the repeating structural unit corresponds to the functional unit of inhibition. Furthermore, we have recent evidence that the erythrocyte inhibitor (M_r 70000), corresponding to the C-terminal three quarters (residues 290–718 of the translation product) of the liver inhibitor and containing three repeating units, inhibits 3 mol CANP (to be published elsewhere). Taken together, each of the four tandemly repeated structural units in the liver inhibitor is active and expresses its activity independently. Preliminary characterization of truncated fragments corresponding to each of the four domains is consistent with this prediction. The CANP inhibitors are degraded easily during purification [10,17]. Thus, inhibitors with M_r values clearly smaller than 110000 have been reported [6]. These inhibitors, however, show similar inhibitory activities [17,18], supporting the assumption that the presumed multidomains for inhibition have similar and independent inhibitory activities.

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