Amino acid sequence around the active site cysteine residue of calcium-activated neutral protease (CANP)

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Received 13 December 1982

The active site of calcium-activated neutral protease (CANP) was specifically labeled with iodoacetic acid. The active site hepta peptide was isolated from carboxymethylated CANP after digestion with proteases and the sequence was determined. The sequence indicates that CANP is a thiol protease and its comparison with other thiol proteases is described.

Ca^{2+}-Activated neutral protease *Thiol protease* *Amino acid sequence* *Active site* *Carboxymethylation*

1. INTRODUCTION

Calcium-activated neutral protease (CANP, EC 3.4.22.17), a typical intracellular protease, is classified as a thiol protease from the effect of various inhibitors for thiol proteases [1,2]. However, direct evidence supporting this classification has not been reported and it is not clear whether CANP is related to thiol proteases or metal proteases.

Recently we reported specific modification of the active site of CANP with iodoacetic acid (IAA) or an epoxysuccinyl derivative (E64 or E64c) in the presence of Ca^{2+} [3,4]. Here, the amino acid sequence around the active site, i.e., carboxymethylated site, of CANP was examined. This is the first report proving that CANP is a thiol protease.

2. MATERIALS AND METHODS

2.1. *Materials*

Chicken muscle CANP was purified as in [5,6]. The following were obtained from the sources indicated: Iodo[2-^{14}C]acetic acid (55 μCi/μmol), Radiochemical Centre; TPCK-trypsin, Worthington; α-chymotrypsin (type II), Sigma; thermolysin (3 × crystallised), Daiwa Kasei (Osaka); Bio-Gel P-4 (200–400 mesh), Bio-Rad Labs; DEAE-Sephacel and Sephadex G-25 (superfine), Pharmacia; reagents for sequence analyses, Wako Pure Chem. (Tokyo).

2.2. *Carboxymethylation of CANP*

Purified CANP (25 mg (0.30 μmol), spec. act.: ≥ 400 units/mg) was dissolved in 50 mM Tris–HCl, 2 mM 2-mercaptoethanol (pH 7.5) at 0.5 mg/ml. [^{14}C]IAA (20-fold molar excess) was added to the CANP solution and carboxymethylation was started by addition of 2 mM Ca^{2+}. The reaction was stopped after 2 h at 0°C by addition of EDTA (5 mM). CANP was collected by precipitation at 70% ammonium sulfate and dialysed against 50 mM Tris–HCl, 5 mM 2-mercaptoethanol, 5 mM EDTA (pH 7.5). The CANP preparation (0.28 μmol) was inactive and incorporation of carboxymethyl groups was 0.77 mol/mol CANP. CANP was then treated with cold IAA for complete carboxymethylation, essentially as in [7], and dialysed against 50 mM ammonium bicarbonate (ambic) (pH 7.8) for digestion with trypsin.

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

2.3. *Preparation and purification of modified active site peptide*

Carboxymethylated CANP was digested with trypsin (molar ratio, 1:30) for 24 h at 30°C. The lyophilised digest was applied to a Bio-Gel P-4 column. The column was developed with 1% ambic (pH 7.8) and 1.2-ml fractions were analysed for absorbance at 280 nm and radioactivity. The main radioactive fractions (0.17 μmol) in fig.1A were pooled, digested with chymotrypsin (molar ratio, 1:35; at 30°C for 24 h), and separated as above. The radioactive fractions (0.16 μmol) were pooled and digested with thermolysin (molar ratio, 1:55) at 40°C for 8 h in the presence of 1 mM Ca^{2+}. Separation of the lyophilised thermolysin digest is shown in fig.1B. The peak indicated (0.14 μmol) was applied to a DEAE-Sephacel column (0.6 × 30 cm) equilibrated with 10 mM ambic (pH 7.8) and peptides were eluted with a 200 ml, 10–500 mM ambic concentration gradient at pH 7.8 (fig.1C). The main fraction (75 nmol) was lyophilised and applied to a Sephadex G-25 column (1 × 200 cm). A single symmetrical peak was obtained on elution with 0.1 N acetic acid. This fraction (44 nmol) was dried and used for sequence analyses.

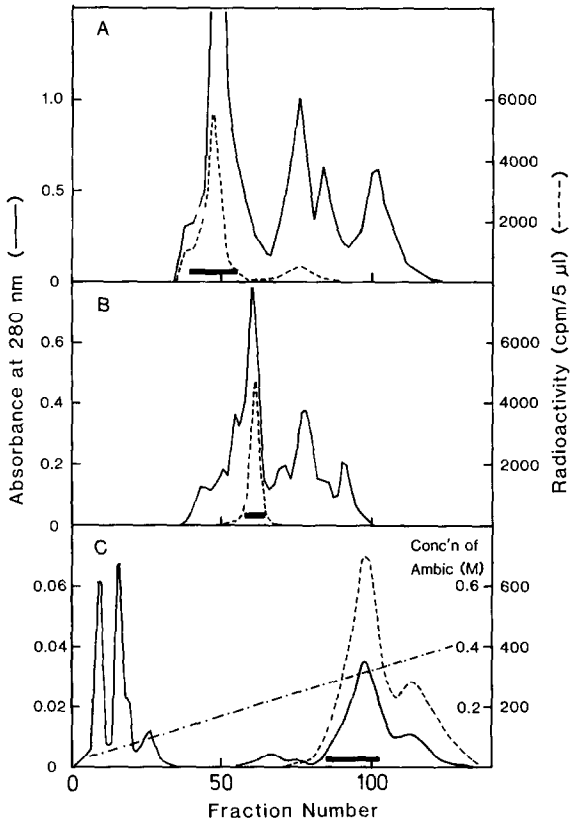

Fig.1. Purification of the active site peptide of CANP: (A) Separation of trypsin peptides of ^{14}C-carboxymethylated CANP on a Bio-Gel P-4 column (1 × 200 cm). Fractions indicated by the horizontal bar were pooled; (B) Separation of thermolysin peptides on a Bio-Gel P-4 column as in (A); (C) Further fractionation of the pooled fraction in (B) on a DEAE-Sephacel column (0.6 × 30 cm). Fractions of 1.2 ml were analysed as above. For details see text.

2.4. *Sequence analysis*

The amino acid sequence of the peptide was analysed with a JEOL 47KS Sequence Analyzer using polybrene [8] and a program recommended by the manufacturer with slight modifications. Thiazolinones collected were converted to phenythiohydantoins (PTH-amino acids) as in [9]. PTH-amino acids were analysed with a Hitachi 638-30 high performance liquid chromatograph on Toyosoda-LS 410 (C_{18}) column (0.4 × 15 cm). The column was equilibrated with 15% methanol, 0.01 M sodium acetate (pH 7.0) and PTH-amino acids were eluted with increasing methanol concentrations (15–30%).

2.5. *Other methods*

The concentration and activity of CANP were determined as in [4,5]. Tryptophan was estimated from absorbance at 280 nm using a molar extinction coefficient of 5400. Radioactivity was measured in a toluene-based scintillator with an Aloka LSC-900 Scintillation counter. Amino acid analyses were performed with a Hitachi 835-50 amino acid analyser after hydrolysis with 6N HCl for 24 h at 110°C.

3. RESULTS

3.1. *Purification and amino acid composition of the active site peptide*

The recovery of the peptide was 44 nmol (15%) from 300 nm of CANP. Two minor fractions were detected as in fig.1A and C, but they were not characterized further. Amino acid analysis of the active site peptide indicated that it was composed of 7 amino acid residues: Asp 1.09 (1), Gly 1.04

(1), Ala 0.88 (1), Leu 0.99 (1), CmCys 0.67 (1), and Trp 1.75 (2). This composition showed that it was essentially pure, though small amounts (< 0.2) of Ser, Glu, Tyr and Phe were also detected. Calculated M_r (885) of the peptide for the composition corresponded to the approximate value of 1000 expected from the elution position with a Bio-Gel P-4 column (fig.1B).

3.2. *Amino acid sequence of the active site peptide*

Figure 2 shows the results of sequence analyses of the active site hepta peptide by a sequenator. The sequence was determined from the N-terminus to the end as Ala-Leu-Gly-Asp-CmCys-Trp-Trp. The initial and average repetitive yields of the analysis were about 80% and 70%, respectively. The recovery of CmCys in the 5th cycle agreed with that calculated from radioactivity obtained in the same cycle. The small amount of tryptophan detected in the 8th cycle may be an 'overlap' of the preceding two tryptophan residues. No PTH-amino acid was detected after the 8th cycle.

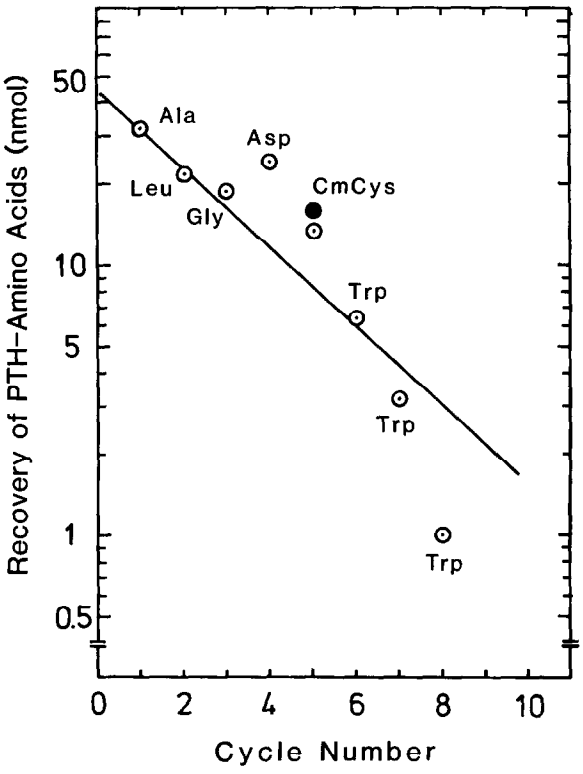

Fig.2. Summarized results of sequence analyses of the active site peptide of CANP. ^{14}C-carboxymethylated CANP (40 nmol) was applied to a Sequence Analyzer. Recovery of PTH-amino acids in each cycle was plotted. (●) recovery of CmCys (carboxymethylcysteine) calculated from radioactivity.

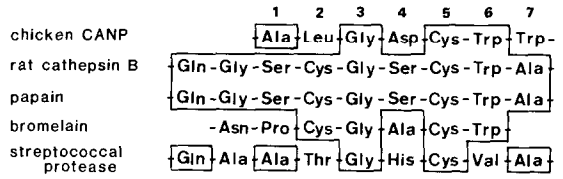

Fig.3. Comparison of the amino acid sequence of CANP with those of other thiol proteases [10,11]. Identical residues are enclosed.

4. DISCUSSION

Figure 3 compares the amino acid sequence around the active site cysteine residue of CANP with those of various thiol proteases [10,11]. Clearly the sequence for CANP resembles those of other thiol proteases. Gly-3 and the active site Cys-5 in CANP are common to all thiol proteases. Further, Trp-6 is found in most thiol proteases. Cathepsin B is very similar to other thiol proteases, especially papain. These thiol proteases are also similar in M_r. CANP is quite different from these thiol proteases in the M_r and requirement of metal ions for activity. Nevertheless, the sequence around the active site cysteine residue of CANP is similar to those in other thiol proteases, suggesting a common evolutionary origin. Thus comparative studies on CANP from various sources are important for clarifying the structure–function relationship and evolution of CANP.

ACKNOWLEDGEMENTS

This study was supported in part by research grants from the Ministry of Education, Science and Culture, and a Grant-in-Aid for New drug development from the Ministry of Health and Welfare of Japan.

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