Reversible interaction between Ca^{2+}-activated neutral protease (CANP) and its endogenous inhibitor

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The interaction between the Ca^{2+}-activated neutral protease (CANP) and its endogenous inhibitor was analyzed. The interaction was completely reversible, and both CANP and the inhibitor regained full activity after dissociation of their complex.

Ca^{2+}-activated neutral protease *Thiol protease* *Protease inhibitor* *Protease-protease inhibitor complex* *Ca^{2+}*

1. INTRODUCTION

The Ca^{2+}-activated neutral protease (CANP) is involved in a variety of physiological processes in vertebrate cells [1–4]. The activity of CANP, as an intracellular protease, is strictly regulated by various mechanisms. Among them, the concentration of Ca^{2+} in the cell is the most important factor. An endogenous proteinaceous inhibitor inhibits CANP non-competitively through the formation of a complex in the presence of Ca^{2+} [5–7]. Melloni et al. [8] reported that CANP and the inhibitor dissociate into 80 kDa and 30 kDa subunits, and 60 kDa subunit, respectively, in the presence of Ca^{2+}, and that the 60 kDa subunit of the inhibitor binds to the 80 kDa subunit of CANP to form a 140 kDa complex. However, little is known about the interaction of CANP with the inhibitor, namely, whether the interaction is reversible or not.

Recently, we purified the inhibitor (M_r = 107000) from human liver, and demonstrated that 1 mol inhibitor inhibits 5 mol CANP from various sources [9]. Here, we analyzed the reaction between chicken CANP and CANP inhibitor from human liver, and revealed that the interaction is completely reversible.

2. MATERIALS AND METHODS

2.1. *Preparation of CANP and its inhibitor*

CANP inhibitor was prepared from human liver as described in [9]. The specific activity was 2500 units/mg. Chicken CANP was prepared from skeletal muscle according to Ishiura et al. [10]. Carboxymethylated-CANP (Cm-CANP) was prepared from purified chicken CANP (spec. act. 490 units/mg) as described in [11] with iodo[2-^{3}H]acetic acid (Radiochemical Centre). Cm-CANP showed no proteolytic activity (spec. act. <0.3 units/mg), and the incorporation of carboxymethyl groups was determined from the incorporated radioactivity to be 1.2 mol/mol CANP.

2.2. *Formation and dissociation of the CANP-inhibitor complex*

The following conditions were used except when otherwise indicated. CANP and the inhibitor were incubated in 50 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol (buffer A) for 20 min at 30°C for formation of the complex. Dissociation of the complex was performed by addition of EDTA (final concentration: 5 mM) to the complex solution followed by incubation of the mixture for 10 min at 30°C.

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2.3. *Separation of CANP from the inhibitor on phenyl-Sepharose CL-4B*

To 50 μl of a mixture of CANP and the inhibitor in the absence of Ca^{2+}, concentrated NaCl solution was added to a final concentration of 0.4 M, and then 50 μl phenyl-Sepharose CL-4B (Pharmacia) swollen in 0.4 M NaCl was added. The mixture was vortex-mixed and centrifuged for 15 s at 10000 × *g*. CANP was adsorbed by the phenyl-Sepharose CL-4B under these conditions, and the supernatant contained only the inhibitor.

2.4. *Other methods*

The activities of CANP and the inhibitor were measured with casein as a substrate as described in [9,10]. One unit of the inhibitor activity was defined as the amount of the inhibitor which completely inactivated one unit of CANP. SDS-polyacrylamide gel electrophoresis (7.5% gel) was carried out according to the method of Laemmli [12], and the gel was stained with Coomassie blue.

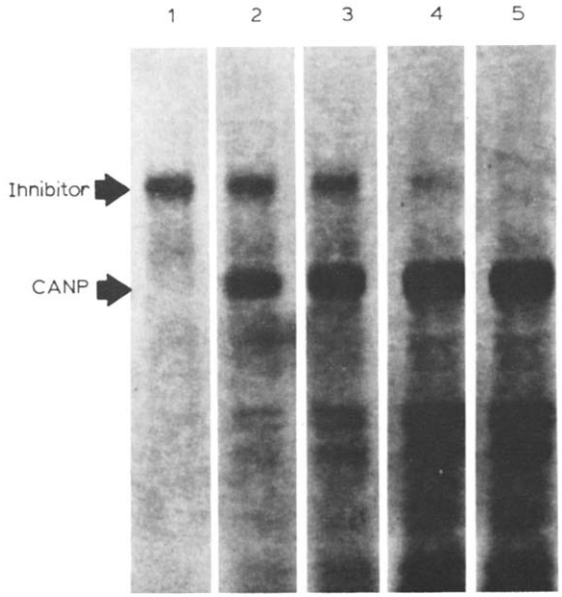

Fig.1. Analysis of interaction between CANP and its inhibitor by SDS-polyacrylamide gel electrophoresis. The inhibitor (2.4 pmol) was incubated in buffer A with various amounts of CANP: (1) none, (2) 6 pmol, (3) 12 pmol, (4) 24 pmol, to form the CANP-inhibitor complex. The complex was dissociated with EDTA and subjected to SDS-polyacrylamide gel electrophoresis. CANP (20 pmol) without incubation with Ca^{2+} is in lane 5. Triplet bands seen below the CANP band were impurities in the CANP preparation used.

3. RESULTS

3.1. *Electrophoretic analysis of the interaction between CANP and the inhibitor*

A fixed amount of the inhibitor was incubated with various amounts of CANP in the presence of 1 mM Ca^{2+}, and each incubated sample was subjected to SDS-polyacrylamide gel electrophoresis (fig.1). When an excess amount of the inhibitor was present (lane 2), neither CANP nor the inhibitor was degraded, as judged by electrophoresis, because CANP was inactivated on the formation of a complex with the inhibitor.

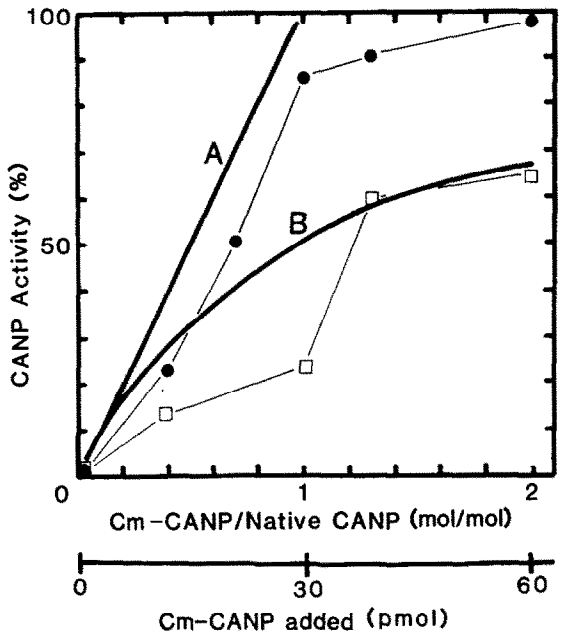

Fig.2. Interaction of Cm-CANP with the inhibitor. Various amounts of Cm-CANP (0–120 pmol) were incubated with 12 pmol inhibitor in buffer A. After incubation (●), native CANP (60 pmol) was added to the mixture and the caseinolytic activity of the added CANP was measured. The results obtained when Cm-CANP and native CANP were added together are also shown (□). The activity was expressed as the % of that measured without the inhibitor. Thick lines indicate theoretical curves when Cm-CANP is added previously (A) and together (B) with native CANP, assuming that Cm-CANP is fully active in the interaction with the inhibitor.

However, the inhibitor was degraded to small fragments when the amount of CANP exceeded that of the inhibitor, and the native inhibitor could hardly be seen (lane 4).

3.2. *Interaction between Cm-CANP and the inhibitor*

Reaction of Cm-CANP with the inhibitor was examined on the basis of the competitive interaction of Cm-CANP and native CANP with the inhibitor. The inhibitor preincubated with Cm-CANP was almost completely inactive with respect to inhibition of native CANP added afterwards (fig.2). However, when Cm-CANP and native CANP were added together, the activity of the inhibitor for native CANP was suppressed to 30–40% in the presence of a 2-fold molar excess of Cm-CANP over native CANP. These results indicate that the interaction of Cm-CANP with the inhibitor was similar to that for native CANP.

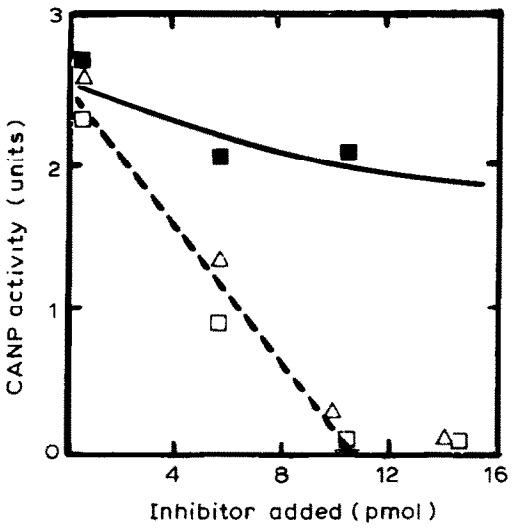

Fig.3. Reversible interaction of CANP with the inhibitor. Various amounts of the inhibitor were incubated with 54 pmol CANP in buffer A containing 3 mg/ml casein at 0°C for 10 min. After incubation, EDTA was added, and the sample was incubated at 0°C for a further 15 min. To the incubated sample, 120 pmol Cm-CANP was added, and the CANP activity was measured (■) on addition of Ca^{2+} again to a final concentration of 5 mM. As a control, the above procedure was carried out without incubation with EDTA (□), and without the addition of Cm-CANP (△).

Table 1

Recovery of the inhibitor after interaction with CANP[a]

Sample	Conditions	Inhibitor activity of the supernatant (%)
Inhibitor plus CANP	–	104
	centrifuged without NaCl[b]	3
	incubated without $CaCl_2$	100[c]
Inhibitor without CANP	–	78
	incubated without $CaCl_2$	108

[a] CANP (200 pmol) was incubated with 200 pmol inhibitor in buffer A and the complex formed was dissociated in EDTA. Then, CANP was removed by the phenyl-Sepharose CL-4B method, and the inhibitor activity of the supernatant was measured

[b] See section 2.3

[c] Value taken as 100%

3.3. *Activity of CANP recovered from the CANP-inhibitor complex*

CANP was incubated with various amounts of the inhibitor in buffer A. The CANP-inhibitor complex was dissociated by the addition of EDTA and the recovery of the CANP activity was examined in the presence of a 2-fold molar excess of Cm-CANP which was added to prevent reformation of the native CANP-inhibitor complex (fig.3). The recovery was about 70%, indicating that CANP was fully active after dissociation from the CANP-inhibitor complex based on the results described above.

3.4. *Recovery of inhibitor activity from the CANP-inhibitor complex*

The CANP-inhibitor complex was dissociated in EDTA, and the inhibitor activity was examined after removal of CANP with phenyl-Sepharose CL-4B. As shown in table 1, all the inhibitor activity was recovered in the supernatant fraction after dissociation from the inhibitor-CANP complex.

4. DISCUSSION

CANP inhibitor has been purified from various animal tissues [13]. However, there have been few reports describing the mode of interaction between CANP and the inhibitor. Cottin et al. [5,6] investigated the interaction by using CANP and the inhibitor immobilized in agarose. Melloni et al. [8,14] isolated the CANP-inhibitor complex by gel filtration in the presence of Ca^{2+}, and observed that the interaction is Ca^{2+}-dependent. These methods, however, are not suitable for analyzing quantitatively the interaction of CANP with the inhibitor on a molar basis. As shown in fig.1, excess CANP digests the inhibitor. Therefore, stoichiometric analysis of the interaction is necessary. In the present study the interaction between CANP and the inhibitor was analyzed quantitatively in vitro. Both CANP and the inhibitor regained full activity after dissociation of their complex. Furthermore, no changes were observed in their electrophoretic mobilities after formation of the complex. These results indicate that the in vivo activity of CANP is reversibly regulated by the inhibitor, and that the interaction is regulated solely by Ca^{2+}. Thus, the intracellular balance of their amounts is a very important factor for the regulation of CANP activity. Several investigators stated that CANP inhibitor is an oligomeric protein, judging from its elution position on gel filtration [8,14–16]. Melloni et al. [8] reported that the inhibitor is dissociated into subunits in the presence of Ca^{2+}. The inhibitor preparation used here is monomeric and we did not observe any change in the molecular mass as estimated by gel filtration after dissociation from the complex (not shown).

In this study CANP and the inhibitor purified from different sources were used. However, the same results were obtained with CANP and the inhibitor of the same source; the inhibitor equally inhibits CANP regardless of the origin.

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