SYNERGISTIC ACTIVATION OF CALCIUM-ACTIVATED NEUTRAL PROTEASE BY Mn^{2+} and Ca^{2+}

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Received 1 February 1982

1. Introduction

Calcium-activated neutral protease (CANP, EC 3.4.22.17) is ubiquitous in cells and probably controls the initial steps of intracellular protein degradation [1]. CANP prepared from various sources usually requires mM Ca^{2+} for activation (called mCANP) [2–4], although another species of CANP (μCANP) highly sensitive to Ca^{2+} and active at μM Ca^{2+} was found [5,6] and was prepared by autolysis of mCANP [7,8]. Since free Ca^{2+} in cells is in the μM order, the role of mCANP in vivo remains unclear. In the cells various ions may act on CANP simultaneously with Ca^{2+}. In the following studies the effect of various metal ions on CANP was examined and it was revealed that Mn^{2+} and Ca^{2+} activate CANP in a concerted manner at a concentration where Mn^{2+} or Ca^{2+} alone is not effective.

2. Materials and methods

mCANP was purified from chicken skeletal muscle [3] and its sensitized form (μCANP) was prepared by autolysis of mCANP as in [7,9]. Standard assays (Ca^{2+} assay) for mCANP and μCANP were performed with 6 mM Ca^{2+} as in [3]. The assay with Mn^{2+}–Ca^{2+} (Mn^{2+}–Ca^{2+} assay) was performed in 2 mM Mn^{2+}–100 μM Ca^{2+} for mCANP and in 1 mM Mn^{2+}–20 μM Ca^{2+} for μCANP. Other conditions for assays were the same as in [3]. The effect of metal ions added to the assay mixture was examined at a fixed $[Ca^{2+}]$; i.e. 100 μM and 20 μM for mCANP and μCANP, respectively, where both CANPs are almost inactive. Metal ion solutions were prepared with their chlorides. Inhibitors of CANP, E-64, E-64c, leupeptin, and antipain, were obtained as in [10].

3. Results

3.1. *Effect of various metal ions on the activity of CANP*

Various metal ions (1.2 mM) were added to the assay mixture together with 100 μM Ca^{2+} where mCANP is almost inactive to determine whether they activated CANP. As reported [3], no single metal ion except for Ca^{2+} activated mCANP at 1.2 mM in the absence of Ca^{2+}. However, Mn^{2+} and Ba^{2+} clearly activated mCANP when they were added together with 100 μM Ca^{2+} (table 1). This indicates that Ca^{2+} can act on CANP cooperatively with such metal ions as Mn^{2+} or Ba^{2+}. Sr^{2+} was slightly effective for the activation. Mg^{2+} and Cd^{2+} had almost no effect on the activity of CANP and could not replace the effect of Mn^{2+} and Ba^{2+}. The effect of Mn^{2+} and Ba^{2+} on mCANP was apparently additive, though further examination was not performed.

Table 1
Effect of metal ions on the activity of mCANP in the presence of Ca^{2+}

Metal ions[a] added	Activity[b] (%)	Metal ions[a] added	Activity[b] (%)
None	2.8	Cd + Sr	3.3
Mn	59	Cd + Ba	36
Mg	0	Mg + Mn	57
Sr	6.1	Mg + Sr	4.7
Ba	38	Mg + Ba	42
Cd	0	Mn + Sr	51
Cd + Mg	0	Mn + Ba	82
Cd + Mn	47	Sr + Ba	43

[a] Each metal ion was added at 1.2 mM to the assay mixture with 100 μM Ca^{2+}

[b] Activity determined by the Ca^{2+} assay (in 6 mM Ca^{2+}) was taken as 100%

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

When 3 kinds of metal ions listed in table 1 were added in various combinations to the assay mixture with 100 μM Ca^{2+}, the activity of mCANP was observed only when Mn^{2+} and/or Ba^{2+} was present. The effect of various metal ions on μCANP was similar to that on mCANP, and μCANP was activated by Mn^{2+} and Ba^{2+}. Further studies on the effect of metal ions on CANP were performed with Mn^{2+}.

3.2. *Optimum [Mn^{2+}] for activation of CANP*

Mn^{2+} activated CANP only in the presence of Ca^{2+}. The [Ca^{2+}] required for this activation was quite low and could not activate CANP in the absence of Mn^{2+}. The optimum [Mn^{2+}] for activation of CANP was 1.5–2 mM for mCANP and 0.5–2 mM for μCANP (fig.1). The [Mn^{2+}] for 50% activation (K_a) was 550 μM and 70 μM for mCANP and μCANP, respectively. These K_a-values are similar to those obtained with Ca^{2+} in the absence of Mn^{2+}; i.e., 410 μM and 70 μM for mCANP and μCANP, respectively (see fig.2).

3.3. *Activation of CANP by Ca^{2+} in the presence of Mn^{2+}*

The results in table 1 suggest that Mn^{2+} enhances the sensitivity of CANP to Ca^{2+}. Accordingly, activation of mCANP and μCANP by Ca^{2+} was examined with and without Mn^{2+} (fig.2). In the presence of Mn^{2+}, half-maximum activation was observed at 40 μM and 15 μM for mCANP and μCANP, respectively, while without Mn^{2+}, the K_a-values were 410 μM and 70 μM, respectively. Mn^{2+} enhanced the sensitivity of both mCANP and μCANP to Ca^{2+} ≳5-times, though absolute K_a-values may not be accurate because Ca^{2+}-buffer was not used. The maximum activity obtained with the Mn^{2+}–Ca^{2+} assay was 60–80% of that determined by the Ca^{2+} assay.

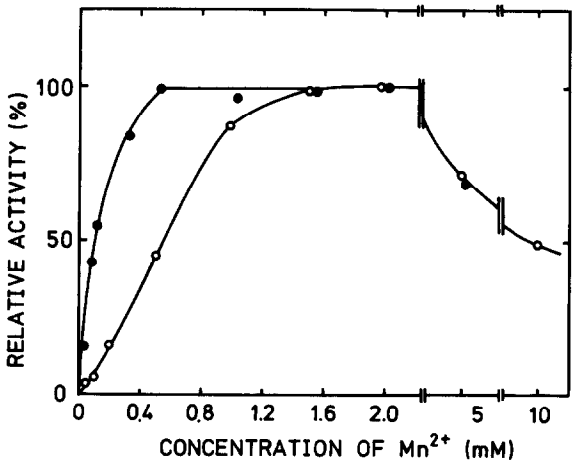

Fig.1. Optimum [Mn^{2+}] for activation of CANP. The activity was measured with various concentrations of Mn^{2+} in 100 μM and 20 μM Ca^{2+} for mCANP and μCANP, respectively. The maximum activity was taken as 100%. (○) mCANP; (●) μCANP.

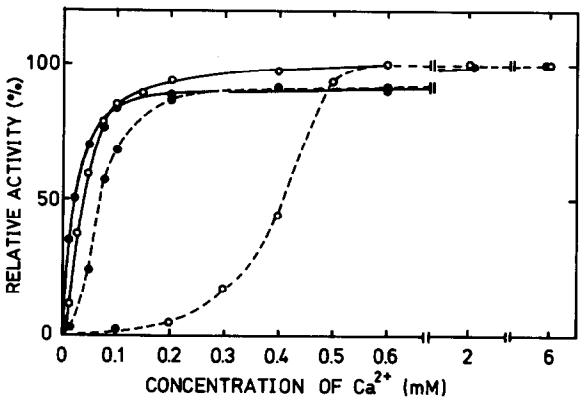

Fig.2. Activation of CANP by Ca^{2+} in the presence of Mn^{2+}. Activity was measured at various concentrations of Ca^{2+} with (——) and without (– – –) Mn^{2+}; 2 mM and 1 mM Mn^{2+} for mCANP and μCANP, respectively. (○) mCANP; (●) μCANP.

3.4. *Kinetic properties of CANP measured by the Mn^{2+}–Ca^{2+} assay*

The optimum pH-values for the activation of mCANP and μCANP by Mn^{2+} were both 7.5–8.0, which are identical to those determined by the Ca^{2+} assay [3]. K_m-Values of mCANP for casein were 1.2 mg/ml and 0.90 mg/ml for the Ca^{2+} and Mn^{2+}–Ca^{2+} assays, respectively. The activities of both CANPs were strongly inhibited by leupeptin, antipain, E-64 and E-64c. The molar ratios of inhibitor to mCANP for 50% inhibition of activity (ID_{50}) were 4, 15, 11 and 8, respectively, which are almost identical to those determined by the Ca^{2+} assay [10]. When mCANP was incubated in 2 mM Mn^{2+}–100 μM Ca^{2+} (pH 7.5), autolysis occurred as in Ca^{2+} [7]. During this autolysis, CANP was converted to its sensitized form to Ca^{2+} and the activity at 100 μM Ca^{2+} appeared (fig.3). SDS–polyacrylamide gel electrophoresis indicated that the M_r-value of mCANP changed as follows: 82 000 (mCANP) → 78 000 → 57 000 → →. The newly formed 2 species (M_r 78 000 and 57 000) active at 100 μM Ca^{2+} were identical to μCANP I and II, respectively, obtained in Ca^{2+} [7]. Though the rate of

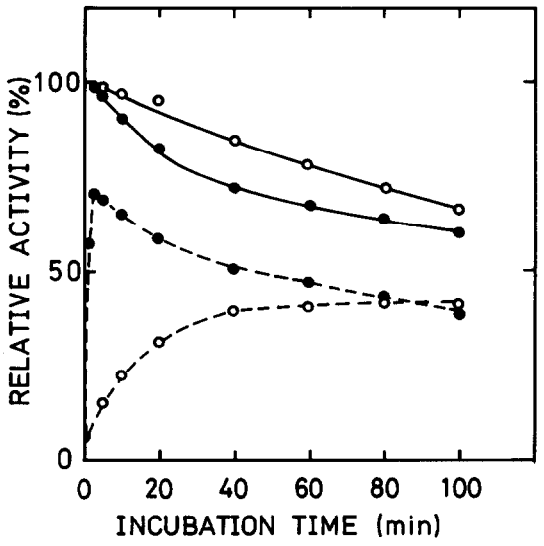

Fig.3. Autolysis of CANP in Mn^{2+}–Ca^{2+}. mCANP (0.5 mg/ml) was incubated at 0°C in 50 mM Tris–HCl, 2 mM 2-mercaptoethanol (pH 7.5) containing 2 mM Mn^{2+}–0.1 mM Ca^{2+} (○). At intervals aliquots were assayed with 6 mM (——) and 100 μM (– – –) Ca^{2+}. Similar incubation was performed in 1 mM Ca^{2+} as a control (●) and aliquots were analyzed as above.

autolysis in Mn^{2+}–Ca^{2+} was slower than that in Ca^{2+}, probably due to the difference in the maximum activities, similar overall features of autolysis indicate the same substrate specificity in Mn^{2+}–Ca^{2+}.

These results show that the properties of CANP measured by the Mn^{2+}–Ca^{2+} assays were essentially the same as those measured by the Ca^{2+} assays.

4. Discussion

In the presence of 1–2 mM Mn^{2+}, the Ca^{2+} sensitivity of CANP is significantly enhanced and both mCANP and μCANP are active at a physiological [Ca^{2+}]. However, since [Mn^{2+}] in vivo is $\leqslant 10$ μM, this activation by Mn^{2+} may not be of physiological importance. Nevertheless, this fact suggests that the sensitivity of CANP to Ca^{2+} may be modulated in vivo by other metal ions to become active at physiological [Ca^{2+}]. If so, not only μCANP but also mCANP may play an important role in the degradation of proteins. The enhancement of Ca^{2+} sensitivity of CANP by Mn^{2+} and other metal ions will be common to CANPs from other sources, because rabbit muscle CANP was also activated by Mn^{2+}, Ba^{2+} and Sr^{2+}.

We suppose that at least 2 molecules of Ca^{2+} bind to CANP. One with a dissociation constant (K_d) of μM order directly participates in the enzyme catalysis and the other with a K_d-value of mM order induces the active conformation of CANP. Only the latter can be replaced by other metal ions like Mn^{2+}. In the case of μCANP, the K_d-value of the latter is lowered by some modification [6,7], though the former site is unchanged. As the K_d-value of the former site is smaller than that of the latter site, the sensitivity of CANP to Ca^{2+} and other metal ions is determined mainly by the latter site. This hypothesis explains the results so far obtained concerning the effect of Ca^{2+} and other metal ions on the structure and activity of CANP [3,10,11]. Preliminary results that the conformation of mCANP in 2 mM Mn^{2+} is indistinguishable from that of carboxymethylated mCANP in 6 mM Ca^{2+} also support this model.

Acknowledgement

This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan.

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